Zur Homepage der Dissertation

Heterotrophe Bakteriengemeinschaften des Deutschen Wattenmeeres- Diversität, Dynamik und Abundanz

Communities of Heterotrophic Bacteria in the German Wadden Sea- Diversity, Dynamics and Abundance

Dissertation

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Erklärung

Teilergebnisse dieser Arbeit sind als Beiträge bei den genannten Fachzeitschriften eingereicht oder werden eingereicht. Mein Beitrag an der Erstellung der verschiedenen Manuskripte wird im folgenden erläutert:

Stevens, H., Simon, M., Brinkhoff, T. 2004. Phylogeny of *Proteobacteria* and *Bacteroides* from oxic habitats of a tidal flat ecosystem. In Vorbereitung zum Einreichen bei Envir. Mirobiol.
Konzeptentwicklung und Durchführung der praktischen Arbeiten durch H. S. Erstellung der ersten Fassung des Manuskriptes durch H. S., Überarbeitung durch T. B., H. S. und M. S.

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Stevens, H., Brinkhoff, T., Simon, M. 2004. Composition and seasonal dynamics of free-living, aggregate- and sediment surface- associated bacterial communities in the German Wadden Sea. Überarbeitete Version eingereicht bei Aquat. Microb. Ecol.
Konzeptentwicklung durch H. S. Durchführung der praktischen Arbeiten durch H. S. Erstellung der ersten Fassung des Manuskriptes durch H. S., Überarbeitung durch M. S., H. S. und T. B.

Weitere Veröffentlichungen

Martens, T., **Stevens, H**., Simon, M., Brinkhoff, T.
Screening of marine Gram-positive bacteria for secondary metabolite production. In Vorbereitung.

Karaman, M., **Stevens, H.**, Brinkhoff, T., Simon, M., Liang, L., Bach, G.
Zeeck, A., Große, T., Lang, S. 2004. New Hydroxyphenylpyrroles from a marine *Halomonas* sp. In Vorbereitung.

Kuever, J., Sievert, S., **Stevens, H.**, Brinkhoff, T., Muyzer, G. 2002. Microorganisms of the oxidative and reductive part of the sulfur cycle at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece), Cahiers Biologie Marine 43: 413 – 416

Folgende Arbeiten resultierten (zu Teilen) aus der hier vorliegenden Studie:

1. Daniel Kessler (2003). Mikro- und Molekularbiologische Untersuchungen zum Sekundärstoffwechsel bei Bakterien der *Roseobacter*-Gruppe. Diplomarbeit am Institut für Chemie und Biologie des Meeres (ICBM) der Carl von Ossietzky Universität Oldenburg.

2. Thomas Große (2003). Untersuchungen zur Nährstoffbildung an neu isolierten Bakterien aus der Nordsee. Diplomarbeit am Institut für Biochemie und Biotechnologie der Technischen Universität Braunschweig.

3. Lanfang Liang (2003) Investigation of secondary metabolites of North Sea bacteria: fermentation, isolation, structure elucidation and bioactivity. Dissertation an der Georg-August-Universität zu Göttingen.

4. Torben Martens. Molekularbiologische Untersuchungen zur Naturstoffproduktion neu isolierter Bakterien. Dissertation am Institut für Chemie und Biologie des Meeres (ICBM) der Carl von Ossietzky Universität Oldenburg. Abschluß voraussichtlich 3. Quartal 2004.

5. Antje Gittel. Untersuchung der bakteriellen Manganoxidation im Deutschen Wattenmeer. Laufende Diplomarbeit am Institut für Chemie und Biologie des Meeres (ICBM) der Carl von Ossietzky Universität Oldenburg. Abschluß voraussichtlich 2. Quartal 2004.

6. Myriam Kruse Entwicklung spezifischer Nachweismethoden für abundante Bakteriengruppen des Deutschen Wattenmeeres. Laufende Diplomarbeit am Institut für Chemie und Biologie des Meeres (ICBM) der Carl von Ossietzky Universität Oldenburg. Abschluß voraussichtlich 2004.

Tagungsbeiträge

Teile dieser Arbeit wurden von mir als Vortrag auf nationalen und internationalen Kongressen und Treffen präsentiert

- **Stevens, H.**, Brinkhoff, T.; Martens, T.; Schlingloff, A.; Grossart, H.P.; Liang, L. and M. Simon. 2002. The bacterial community of the German Wadden Sea and the challenge to find its secondary metabolites. 7th Symposium on Bacterial Genetics and Ecology, 15. – 19. Juni, Bergen, Norwegen.

- **Stevens, H.**, Schlingloff. A., Brinkhoff, T, Grossart, H. P., Simon, M. 2000 Isolation of novel marine microorganisms. Meeting Marine Biotechnology, 18. Dezember , Braunschweig, Deutschland.

Angemeldet:

- **Stevens, H.**, Brinkhoff, T. 2004 Studies on the microbial community in intertidal mudflats. International Symposium on Microbial Ecology, (ISME-10) 22. – 27. August, Cancun, Mexiko.(Vortrag /Poster).

Weitere Tagungsbeiträge

Rink, B., Lunau, M., Seeberger, S., **Stevens, H.**, Brinkhoff, T., Grossart, H.-P. and Simon, M. 2003. Different diversity patterns of particle – associated and free – living bacterial communitiesin the German Wadden Sea. Workshop on BioGeoChemistry of tidal flats, 14. –17. Mai, Hansewissenschaftskolleg Delmenhorst, Deutschland; Reports: Forschungszentrum Terramare 12:96-98 (Poster und Extended Abstract).

Simon, M., Lunau, M., Brinkhoff, T., **Stevens, H.**, Rink, B., Duerselen, C. and Grossart, H. – P. 2003. Tidal and seasonal variations in dynamics of microaggregates and associated bacterial communities in the German Wadden Sea. Workshop on BioGeoChemistry of tidal flats, 14. –17. Mai, Hansewissenschaftskolleg Delmenhorst, Deutschland; Reports: Forschungszentrum Terramare 12:105-108 (Vortrag und Extended Abstract).

Martens, T., **Stevens, H.**, Grossart, H. – P., Selje, N., Simon, M., Brinkhoff, T. 2003. Screening of marine bacteria for new secondary metabolites. Jahrestagung der VAAM, 23. – 26. März, Göttingen, Deutschland (Vortrag).

Rink, B., **Stevens, H.**, Lunau, M., Brinkhoff, T.; Simon, M. 2003. Completing the picture of natural habitats. The use of specific primer sets in DGGE. Annual Meeting of the VAAM, 23. – 26. März, Göttingen, Deutschland (Poster).

Stevens, H., Brinkhoff, T., Schlingloff, A. and Simon, M. 2003. Cultivable bacteria from the Wadden Sea: who is where and what is on the menu? Annual meeting of the VAAM, March 23 – 26. Munich, Deutschland (Poster).

Martens, T., **Stevens, H.**, Grossart, H. – P., Selje, N., Simon, M., Brinkhoff, T. 2002. PCR-based screening for novel secondary metabolites from marine bacteria. 3rd European Conference on Marine Natural Products, 15. – 20. September Schloß Elmau, Bayern, Deutschland (Poster).

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Stevens, H., Brinkhoff, T., Schlingloff, A., Grossart, H. – P., Liang, L., Simon, M. 2002. Bacteria of the German Wadden Sea – a pool of secondary metabolites to come? 3rd European Conference on Marine Natural Products, 15. – 20. September, Schloß Elmau, Bayern, Deutschland (Poster).

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Stevens, H., Brinkhoff, T., Grossart, H – P, Schlingloff, A, Simon, M. 2001. Secondary metabolites produced by bacteria isolated from the Wadden Sea, North Sea (Germany). International Symposium on Microbial Ecology, (ISME-9) 26. – 31. August, Amsterdam, Niederlande (Poster).

Stevens, H., Schlingloff, A., Brinkhoff, T., Simon, M. 2000. Heterogeneities of bacterial communities in sediments, water and aggregates of the Wadden Sea (Germany). Jahrestagung derVAAM, 12. – 16. März, München, Deutschland (Poster).

Stevens, H., Schlingloff, A., Brinkhoff, T., Grossart, H. – P., Simon, M. 2000. Seasonal dynamics of particle – associated, free-living and sediment-associated bacterial communities in the German Wadden Sea. 12. – 13. September, Workshop marine Biotechnologie. Braunschweig, Deutschland (Poster).

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Abkürzungsverzeichnis

ANOVA	analysis of variance
BAC	bacterial artificial chromosome
CARD-FISH	catalysed reporter deposition FISH
CO_2	Kohlendioxid
COSY	correlation spectrography
DAPI	4´,6´-Diamino-2-phenylindol
DC	Dünnschichtchromatografie
DGGE	Denaturierende Gradienten Gelelektrophorese
DMSO	Dimethylsulfoxid
DNA	desoxy ribonucleic acid
DOM	dissolved organic matter
EI-MS	Elektonenionisationsmassenspektroskopie
ESI	electronspray ionisation
et al.	et alii
FISH	fluorescence *in situ* hybridization
gram+	Gram positive
hgc	high guanosine-cytosine content
HMBC	heteronuclear multiple bond connectivity
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
lgc	low guanosine-cytosine content
MAR	microautoradiography
max.	maximal
MB	Marine Broth
MICRO-FISH	MAR and FISH
MPN	most probable number
MS	mass spectroscopy
MW	molecular weight
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance
O_2	Sauerstoff
PCR	polymerase chain reaction
PFGE	post field gel electrophoresis
POM	particulate organic matter
psu	practical salinity unit
RNA	ribonucleic acid
RP	reverse phrase
rt	Rolltank
STARFISH	substrate-tracking autoradiographic FISH
UF	Unterfraktion
V(B)NC	viable (but) non-culturable
vs.	versus

ZUSAMMENFASSUNG

Das Wattenmeer ist als Teil der südlichen Nordsee ein nährstoffreiches küstennahes Flachmeer, das sich von den Niederlanden (den Helder) über Deutschland bis nach Dänemark (Ejsberg) erstreckt. Beeinflusst wird dieses Ökosystem besonders durch tidale Strömungen und einen Eintrag von organischen und anorganischen Nährstoffen vom Land und aus der angrenzenden Nordsee. Obwohl mikrobielle Umsetzungen in heterotrophen Systemen von großer Bedeutung sind, wurde die Zusammensetzung der Bakteriengemeinschaften in diesem Ökosystem nur wenig untersucht. In der vorliegenden Arbeit wurde zum ersten Mal die Struktur und die saisonale Dynamik der Bakteriengemeinschaften in verschiedenen Kompartimenten, d.h. freilebend in der Wassersäule sowie an Aggregaten und der Sedimentoberfläche assoziiert in einem polyphasischen Ansatz untersucht. Genfragmente der 16S rRNA wurden anhand der Denaturierenden Gradientengelelektrophorese (DGGE) und anschließender Clusteranalyse untersucht. Die aus der DGGE resultierenden Banden wurden sequenziert und eine 16S rDNA Klonbibliothek konstruiert. Aus Verdünnungskulturen mit verschiedenen Medien supplementiert mit unterschiedlichen Biopolymeren wurden ungefähr 250 Bakterienstämme isoliert und phylogenetisch identifiziert. Alle erhaltenen Sequenzen wurden in einer umfangreichen phylogenetischen Analyse ausgewertet. Als mögliche Einflussfaktoren der Bakteriengemeinschaft wurden Abundanz und Diversität des Phytoplanktons sowie verschiedene physico-chemische (z.B. Sauerstoffgehalt, Salinität, pH-Wert, Temperatur und Windrichtung und –geschwindigkeit) und biologische (Chlorophyll und Phaeopigmente, gelöster organischer und anorganischer Kohlenstoff) Parameter bestimmt. Zusätzlich wurden einige Stämme auf ihr biotechnologisches Potential hin untersucht, die von ihnen produzierten Naturstoffe isoliert und ihre Struktur aufgeklärt.

Die Hauptaussagen dieser Arbeit können wie folgt zusammengefasst werden:

- Die Struktur der Bakteriengemeinschaften in den drei Kompartimenten unterscheidet sich. Die mit unseren Methoden detektierbare freilebende Artenvielfalt unterscheidet sich signifikant von der sediment-assoziierten (ANOVA-test). Eine Clusteranalyse zeigte keine Überschneidungen der Freiwasser- mit der Sedimentfraktion. Eine Analyse der Aggregatfraktion ergab Überschneidungen mit beiden anderen Kompartimenten, ein Hinweis auf ihre mögliche Funktion als Bindeglied zwischen mikrobiellen Prozessen in den beiden anderen Fraktionen.
- Aus den Isolierungsansätzen konnten Bakterien aus 7 verschiedenen phylogenetischen Klassen isoliert werden, auch dominante Vertreter der unterschiedlichen Kompartimente. Einige Stämme zeigten äußerst geringe 16S rDNA Ähnlichkeit mit validiert beschriebenen Spezies aus den Datenbanken (≤90 %) und bilden höchstwahrscheinlich neue Gattungen. Biotechnologische Untersuchungen ergaben, dass einige der marinen Bakterien eine Vielfalt von Naturstoffen in geringen Mengen produzieren.
- Auf der Basis der mit den unterschiedlichen Herangehensweisen erhaltenen Sequenzen konnten 3 neue phylogenetische Cluster definiert werden, die für das Wattenmeer charakteristisch sind: 2 Cluster aus der phylogenetischen Klasse der α-*Proteobakterien* und einer aus der Klasse der γ-*Proteobakterien*. Letzteres besteht ausschließlich aus Sequenzen unkultivierter mariner Bakterien, die an Oberflächen angehefetet leben.

- Obwohl die Zusammensetzung der Phytoplanktongemeinschaft und die Konzentration und Zusammensetzung des organischen Kohlenstoffes über das Jahr stark schwankten, gab es keine ausgeprägten saisonalen Veränderungen in der Zusammensetzung der Bakteriengemeinschaften. Diese schienen gut an das hochdynamische System angepasst zu sein. Statistische Analysen zeigten, dass Änderungen der Bakterienzellzahl über das Jahr auf die Windgeschwindigkeit zurückzuführen sind. Die Windrichtung schien dabei der Hauptfaktor zu sein, da sich mit ihr die Wellendynamik und damit die Resuspension des Sedimentes stark verändern. Das Wattenmeer zeigt sich also als hoch komplexes System, das bezogen auf die Bakteriengemeinschaft in erster Linie von physikalischen Parametern abhängig ist.

Insgesamt zeigte sich für die Analyse der Bakteriengemeinschaft im Wattenmeer die Kombination von kultivierungsabhängigen und –unabhängigen Methoden sehr gut geeignet. Ergebnisse konnten durch die Vielfalt der Methoden entweder bestätigt werden oder durch Diskrepanzen in den Ergebnissen der verschiedenen Methoden die Beurteilung der methodenspezifischen Verzerrungen möglich machen.

SUMMARY

The Wadden Sea is a shallow and nutrient-rich coastal ecosystem of the southern North Sea stretching from the Netherlands (Den Helder) over Germany to Denmark (Esbjerg). Tidal dynamics and inputs of organic and inorganic nutrients from land and the North Sea highly affect this habitat in which microbial processes are of major importance. Only limited information, however, is available about the composition of the bacterial communities. This thesis addressed for the first time questions about the structure and seasonal dynamics of the heterotrophic bacterial community in this habitat using a polyphasic approach. Investigations included the analyses of three different habitats, i.e. the water column (free-living bacteria), suspended aggregates and the sediment surface. Denaturing gradient gel electrophoresic analysis of 16S rRNA gene fragments, a subsequent cluster analysis and sequencing of bands were performed and a 16S rDNA clone library was constructed. Around 250 bacterial strains were isolated using dilution cultures with a variety of media supplemented with various biopolymers. All obtained sequences were included in an extensive phylogenetic analysis. Phytoplankton counts and diversity and various physico-chemical parameters (e.g. oxygen, salinity, pH, temperature and wind speed and direction) and biological parameters (chlorophyll and phaeopigments, dissolved organic and inorganic carbon), were determined as possible factors influencing the microbial community. In addition, some strains were screened for their biotechnological potential, their natural products were isolated and the structure elucidated. The mayor findings of the thesis can be summarized as follows:

- The community structure of the three habitats differed from each other. The detectable species richness of the free-living bacterial community and the sediment-associated differed significantly and the cluster analysis showed no overlaps. The aggregate-associated bacterial community showed overlaps with the other habitats indicating its mediating position between processes in the water column and on the sediment surface.
- With the isolation approaches bacteria affiliating to seven different classes were obtained, including numerically abundant members of this habitat. Several strains showed very low 16S rRNA similarity to the next related validly described species (≤90 %) and presumably can be assigned as new genera. Biotechnological investigations of new strains showed that they produce a variety of natural products in low amounts.
- Three new clusters which are considered as characteristic for the German Wadden Sea could be defined on the basis of sequences derived from the various approaches: two clusters affiliate within the α-*Proteobacteria* and one within the γ-*Proteobacteria*, the latter comprised only sequences from uncultured surface-associated marine bacteria.

- Seasonally, the overall bacterial community in all three habitats was stable despite pronounced changes of the phytoplankton community and organic carbon concentration and composition. This suggests that the bacterial communities were adapted to this highly dynamic system. Statistical analysis showed that annual changes in total cell counts were most likely caused by wind speed. Wind direction appears to be the main factor as it influences wave action of water masses and thus sediment resuspension. Hence, physical parameters appear to be important in controlling dynamics of the bacterial community in the Wadden Sea.

Overall, the combination of culture-dependant and -independent approaches proved powerful to analyze the bacterial communities in this ecosystem. While certain findings could be confirmed by congruent results of different methods, discrepancies between methods allowed the detection of methodological biases

Inhaltsverzeichnis

I

EINLEITUNG

I.1 MARINE HABITATE UND DIE BEDEUTUNG HETEROTROPHEN BAKTERIOPLANKTONS

Aquatische Habitate dominieren die Erde. Die Erdoberfläche z.B. ist größtenteils, nämlich zu etwa 70 %, mit Wasser bedeckt, so dass die Erde vom All aus gesehen als blauer Planet erscheint. Der Hauptanteil des gesamten Wassers unseres Planeten liegt chemisch gebunden im heißen Erdkern vor, Wasser an und auf der Erdoberfläche ist nur zu 3 % an Land gebunden (Eis, Grundwasser, Seen und Flüsse, Boden- und atmosphärische Feuchte), 97 % bilden das Weltmeer[1]. Im Meer und an dessen Rändern findet man unterschiedliche physikalisch-chemische Gegebenheiten, die zur Ausbildung verschiedener Habitate und Organismengesellschaften führten. Neben vielen anderen möglichen Gliederungen, z.B. der horizontalen physikalisch-chemischen Einteilung des Weltmeeres in Pelagial (freies Wasser) und Benthal (Boden und Ränder), das wiederum vertikal unterteilt werden kann (Littoral, Bathyal, Abyssal und Hadal, vgl. einführende Literatur, z.B (Sommer, 1998), kann eine Einteilung nach Lebensgemeinschaften vorgenommen werden. So gibt es die Gemeinschaften des offenen Ozeans, die Tiefseegemeinschaften inkl. der Gemeinschaften an Hydrothermalquellen, die Planktongemeinschaften, die polaren Gemeinschaften, Gemeinschaften an Korallenriffen, an Fels-, Kies-, und Sandküsten und die Gemeinschaften der Ästuare und Salzmarschen (vgl. einführende Literatur; z.B. Sommer, 1998; Garrison, 1999). Obgleich man geneigt ist, Lebensgemeinschaften anhand „großer“, das heißt hier makroskopischer, Organismen zu charakterisieren, sind es jedoch Mikroorganismen, die den größten Anteil der gelösten und partikulären Stoffe umsetzen und denen somit eine globale Bedeutung in allen biogeochemischen Stoffkreisläufen zukommt (z.B. in aquatischen Habitaten bewirken Mikroorganismen den größten Teil (90 %) der Stoffumsetzungen (Löffler, 2001) und sind für Kreisläufe nahezu aller Elemente von großer Bedeutung; z.B. Schlegel, 1992; Madigan *et al.*, 2003.

Der größte aller biogeochemischen Kreisläufe ist der des Kohlenstoffs, der sich aus heutiger Sicht eher als ein ineinandergreifendes Muster von Fraß- und Detritus-Nahrungsketten, als Nahrungsnetz darstellt (Abb. I.1; nach Azam, 1998).
Dieses Nahrungsnetz besteht aus dem „klassischen“ Teil, der eine Fraßkette beschreibt (*grazing food chain*, s. Abb.2) in die ein Teil der Primärproduktion durch Phytoplanktona und Cyanobakterien eingeht (Conan *et al.*, 1999) und der betreffende Kohlenstoff über mehrere Trophiestufen den Endkonsumenten zugeführt wird. Dabei bildet das Phytoplankton das partikuläre organische Material (POM), setzt aber auch gelöstes organisches Material (dissolved organic matter, DOM) frei (Bjørnsen, 1988). POM kann in der Wassersäule aggregieren und absedimentieren (*sinking flux*).

Im Nahrungsnetz kommt heterotrophen Bakterien eine zentrale Rolle zu: Sie nehmen das gelöste Material auf, das für höhere Trophiestufen unzugänglich ist, es wird in der bakteriellen Sekundärproduktion[2] (Wachstum) verwertet. Abgestorbene Zoo- und

[1] Die traditionelle Unterscheidung des Weltmeeres in verschiedene Ozeane, z.B. Pazific, Atlantic, Baltic etc. ist eine künstliche, die anhand der Kontinentalgrenzen und imaginärer Linien, wie z.B. dem Äquator erfolgte. Sie dient nur der Orientierung. Erdgeschichtlich gesehen ist das Auftreten dieser “Einzelozeane” nur eine temporäre Erscheinung eines großen Weltmeeres (Garrison, 1999)

[2] Der Begriff “bakterielle Produktion” meint hier natürlich nicht, dass ein Produktionsvorgang bakteriell verseucht ist, oder ein bakterielles Produkt erzeugt wird, sondern er meint die Produktion

Phytoplankter werden hydrolisiert (Itturriaga und Hoppe, 1977) und organisches Material auf Aggregaten (aggregiertes POM und z.B. Fäzes) umgesetzt (Simon *et al.*, 2002). Somit werden Nährstoffe für andere Trophiestufen verfügbar gemacht: zum einen für bacteriovore Protisten (Cilliaten und mixo- und heterotrophe Flagellaten; Azam *et al.*, 1983; Fuhrman und Noble, 1995; Zubkov *et al.*, 1998; Suzuki, 1999), dann durch bakterielles Wachstum, zum anderen für weitere Trophiestufen durch die Lösung anorganischer Nährstoffe, deren Verfügbarkeit dadurch erhöht wird. Damit wird innerhalb des Nahrungsnetzes ein Kreis, der *microbial loop*, geschlossen.

Weitere Einflussfaktoren des Systems sind Viren, die durch ihre Lyse von Bakterioplankton und Phytoplankton einen erheblichen Anteil zum DOM-Pool beitragen (Proctor und Fuhrman, 1990; Fuhrman, 1999).

Das Nahrungsnetz im Benthos ist abhängig von der Bereitstellung von POM durch das pelagische Nahrungsnetz (*sinking flux*, Deming, 1993). Über die Einflussgrößen des benthischen Nahrungsnetzes gibt es nur wenige Studien, die gleichzeitig alle Komponenten betrachten (Epstein, 1997a, 1997b; Dietrich und Arndt, 2000). Beide Netze ähneln sich bezüglich der Einflussfaktoren (Deming, 1993; Dietrich und Arndt, 2000), die Einflussgrößen unterscheiden sich jedoch.

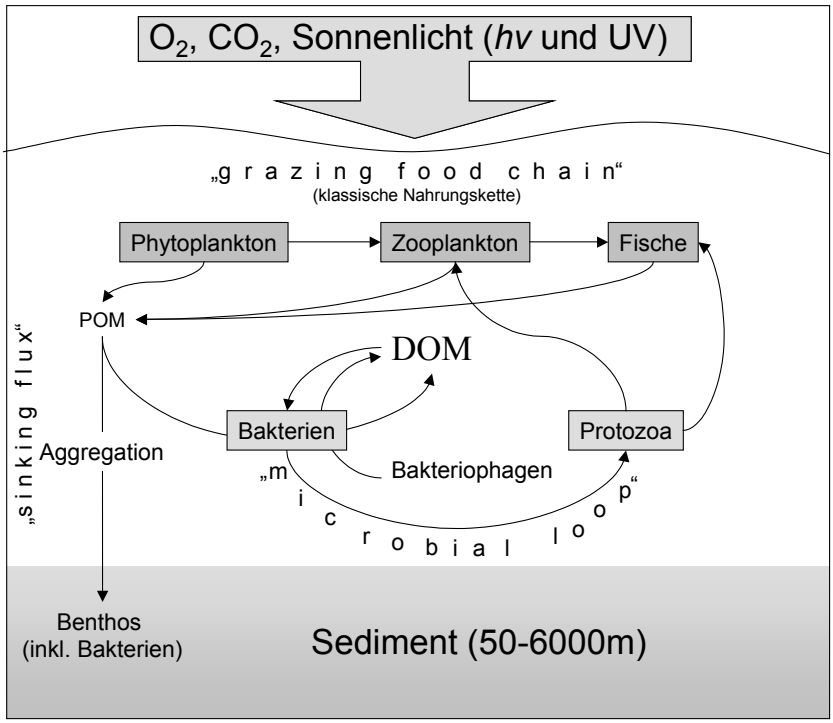

Abb. I.1. Vereinfachte Darstellung des *microbial loop* im pelagischen Nahrungsnetz der euphotischen Zone (nach Azam 1998). DOM = *dissolved organic matter* (gelöstes organisches Material); POM = *particulate organic matter* (partikuläres organisches Material)

Anders als im Pelagial scheinen bakteriovore Protisten im Sediment nur geringe Auswirkungen auf die bakterielle Produktion zu haben (Kemp, 1988; Epstein, 1997a; Wieltschnig *et al.*, 2003). Bakterielle Produktionsraten und Verdopplungszeiten haben in beiden Systemen die gleiche Größenordnung. Trotzdem bleibt letztendlich ungeklärt, inwieweit der *microbial loop* des Benthos dem des Pelagials ähnelt und ob er eine Quelle oder Senke für organisches Material ist (Bak *et al.*, 1995; Dietrich und Arndt, 2000). Unklar bleibt auch die weitere Rolle bakterieller Produkte und bakterieller Biomasse in diesen Systemen (Wieltschnig *et al.*, 2003).

von Biomasse durch Bakterien. Da der Begriff jedoch allgemein angewandt (vgl. einschlägige Literatur) wird und sich als praktikabel anwendbar herausstellt, wird er hier wie vorgestellt benutzt

I.2 DER KAMPF DER GIGANTEN: KLASSISCH *VS*. MOLEKULAR - EIN WETTSTREIT UM DIE AUFKLÄRUNG DER STRUKTUR DER BAKTERIENGEMEINSCHAFT?

Im vorangegangenem Kapitel wurde die zentrale Rolle pelagischer und benthischer heterotropher Bakteriengemeinschaften in der Stoffumsetzung aquatischer Systeme erläutert. Zur genaueren Untersuchung des Bakterioplanktons wurden verschiedene Studien angefertigt, die sich mit der Bestimmung von Wachstum, Aktvitäten und Stoffumsatzraten in unterschiedlichen marinen Habitaten befassen (Joint und Morris, 1982; Staley und Konopka, 1985; Cole *et al.*, 1988). Bis in die 80er Jahre hinein wurden heterotrophe Bakterien in der ökologischen Mikrobiologie ausschließlich als funktionelle Einheit, als *black box*, gesehen und somit die große phylogenetische und physiologische Vielfalt heterotropher Bakterien außer Acht gelassen. Auf wertvolle Informationen über die Ökologie des Habitates musste infolgedessen verzichtet werden, wie z.B. auf die nähere Bestimmung der „Hauptakteure", d.h. der stoffwechselaktivsten Bakterien im zentralen *microbial loop* (s. Abb. I.1). Durch die Aufklärung der Zusammensetzung der Bakterienpopulation, ihrer Dynamik, ihrer Substratanforderungen und anderer Parameter wie Fraßfeinde und virale Infektion können Rückschlüsse auf die Qualität und Quantität des von Bakterien produzierten gelösten organischen Materials (DOM) und freigesetzter anorganischer Stoffe gezogen werden. Für die Beschreibung eines Habitates und den darin ablaufenden Zusammenhängen ist dies essentiell. Die Anforderungen spezifischer Bakteriengruppen spiegeln also die Besonderheiten des Habitats wider.

Doch die geringe Größe und das relativ einheitliche Aussehen der Individuen von Bakteriengemeinschaften erschweren, anders als bei Makroorganismen in der Botanik oder der Zoologie, deren Erforschung (Sieburth *et al.*, 1978). Die Morphologie von Bakterienzellen ist nur gering variabel (Fuhrman, 2003), sie erscheinen als Kokken, Stäbchen, Spirillen, Vibrionen oder Spirochäten, die manchmal zu Ketten, Päckchen (Sarcinen) oder Zellhaufen zusammengeheftet sind (Schlegel, 1992). Selbst Kulturen auf Agarmedien (Zellkolonien) sind in ihrem Erscheinungsbild für eine verlässliche Identifizierung nicht geeignet, da eine Vielzahl von Faktoren (von der Wahl des Mediums bis hin zur Dicke der Agarschicht) Farbgebung und Kolonieform in erheblichem Maße beeinflussen. Unterschiedliche phylogenetische Gruppen können sich in ihrem Erscheinungsbild ähneln oder sogar gleichen. So wurden Bakterien in den Anfängen zwar auch phänotypisch, vor allem aber physiologisch, nach ihren Stoffwechselleistungen, klassifiziert und identifiziert. Diese dazu auszuführenden Versuche waren meist sehr zeitaufwendig und ohne eine Kultivierung von Reinkulturen im Labor nicht möglich. Zudem war eine Klassifizierung oft ungenau, da in verschiedenen Labors unterschiedliche Versuche ausgeführt und Versuchsergebnisse oft divergent bewertet wurden.

Nach den ersten Isolierungen von ZoBell in den 40er Jahren des 20. Jahrhunderts (Zobell und Upham., 1944) wurde zunächst das von ihm gefundene Gattungsspektrum nur wenig erweitert (Baumann *et al.*, 1972). Man schloss daraus, dass sich nur ein kleiner Teil der im Mikroskop sichtbaren Bakterien aus einem Habitat in Kultur bringen lässt (Jannasch, 1958), was später als *great plate count anomaly* (ugf. „Plattenzellzahlanomalie") bezeichnet wurde (Staley und Konopka, 1985). Hinter diesem nur schwer in einem Wort zu übersetzenden Begriff verbirgt

sich die Tatsache, dass die Anzahl der Bakterienarten im Habitat (*in-situ*) größer ist als die Anzahl der Kolonien, die sie auf festem Medium (Agarplatten) bilden. Bis heute schätzt man die allgemeine (also nicht nur auf Festmedien bezogene) Kultivierungseffizienz auf lediglich um die 1 %[3] der gesamten bakteriellen Gemeinschaft eines Habitates (Amann *et al.*, 1995), nur 26 der mit kultivierungsunabhängigen Methoden postulierten 52 Phyla der Domäne der Bakterien enthalten kultivierte Stämme (Rappé und Giovannoni, 2003). Wagner und seine Mitarbeiter fanden 1993 heraus, dass auch die Abundanz der Bakterien verschiedener Habitate nicht durch ihre Kultivierungshäufigkeit widergespiegelt wird (Wagner *et al.*, 1993). Hierfür gibt es mehrere und vielschichtige Gründe: Zum einen ist es aufwendig, wenn nicht unmöglich, im Labor die Bedingungen natürlicher Habitate zu simulierten (Strömungen, Konkurrenzdruck, Temperatur-, Licht- und Nährstoffschwankungen), so dass viele Organismen unter den habitatfremden Laborbedingungen nicht gedeihen.

Zum anderen können Zellen, die ihre Teilungsfähigkeit verloren haben, abgestorben oder fast abgestorben (*sublethal*; Dodd *et al.*, 1997) sind, zwar zählbar sein, kultivieren kann man sie jedoch natürlich nicht[4] (Bloomfield *et al.*, 1998; Nyström, 2001).

In der Mitte der 80er Jahre wurden molekularbiologischen Methoden für die Anwendung in der mikrobiellen Ökologie entwickelt, anhand derer es möglich war, die Bakterienpopulation *in situ* ohne Kultivierung zu untersuchen (Olsen *et al.*, 1986; Pace *et al.*, 1986). Obschon Zuckerkandl und Pauling schon 1965 über die Nutzung biologischer Moleküle zur Aufklärung der evolutiven Entwicklung berichteten (Zuckerkandl und Pauling, 1965), wurden sie erst Ende der 70er / Anfang der 80er Jahre des letzten Jahrhunderts in weiten Kreisen zur phylogenetischen Einordnung von Prokaryoten genutzt (Fox *et al.*, 1980). Woese und seine Mitarbeiter führten bis 1976 die ribosomale RNA (5S, 16S rRNA) als in genügendem Maße konserviertes Molekül ein, Bakterien voneinander zu unterscheiden (Woese *et al.*, 1976). Ihr Sequenzvergleich ermöglichte eine relativ stabile und sichere Klassifizierung der schon vorhandenen beschriebenen Arten und die Einordnung neuer Bakterien. Mitte bis Ende der 80er Jahre des vorherigen Jahrhunderts fanden diverse molekulare Techniken meist auf 16S rRNA Ebene in der mikrobiellen Ökologie Anwendung. So ist es z.B. möglich, bakterielle DNA direkt aus dem Habitat zu extrahieren und damit die Artenvielfalt („richness“) der Bakterienpopulation anhand von Klonbibliotheken[5] (auch mit dem Gesamtgenom von Bakterien: BAC[6]-libraries (Béjà *et al.*, 2000) oder anhand eines Bandenmusters resultierend aus Denaturierender Gradienten Gelelektrophorese (DGGE, Muyzer *et al.*, 1993) zu untersuchen. Durch Sequenzierung der Klone oder der DGGE Banden können Bakterien der

[3] Bei nährstoffarmen Habitaten zw. 0.001-0.1 % (Kogure *et al.*, 1980; Ferguson and Sunda, 1984), bei nährstoffreichen bis zu 15 % (Wagner *et al.*, 1993). Nur in Einzelfällen wurden höhere Kultivierugseffizienzen auch aus nährstoffarmen Habitaten erreicht (bis 100 % bei Bruns *et al.*, 2002, s. dazu die Diskussion in Kapitel III)

[4] Der für diese Zellen gebräuchliche Begriff *viable but non-culturable* (VNC oder VBNC) ist umstritten, da allein das Faktum, dass ein Organismus (noch) nicht kultiviert ist, einen Stamm oder ein Bakterium noch nicht zu einem generell Unkultivierbaren macht (Barer and Harwood, 1999). So können VNC-Organismen z.B. ihre Teilungsfähigkeit wiedererlangen (Bloomfield *et al.*, 1998; Nyström, 2001). Oft wird stattdessen der Begriff *uncultivated* oder *not yet cultivated* benutzt .

[5] Die Methoden wurden häufig aus anderen Fachgebieten adaptiert, in der Medizin z.B. gab es Klonbibliotheken schon viel früher (1979, für das Gesamtgenom der Maus; Kemp *et al.*, 1979).

[6] BAC = *bacterial artificial chromosome*. Plasmid zum Klonieren >300kb großer DNA-Fragmente (Shizuya *et al.*, 1992)

Umweltprobe identifiziert werden. Dadurch werden neue Sequenzen erhalten, die in der Regel nicht identisch mit denen bereits in den Genbanken hinterlegter kultivierter Organismen waren. Durch die große Anzahl neu hinzugekommener 16S rRNA Sequenzen konnten markierte Sonden entwickelt werden (z.B. mit einem Fluoreszenzfarbstoff bei der Fluoreszenz-*in*-*situ*-Hybridisierung, FISH) und mit denen man spezifische Bakteriengruppen direkt im Habitat nachweisen kann (z.B. Giovannoni *et al.*, 1988; Holben *et al.*, 1988; Stahl *et al.*, 1988; Amann *et al.*, 1990). So erfuhren Phylogenie und mikrobielle Ökologie durch die neuen Methoden einen Aufschwung; eine stabile Einordnung schon beschriebener und neuer Isolate in phylogenetische Stammbäume wurde möglich und das Wissen über die Diversität von Bakterien in verschiedenen Habitaten konnte erheblich erweitert werden. Für eine möglichst realistische Beurteilung eines Habitates ist es unbedingt notwendig, die abundanten Bakterien zu identifizieren[7], ein Ziel, den man mit dem Aufkommen der molekularen Methoden deutlich näher gerückt ist.

So ist auch die Euphorie zu verstehen, mit der dann letztendlich nach einer langen Anlaufzeit die Molekularbiologie Mitte der 90er Jahre des letzten Jahrhunderts enthusiastisch gefeiert wurde (ein gutes Beispiel hierfür ist der Minireview von Olsen *et al.*, 1994). Infolgedessen verschwand der kultivierungsabhängige Ansatz jahrelang (von Ende der 80er bis Ende der 90er Jahre) aus dem Bewusstsein vieler „moderner“ Mikrobiologen. Erst, als sich die Erkenntnis durchsetzte, dass sich über die Sequenz allein keine Aussagen über die Stoffwechselaktivitäten machen lassen, vor allem nicht, wenn der nächste beschriebene verwandte Organismus nur geringe Sequenzähnlichkeit aufweist, wurde wieder über neue Kultivierungsstrategien und damit auch der Erhöhung der Kultivierungseffizienz nachgedacht. Auch hat jede molekularbiologische Methode ihre eigenen systematischen Fehler. Der Vergleich der einzelnen Methoden zeigt z.T. große Abweichungen (z.B. Muyzer *et al.*, 1993; Amann *et al.*, 1995; Suzuki *et al.*, 1997; Head *et al.*, 1998; Cottrell und Kirchman, 2000). Man geht jedoch davon aus, dass die (inhärent unbekannte) *in situ* Gegebenheit durch diesen Vergleich abgeschätzt werden kann.

Seit ZoBell gibt es inzwischen eindeutig verbesserte Kultivierungsansätze, wie z.B. die Verdünnungsreihe bei der abundante Organismen in den höchsten Verdünnungsstufen zu finden sind[8], die Wahl von Medien, deren Zusammensetzung

[7] Hierbei stellt sich die Frage, ob abundante Bakterien auch immer die für das Habitat relevanten darstellen, also den höchsten Stoffumsatz haben, oder ob es sich um abgestorbene oder ruhende Zellen handelt (Zobell, 1946; Bloomfield *et al.*, 1998). Nun ist es so, dass abundante Bakterien offensichtlich einem geringeren Fraßdruck ausgesetzt sind, d.h. entweder einen Fraßschutz (Schleime, Kettenbildung) oder eine hohe Wachstumsrate entwickelten, beides Indizien für ein prosperierenden Stoffumsatz (auch die Bildung von Schleimen setzt einen gute physiologischen Zustand voraus). Abundante Bakterien müssen also gut an das Habitat angepasst sein, was sie zu geeigneten Markern für Rückschlüsse auf das Habitat macht. Nach spezifischen Färbemethoden (DAPI) waren über 90 % aller Zellen in aquatischen Habitaten stoffwechselaktiv (Zweifel and Hagström, 1995).

dem jeweiligen Habitat angepasst sind, bis hin zur Imitierung des Habitats mit Mikrokosmen (Federle *et al.*, 1986; Bordalo, 1993; Chin *et al.*, 1999; Almeida *et al.*, 2001), die Zugabe von Signalmolekülen (Bruns *et al.*, 2002), die Verwendung von Verdünnungsreihen und dem Habitat angenäherte Medien (Selje und Simon, 2004b) u.v.m. Jedoch gibt es bis heute Anhäufungen im phylogenetischen Stammbaum (*cluster*) von molekularbiologisch erhaltenen Sequenzen, in die z.T. keine Sequenz von isolierten Bakterien fällt (SAR202; Giovannoni *et al.*, 1996, SAR 324; Wright *et al.*, 1997, SAR11; Giovannoni *et al.*, 1990, SAR406; Gordon und Giovannoni, 1996)[9].

Inzwischen können mit molekularbiologischen Methoden auch Hinweise über die Physiologie abundanter Bakterien erhalten werden: Eine Kombination aus FISH und Microautoradiographie (MAR), bei der (wie bei FISH) der Anteil verschiedener Bakteriengruppen im Habitat ermittelt werden kann und (wie bei MAR) der Anteil, der ein bestimmtes radioaktiv markiertes Substrat aufnimmt (Lee *et al.*, 1999; Ouverney und Fuhrman, 1999: STARFISH; Cottrell und Kirchman, 2000: MICRO-FISH). Über Substratspektren einzelner phylogenetischer Gruppen ist so eine nähere Bestimmung der „Hauptakteure", d.h. der stoffwechselaktivsten Bakterien im zentralen *microbial loop* möglich (wie am Anfang des Kapitels gefordert).

Auch durch die oben erwähnten BAC-libraries können über das Vorhandensein bestimmter Gene Rückschlüsse auf die physiologischen Fähigkeiten gezogen werden.

Erfolgversprechend ist ein polyphasischer Ansatz, der Kultivierungsansätze und molekularbiologische Herangehensweisen gleichermaßen einbezieht und immer häufiger zur Untersuchung der Bakteriengemeinschaft, aber auch zur Charakterisierung neuer Isolate gewählt wird (Spring *et al.*, 1996; Vancanneyt *et al.*, 1996; Bowman, 1997; Brinkhoff und Muyzer, 1997; Heuer, 1997 Brinkhoff *et al.*, 1998; Wang *et al.*, 1999; Ivanova *et al.*, 2001; Rossello-Mora und Amann, 2001; Yoon *et al.*, 2001; Young, 2001; Gillis *et al.*, 2002; Kapitel V).

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[8] Eine spezifische Anwendung der Verdünnungsreihen stellt die Methode der größten wahrscheinlichen Zahl (*most probable number*, MPN) dar, die zunächst zur Quantifizierung spezifischer physiologischer Bakteriengruppen (Trolldenier, 1993; Brinkhoff *et al.*, 1998; Sievert *et al.*, 1999), dann aber auch zur Bestimmung der Gesamtkeimzahl in natürlichen Habitaten genutzt wurde (Bruns *et al.*, 2002; Kapitel III) entwickelt wurde. Dabei wurden mehrere parallele Verdünnungsreihen mit (meist) spezifischen Medien unter bestimmten Bedingungen inkubiert und statistisch über die Höhe der Verdünnungsstufe mit positivem Wachstum die wahrscheinliche Zahl von Bakterien dieser Gruppe innerhalb bestimmter Grenzen (meist 90 oder 95 % Konfidenzintervall) bestimmt. Hinsichtlich von Kultivierungserfolgen abundanter Organismen wird die MPN Methode sehr unterschiedlich beurteilt. Bei Bruns *et al.*, 2001 wurden kaum, bei Selje et al. wurde hingegen einige abundante Vertreter isoliert (Selje and Simon, 2004b). Ein Grund für diese Diskrepanz kann in der Wahl unterschiedlicher Medien und Inkubationsbedingungen oder am Volumen des Inokulums liegen (Bruns et al haben die Micro-Drop Methode angewandt (Kulturvolumen von 20µl, Selje et al eher den klassischen Ansatz mit einem Kulturvolumen von 10ml verfolgt.

[9] Für einige *cluster* gibt es heute schon einige Isolate: die Isolate, die in das SAR 11 *cluster* fällt, gab es erst 12 Jahre später (Rappé *et al.*, 2002), für *cluster* OM43, SAR92, and OM60/OM241 wurden einige Isolate mit einem Isolierungskonzept gewonnen, das hohe Durchsatzzahlen zum Prinzip hat (*high-troughput isolation*; Connon and Giovannoni, 2002).

I.3 DAS UNTERSUCHUNGSGEBIET: DAS WATTENMEER DER SÜDLICHEN NORDSEE

Geographie und Entstehung

Wattenmeere sind von den Gezeiten geprägte Naturräume, die in Mauritanien, Bangladesh, Australien, Süd-Korea und auch in der südlichen Nordsee vorkommen. Das Wattenmeer der südlichen Nordsee ist mit fast 7500 km^2 die größte zusammenhängende Wattfläche der Welt und reicht mit einer Gesamtlänge von 500km von Esbjerg (Dänemark) bis Den Helder (Niederlande). Der deutsche Teil der Nordseewattlandschaft umfasst das schleswig-holsteinische, das hamburgische und das niedersächsische Wattenmeer mit einer Fläche von 1750 km^2.

Der Begriff Wattenmeer ist (noch) nicht eindeutig definiert. Aufgrund des Gebietes, das durch den Nationalpark Niedersächsisches Wattenmeer ausgewiesen wurde, sind folgende Gebiete einbezogen:

- das Watt mit dem Rinnensystem: Priele, Baljen, Seegats.
- die Salzwiesen.
- die Inseln mit den Dünen, dem Strand sowie dem Vorstrand bis 10 m Wassertiefe.

Das Nordseewatt umschließt also den schmalen Streifen sogenannter mariner oder "offener" Watten, die vor den vorgelagerten Inseln liegen und den gesamten Raum zwischen der Festlandslinie (Innenküste) und den Inseln (Außenküste), den man als Rückseitenwatt bezeichnet, einschließlich der Brackwasserwatten der Flußästuare (z.B. Ems, Weser und Elbe) und der Buchtwatten in Dollart, Leybucht und Jadebusen (Abb. I.3.).

Das Wattenmeer der südlichen Nordsee verdankt seine Entstehung in erster Linie dem Anstieg des Meeresspiegels nach der letzten Eiszeit, zu der große Flächen des Nordseebodens durch Bindung des Wassers in Eis und zusätzlichen Abfluß des Wassers trockenfielen (Figge, 1980; Streif, 1990). Zu Beginn des Holozän (zur Warmzeit vor 10.000 Jahren) wurden diese Flächen insbesondere bei Sturmfluten wiederholt überflutet (Eisma *et al.*, 1981). Durch sukzessive Absedimentation von Tonen und Silten währen der Überflutungen entstanden Flachwasserzonen, die bei Ebbe trocken fallen - das Watt.

Natürlich wurde die Küstenlinie noch durch weiteren Meeresspiegelanstieg und Ablagerungen von Torfen und Brackwasserablagerungen modifiziert, so dass die heutige Küstenlinie erst vor ca. 7500 Jahren entstand (Streif, 1999).

Physico-chemische Besonderheiten

Zeitlich und räumlich begrenzte Untersuchungen (mehrere Stunden bis Tage und ein paar Meter bis Kilometer) im Wattenmeer ergaben große Schwankungen in den biotischen und abiotischen Faktoren, diese hohe temporäre und räumliche Variabilität stellen das Kennzeichen des Habitats dar (Dittmann, 1999). Die Hydrodynamik ist hierbei das wohl das augenfälligste Merkmal. Durch die Gezeiten werden große Flächen des Nordsee-Wattenmeeres einem ca. 12-h-Zyklus von Überflutung und Trockenfallen unterworfen.

Abb. I.3. Geographische Übersicht über den Wattenmeerraum der südlichen Nordsee (verändert nach www.nationalpark-hamburgisches-wattenmeer.de)

Die dabei auftretenden Fließgeschwindigkeiten betragen 1,8 – 7,2 km/h, wobei die höchsten Geschwindigkeiten in den Prielen zu messen sind. Der Salzgehalt beträgt im Schnitt 25-30 psu, durch hohe Niederschläge kann aufgrund der geringen Tiefe der Wassersäule der Verdünnungseffekt sehr hoch sein, so dass der Salzgehalt auf 20 psu (vgl. Kapitel II) bis 10 psu absinken kann. Man kann Wattenmeersedimente nach ihrer Korngröße in Sandwatt (durchschnittliche Korngröße von über 0.1 mm), Mischwatt (Übergangsgebiet; Korngröße liegt zw. 0.06 mm und 0.1 mm) und Schlickwatt (Korngröße < 0.06 mm) unterscheiden[10], wobei die Sauerstoffverfügbarkeit im grobkörnigem Sandwatt der größeren Eindringtiefe wegen (die Interstitialräume in grobkörnigen Sedimenten sind größer) höher ist. Betrachtet man jedoch Zeiträume über mehrere Jahre oder Jahrzehnte und das Wattensystem als Ganzes, lässt sich das Wattenmeer als ein konstantes Ökosystem beschreiben, vergrößert man den Fokus um weitere Jahrzehnte und nimmt das gesamte Watt als Beobachtungseinheit kann man sogar von einem persistenten Habitat sprechen (Grimm *et al.*, 1999).

Extreme Ereignisse, z.B. Stürme, haben kurzzeitig große Auswirkung auf das Wattenmeer (Nehls *et al.*, 1998, vgl Kapitel II) Gründe hierfür ist wohl die geringe Tiefe der Wassersäule über weite zeitliche und räumliche Strecken, die z.B.

[10] Auch andere Unterscheidungen sind möglich, z.B. nach dem Bewuchs in Quellerwatt, auf Miesmuschelbänken und Muschelschillfeldern oder nach den Torf- und Kleigehalten.

klimatische Veränderungen in geringerem Maße ausgleicht als ein gewaltigerer Wasserkörper. Dies geht einher mit der starken Hydrodynamik, die große Resuspensions- und Sedimentatonsereignisse (van Leussen, 1996; Jago *et al.*, 2002) und Ströme gelöster organischer (Poremba *et al.*, 1999a) und anorganischer Stoffe bewirkt (Asmus *et al.*, 1998). Aber dieser Dynamik, die über die Gezeiten regelmäßig auftritt, ist wahrscheinlich auch die hohe Belastbarkeit des Habitats bei extremen Ereignissen zuzuschreiben. Die Belastbarkeit eines Habitats bezeichnet dessen Rückkehr zum Ursprungszustand oder zur Ursprungsdynamik nach einer Störung. Organismen in diesem Habitat zeichnen sich durch hohe taxonomische und funktionelle Diversität, Mobilität und meist hohe Wachstumsraten aus. Benthische Organismen begegnen extremen Störungen mit Anpassung durch ihre Physiologie (*resilience in-situ*) oder mit vertikaler oder horizontaler Migration (*resilience by migration*; Dittmann und Grimm, 1999).

Die Problematik, die jedem Stabilitätskonzept zugrunde liegt, sollte man hier nicht aus den Augen verlieren. Zum einen kann „Stabilität" nicht als objektiv messbares Ereignis wahrgenommen werden, sondern definiert sich immer nur über den Vergleich mit anderen „nicht stabilen" Ereignissen und (in der Ökologie) unterschiedlichen Habitaten. Dazu kommt, dass zur Erfassung von „Stabilität" je nach ökologischer Fragestellung und zu untersuchendem Gebiet unterschiedliche Parameter zur Bestimmung von „Stabilität" gemessen und herangezogen werden und es somit zur Festlegung unterschiedlichster Maßgaben kommt (Connell und Sousa, 1983; Williamson, 1987; Pimm, 1991; Grimm, 1996; Gigon und Grimm, 1997).

Tabelle 1: Übersicht über die wichtigsten Stabilitätskonzepte und –eigenschaften in der Ökologie (nach Grimm *et al.*, 1992, 1996).

Stabilitäts-Eigenschaften	Definition
Konstanz	im Wesentlichen unverändert bleiben
Resistenz	im Wesentlichen unverändert bleiben trotz des Vorkommens von Störungen
Beständigkeit	zum Ausgangszustand oder Ausgangsdynamik zurückkehren nach einer temporären Störung
Elastizität	Geschwindigkeit mit der nach einer temporären Störung zum Ausgangszustand oder zur Ausgangsdynamik zurückgekehrt wird
Fortdauer	zeitliche Fortdauer eines Ökologischen Systems

Makroskopische und Mikroskopische Besiedlung

(an Wattenmeere sind aufgrund ihrer hydrodynamischen Beeinflussung, der wechselhaften Wasserverfügbarkeit[11], schwankenden Salzgehalten und (bedingt

[11] Die Wasserverfügbarkeit (= Wasseraktivität, a_w) bezeichnet den Betrag des „freien" oder „verfügbaren" Wassers eines Systems und ist definiert als 1/100 der relativen Feuchte (*relative humidity*, RH) bezogen auf das Substrat. Eine RH von 95% ist also eine Wasserverfügbarkeit von $a_w = 0.95$. Für Bakterien liegt das Optimum bei $a_w = 0.99$, bei einigen Halophilen bei 0.75 (Singleton and Sainsbury, 1999).Der a_w von Meerwasser liegt bei $a_w = 0.98$. Errechnet wird die Wasseraktivität mit $a_w = p/p_0$, mit p = Dampfdruck der Lösung (des Substrates) und p = Dampfdruck von reinem Wasser bei gleicher Temperatur.

durch die Flachheit der Wassersäule) starken klimatischen Schwankungen Naturgebiete mit einzigartigen Lebensgemeinschaften. Große Teile des Nordseewattenmeeres sind mittlerweile zum Nationalpark erklärt worden.

Das Sediment ist an der Oberfläche und in den oberen ca. 10 cm von Muscheln, Wattwürmern (im Sandwatt vorwiegend *Arenicola marina*, im Schlickwatt vorwiegend *Nereis diversicolor*, *N. virens* und *Heteromastus filiforsari*), Schnecken (neben *Litorina littorea* noch *Hydrobia ulvae*, dieWattschnecke, die im Sandwatt zu finden sind) und Krebsen (im Sandwatt die Strandkrabbe, *Carcinus maenas*, im Schlickwatt eher der Schlickkrebs *Corophicum volutator*) besiedelt.

Andere Makroorganismen, die sich nicht oder nur temporär auf den Sedimenten aufhalten sind die Vögel (z.B. der Austernfischer, *Haematopus ostralegus*), Fische (das Watt dient als Aufzuchtstation für z.B. die Scholle, *Pleuronectes platessa* und die Seezunge, *Solea solea*) und der wohl berühmteste makroskopische Faunavertreter *Phoca vituliona*, der Seehund, der auf Sandbänken im Rückseitenwatt z.B. vor Wangerooge und Baltrum zu finden ist. Makrophyten auf der freien Wattfläche sind vor allem der Blasentang *Fucus vesiculosus*, der Meersalat (*Ulva lactuca*) und der Darmtang (*Enteromorpha linza*). An die schließen sich Seegraswiesen (*Zostera noltii*) und Quellerwiesen (*Salicornia europaea*) an. Jedes einzelne dieser Organismen ist speziell an die herrschenden Bedingungen (Salz, Strömungen) angepasst.

Über die Zusammensetzung der Makroorganismen im Wattenmeer gibt es also einige Erkenntnis, über die einiger Mikroorganismen allerdings auch. So gab es über viele Jahrzehnte genaue Bestimmungen der Zusammensetzung pelagischer und benthischer Diatomeengemeinschaften (*Bacillariaceae*, z.B. Niesel, 1997; Niesel und Günther, 1999; vgl. Kapitel II) die mit den *Chlorophyta* zusammen die Primärproduzenten im Watt bilden. Benthische Diatomeen kommen in großer Zahl vor (10^4-10^5 Zellen x l^{-1}, in den Planktonblüten bis zu 1.4 x 10^7 Zellen x l^{-1}) und hoher Diversität (66 verschiedene Gattungen mit 162 verschiedenen Arten (Niesel, 1997; Niesel und Günther, 1999) im Wattenmeer vor und sind als braun-grüner Belag auf Wattenmeersedimenten sichtbar. Cyanobakterien kommen hier nur sporadisch vor (Niesel und Günther, 1999).

Über die Zusammensetzung der häufigsten Mikroorganismen, der Bakterien, gibt es bis heute nur wenige Untersuchungen, obwohl ihre essentielle Rolle in marinen Habitaten, speziell auch im Wattenmeer, schon früh erkannt wurde (vgl. Kapitel II). Die Dynamik der gelösten und partikulären Nährstoffe, an deren Umsetzung vorwiegend Bakterien beteiligt sind, und die der Primärproduktion wurde in verschiedenen Regionen des Wattenmeeres untersucht (Admiraal *et al.*, 1983; van Leussen, 1996; Behrends und Liebezeit, 1999; Tillmann *et al.*, 2000; Wolfstein *et al.*, 2000). Auch die bakterielle Produktion und der Umsatz organischen Materials wurde gemessen (Admiraal *et al.*, 1985; van Duyl und Kop, 1988; Poremba *et al.*, 1999b). Diese Messungen zeigten, dass im Wattenmeer heterotrophe Prozesse über autotrophe dominieren und deuten somit die Relevanz heterotroph lebender Bakterien an. Villebrandt *et al*. (Villbrandt *et al.*, 1999) unterteilen die an den Stoffumsätzen beteiligten Bakterien in physiologische Gruppen, bedauerlicherweise beruhen die meisten ihrer Ergebnisse auf unveröffentlichten Daten, sind also nicht nachvollziehbar. Sie fanden auf Muschelbänken vorwiegend copiotrophe Bakterien hohe Nährstoffkonzentrationen angepasst) und auf Wattflächen als Gesamtpopulation spezialisierter erscheinende oligotrophe Bakterien (an geringe Nährstoffkonzentrationen angepasst).

Frei oder gebunden: die mikroskopische Besiedlung der Wassersäule, der Aggregate und der Sedimente

Schon Anfang der 50er Jahre des letzten Jahrhunderts wurden makoskopische, die Trübung der Wassersäule mariner Habitate verursachende Partikel untersucht[12]. Das langsame Absedimentieren dieses partikulären Materials erinnerte die *in-situ* Beobachter an herunterschwebende Schneeflocken, so dass der Begriff des *marine snow,* „Meeresschnee" geprägt, wurde (Suzuki und Kato, 1953). Heute gelten als *marine snow* fragile, amorphe, makroskopische Partikel mariner Wassersäulen mit einer Größe von ≥0.3 mm (Alldredge und Cox, 1982; Alldredge und Gotschalk, 1988). Diese Partikel bilden sich in der Wassersäule durch Zusammenwirken von physikalischen, chemischen und biologischen Parametern wie Strömungen, Gezeiten, Resuspension von Sedimenten und Verklumpung von Mikroaggregaten (Alber und Valiela, 1994; Turner, 2002) und werden dort auch wieder physikalisch (durch gemeinen Scherkräfte) oder biologisch (durch Mikroorganismen) abgebaut (Smith *et al.*, 1992; Grossart und Plough, 2000; Plough und Grossart, 2000).

Im Gegensatz zu Partikeln, die rein anorganischer Natur sind, setzen sich Aggregate aus allochtonen und autochtonem partikulärem und auch organischem Material zusammen. Dies können lebende als auch abgestorbene Algen, fädige Cyanobakterien, Kotballen (*fecal pellets*) und auch Protozoen sein (Turner, 2002) Die Nährstoffkonzentration eines Aggregates übersteigt die des Umgebungswassers oft um ein Vielfaches (Paerl und Priscu, 1998; Simon *et al.*, 2002). Aggregate bilden also für Bakterien einen Lebensraum mit sehr guter Substratverfügbarkeit, für heterotrophe Bakterien ein *hot spot* von Nährstoffen (Grossart *et al.*, 2004). Andererseits entwickelt sich gerade an nährstoffreichen räumlich begrenzten Stellen starke Konkurrenz um den Platz und die verfügbaren Ressourcen. Aggregate sind also stark mit Bakterien besiedelt (Kiørboe *et al.*, 2002), deren Zellzahl, wie mikroskopische Beobachtungen zeigen, die des Umgebungswassers oft um das 10-100 fache übersteigt. Bakterien tragen auch vornehmlich zum schnellen Abbau der Aggregate bei (Smith *et al.*, 1992). Ihre hydrolytische Aktivität ist um einige Größenordnungen höher als im Umgebungswasser (Kiørboe *et al.*, 2001; Simon *et al.*, 2002) Enzymmessungen unter Verwendung fluorigener Substrate zeigte, dass Aggregat-assoziierte Bakterien mehr Substrate hydrolysieren als sie selber verwerten können, (Smith *et al.*, 1992). sie setzen also hydrolysierte Produkte frei. Aggregate sind somit eine der Hauptquellen gelösten organischen Materials im Wasser pelagialer Habitate.

Doch nicht nur das hohe Nährstoffangebot, die hohe Bakterienabundanz und die (daraus folgende) hohe mikrobielle Aktivität auf Aggregaten unterscheidet sich von der in der Freiwasserfraktion, auch wurden in verschiedenen phylogenetischen Arbeiten Unterschiede in der Artenzusammensetzung der Bakteriengemeinschaft entdeckt (DeLong *et al.*, 1993; Crump *et al.*, 1999; Fandino *et al.*, 2001; Riemann und Winding, 2001). Deshalb wird angenommen, dass Aggregat-assoziierte Bakterien eine eigenständige Population darstellen, die physiologisch an das Habitat angepasst sind. Allerdings existieren auch Studien, die hohe Austauschraten zwischen beiden Habitaten durch Anheftung und Dissoziation beweglicher Bakterien

[12] Später auch die Partikel in der Wassersäule limnischer Systeme. Hier wurde dann der entsprechende Begriff *river snow* und *lake snow* geprägt (Grossart and Simon, 1993; Böckelmann, 2000). Die Zusammensetzung dieser Aggregate ist bezüglich der Größe als auch der Zusammensetzung der besiedelnden Mikroorganismen, sowie des Anteils anorganischen und refraktärem Materials unterschiedlich zu *marine snow* (Zimmermann, 1997; Simon *et al.*, 2002).

prognostizieren (Kiørboe *et al.*, 2001) oder aber keine phylogenetischen Unterschiede zwischen der Bakteriengemeinschaft auf Partikeln und im Umgebungswasser ausmachen können (Hollibaugh *et al.*, 2000).

Das Deutsche Wattenmeer ist im Gegensatz zum offenen Ozean ein heterotrophes System (Admiraal *et al.*, 1985; van Duyl und Kop, 1988; Poremba *et al.*, 1999a) begründet v.a. durch terrestrische, fluviale Nährstoffeinträge und durch Einträge aus der Nordsee. In Kapitel II wird untersucht, ob sich die Populationen der freilebenden und Aggregat-assoziierten Bakterien auch in heterotrophen Systemen deutlich voneinander unterscheiden. In einem so stark hydrodynamisch beeinflussten Küstengebiet wie dem Wattenmeer sind Aggregate großen Scherkräften unterworfen, was einen Austausch mit der Freiwasserfraktion entgegen käme und die geringe Größe (5-200 µm)[13] der dort dominierenden Aggregate erklärt (McCandliss *et al.* 2002; Lunau *et al.*, 2003). Im Wattenmeer spielen zusätzlich Sedimentations- und Resuspensionseffekte eine zentrale Rolle (Jago *et al.*, 2002; van Leussen, 1996; Wainright, 1990, 1997; Milligan, 1995). Zu dem Austausch der freilebenden mit den Aggregat-assoziierten Bakterien könnte, durch Resuspensionsereignisse hervorgerufen, ein weiterer Austausch mit der Bakteriengemeinschaft der oberen Sedimentschichten stattfinden. Allgemein unterscheidet sich die Abundanz und die Zusammensetzung der Bakteriengemeinschaft in marinen Sedimenten erheblich von der der darüberliegenden Wassersäule. Die Sedimentschicht ist in tidalen Habitaten wie dem Deutschen Wattenmeer schon nach wenigen Millimetern anoxisch, während anoxische Bereiche in dem darüberligen Wasserkörper allerhöchstens in Mikronischen auf Aggregaten zu finden sind (Alldredge und Cohen, 1987; Bianchi *et al.*, 1992). So ist eine Ausbildung von unterschiedlichen physiologischen und phylogenetischen Gruppe nicht weiter verwunderlich (z.B. im Sediment das gehäufte Vorkommen vorwiegend anaerob verstoffwechselnder δ-*Proteobakterien*). Ob diese Unterschiede trotz hoher Dynamik auch zwischen den oderen Sedimentschichten und der darüberliegenden Wassersäule im Wattenmeer ausprägen oder ob ein reger Austausch der Sediment-assoziierten Bakterienpopulation mit der darüberliegenden Wassersäule stattfindet, wird in Kapitel II untersucht.

Der Probenahmeort

Unser Probenahmehort liegt im Rückseitenwatt von Spiekeroog, nahe der Küste von Neuharlingersiel (s. Abb. 1 im Kapitel II). Das Watt gehört zu der Kategorie Mischwatt, die Sauerstoffeindringtiefe beträgt 4 mm. In diesem Bereich treten als Makroalgen vorwiegend Braunalgen (*Phaeophyceae*) *Fucus vesiculosus*, auch „Blasentang“ genannt, auf. Der Beprobungssort ist im Westen durch eine Buhne von Wind- und Wasserströmungen weitgehend geschützt. Physikalisch-chemische Parameterschwankungen und ihre jahreszeitlichen Zusammenhänge mit Phytoplankton- und der Bakterienzusammensetzung werden in Kapitel II genauer dargestellt.

[13]Streng genommen wären diese Partikel zu klein für die o.g. Definition. So werden sie bei McCandliss (McCandliss *et al.;*; 2002) als *suspended particles* bezeichnet, bei Simon *et al.* als „Mikroaggregate“(Simon *et al.*; 2002). Da sie u. a. organisches Material enthalten, werden sie hier ebenfalls als „Mikro**aggregate**“ und nicht als „Mikro**partikel“** geführt.

I.4 BIOTECHNOLOGIE – NATURSTOFFE AUS DEM MEER

Die Naturstoffchemie hat sich während der letzten Jahrzehnte kontinuierlich entwickelt. In der Pharmazeutik haben Naturstoffe aufgrund ihrer hohen chemischen Diversität und Bioaktivität gegen verschiedenste Krankheiten und deren Erreger ihren festen Platz (Faulkner, 1998; Zeeck und Bach, 1999; Kelecom, 2002) und haben mit über 34 % der zwischen 1992 und 1995 eingeführten Medikamente ihre Wirksamkeit bewiesen (Zeeck, 1997). Da immer mehr Resistenzen v.a. gegen Antibiotika auftreten und immer neue Krankheitserreger im Zuge der Globalisierung immer weiter verbreitet werden, sind sie mit ihrer strukturellen Diversität willkommene Ergänzungen der einfacheren Produkte der kombinatorischen Chemie (Zeeck, 1997). Im Zuge der intensivierten Suche nach neuen Wirkstoffklassen und Wirkstoffproduzenten erschien der Ansatz, unüblichere als die zuvor meist beprobten terrestrischen Habitate zu untersuchen, erfolgsversprechend. Das Meer war bis in die frühen sechziger Jahre des vergangenen Jahrhunderts ein hinsichtlich biotechnologischer Untersuchungen kaum beachtetes Habitat, aber bis Anfang dieses Jahrhunderts wurden bereits 10000 neue Verbindungen aus diversen marinen Quellen isoliert (Fusetani, 2000), von denen viele pharmakologische Wirkung zeigten. Die bakterizide Wirkung von Meerwasser war bekannt und wurde der Antibiotikaproduktion durch Algen, aber mehr noch den Bakterien zugeschrieben. Da marine Bakterien jedoch als äußerst schwer zu isolieren und kultivierten galten, gab es zunächst vorwiegend Studien über Naturstoffe aus Schwämmem, Algen und Muscheln (Fenical, 1993).

Seit Mitte der 90er Jahre des vergangenen Jahrhunderts wurden immer mehr Ansätze im Bereich der mikrobiellen marinen Naturstoffforschung betrieben (Fenical, 1993; Jensen und Fenical, 1994), da man die Möglichkeit sah, dass hochwirksame pharmakologische Substanzen aus marinen Schwämmen, Algen und Muscheln von ihnen symbiotisch verhafteten Mikroorganismen stammten (Jensen und Fenical, 1996; Kelecom, 2002).

Marine als auch terrestrische Organismen produzieren eine große Vielfalt von Naturstoffen und auch Sekundärmetaboliten[14], wie z.B. Terpene, Steroide, Polyketide, Peptide, Alkaloide und Porphryne (Fenical, 1993). Die meisten der marinen Metabolite haben ihr Gegenstück in terrestrischen Organismen, einige sind jedoch sehr verschieden von diesen (Faulkner, 2000; Jensen und Fenical, 2000), bisweilen so verschieden, dass sie eine eigene Stoffklasse bilden (z.B. marine Sterole).

In den meisten Fällen kennt man die *in-situ* Funktion der Naturstoffe nicht (Vining, 1992), einige dienen als Kommunikationsmittel (Pheromone, Signalstoffe (Kleerebezem *et al.*, 1997; Shaw *et al.*, 1997), als Schreck- oder (sexuelle) Lockstoffe (Demain, 1992; Maplestone *et al.*, 1992), als Antifouling-Schutz, oder als Fraßschutz (Steinberg *et al.*, 1997). Während man bei der Produktion anitiviraler

[14] Die Begriffe Sekundärmetabolite – Naturstoffe werden in der Literatur häufig synonym gebraucht (Maplestone *et al.*, 1992). Der klassische Ausdruck „Sekundärmetabolite“ steht nach der traditionellen Definition der klassischen Mikrobiologie ausschließlich für vorwiegend in der stationären Phase produzierte Metabolite. Aus den meisten Arbeiten wird nicht ersichtlich, in welcher Entwicklungsphase der Metabolit / der Stoff produziert wird. In dieser Arbeit wird der Begriff „Sekundärmetabolit“ ausschießlich im klassischen Sinne gebraucht, sonst der Begriff „Naturstoff“.

oder antibakterieller Substanzen durch Makro- und Mikroorganismen noch einen Zusammenhang mit der *in-situ* Funktionen herstellen kann, scheinen die gefundenen Wirkungen anderer Produkte mit der *in-situ* Funktion wenig gemein zu haben (antitumor und immunosupressive Wirkungen, Neurotoxine, Hepatotoxine, Herzstimulatien).

Daß immer noch weite Bereiche mariner Organismen nicht weiter untersucht werden und sich viele Biotechnologen, v.a. in den finanzorientieren Industrien, eher andere Schwerpunkte setzen, mag zum einen an logistischen Problemen liegen, die Probenahmen in marinen Habitaten oft mit sich bringen (finanzintensive Schifffahrten, Tauchgerät oder andere Probenahmeapparaturen), zum anderen an der geringen Produktausbeute (komplexe Strukturen lassen sich nur bedingt chemisch synthetisieren) und an den Schwierigkeiten marine Organismen zu kultivieren, was früher auch für Mikroorganismen galt, heute aber vor allem immer noch bei der Kultivierung mariner Invertebraten Gültigkeit hat.

Aber die Erforschung neuer Fermentationswege, neuer Kultivierungstechniken und die Erschließung neuer molekularer Wege, Untersuchungseffizienzen zu erhöhen, wird die in den nächsten Jahren unzweifelhaft intensiv betriebene biotechnologische Forschung an marinen Bakterien zu großem Erfolg bringen.

I.5 ZIELSETZUNG DER ARBEIT UND AUSBLICK

Diese Arbeit wurde im Rahmen des niedersachsenweiten Projektes „Marine Biotechnologie“ von der VW-Stiftung finanziert, dessen Schwerpunkt die Erforschung neuer mariner Naturstoffe aus neuen Bakterien bildet. So ergaben sich von vornherein zwei sich unterscheidende jedoch nicht widersprechende Gesichtspunkte, unter denen die Arbeit gefertigt werden sollte: der biotechnologische und der ökologische Gesichtspunkt, wobei zweiter im Rahmen meiner Dissertation den Schwerpunkt bildet.

Das Interesse an neuen pharmakologisch aktiven Naturstoffen aus marinen Quellen ließ das Teilprojekt 5 des o. g. Biotechnologieprojektes entstehen. So sollten in dieser Arbeit vor allem **„neue“ Bakterien isoliert werden**, die, so erhoffte man sich, die Quellen strukturell neuer Naturstoffe bilden. Das Wattenmeer stellt dafür ein geeignetes Habitat dar, da seine ungewöhnliche Hydrodynamik ein hohes Maß an Anpassung der dort lebenden Organismen erfordert und wahrscheinlich eine spezifische, weitgehend unerforschte Bakteriengemeinschaft birgt. Die biotechnologischen Ergebnisse dieser Arbeit werden im **Kapitel VI** dargestellt.

Die ökologische Zielsetzung der vorliegenden Arbeit war eine möglichst umfassende **Beschreibung der Gemeinschaft heterotropher Bakterien** aus unterschiedlichen Kompartimenten (Wassersäule, Aggregate, obere Sedimentschicht bis in die Transitionsschicht) des Wattenmeeres der südlichen Nordsee mit kultivierungsabhängigen (**Kapitel III**) und –unabhängigen Methoden. Damit sollte die Artenvielfalt und die Abundanz kultivierter und unkultivierter Organismen bestimmt werden (**Kapitel V**).

Abundante Arten sollten bestimmt und isoliert werden und durch eine umfassende phylogenetische Analyse aller erhaltenen Sequenzen sollte eine Abschätzung der Spezifität der Bakteriengemeinschaft und eine Identifizierung für das Habitat wichtiger Sequenztypen erfolgen. Ergebnisse aus diesen Ansätzen sind in **Kapitel IV** und **V** vorgestellt.

Die saisonale Auswirkung der Dynamik des Systems Wattenmeer und die primären Einflussfaktoren auf die Zusammensetzung der Bakterienpopulation in den einzelnen Teilhabitaten sollten auf der Basis unterschiedlicher Umweltparameter interpretiert werden (**Kapitel II**).

Die Kapitel II-IV sind bei internationalen wissenschaftlichen Zeitschriften eingereichte Manuskripte, in denen die Abbildungen in den laufenden Text eingefügt wurden. Das erste Manuskript (Kapitel II) wurde bereits nach den Vorschlägen dreier unabhängiger Gutachter überarbeitet. Das Manuskript, dem Kapitel V zugrunde liegt, befindet sich noch in Vorbereitung. Aus diesem Grund wurden Kapitel II – V in Englisch verfasst.

Literaturverzeichnisse für das jeweilige Kapitel sind an dessen Ende zu finden.

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II

COMPOSITION AND SEASONAL DYNAMICS OF FREE-LIVING, AGGREGATE- AND SEDIMENT SURFACE-ASSOCIATED BACTERIAL COMMUNITIES IN THE GERMAN WADDEN SEA

Composition and Seasonal Dynamics of Free-Living, Aggregate- and Sediment Surface-Associated Bacterial Communities in the German Wadden Sea

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KEY WORDS: Bacteria, Phytoplankton, Aggregates, POC, Sediment, DGGE, Wadden Sea

II.1 ABSTRACT

The Wadden Sea is a tidal flat ecosystem at the land-sea interface of the southern North Sea with a high load of suspended matter and nutrients. Despite the general importance of microbial processes the composition of bacterial communities in this ecosystem has been studied only little. Therefore, we investigated the composition of the bacterial communities freely suspended (FL) in the bulk water, associated to suspended aggregates (AG) and to the oxic sediment surface (SE) over an annual cycle (June 1999 to June 2000) in the East Frisian Wadden Sea, applying denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. Wind strength, suspended matter (SPM), particulate organic carbon (POC), chlorophyll, phaeopigments, and the composition of the phytoplankton community were studied as well. Dynamics of these parameters covaried among each other to various extents, but not systematically with that of bacterial numbers and community composition. A cluster analysis revealed that the DGGE banding patterns of the three communities grouped separately even though band overlaps among the communities occurred. Some identical or highly similar phylotypes were found in the FL- and the AG-associated communities, and in the AG- and the SE-associated communities, as shown by sequence analysis of excised bands. Prominent members of each community occurred over most of or during the entire study period. Dominant phylotypes in the FL-, AG- and SE-associated communities affiliated with α-*Proteobacteria* (mainly *Roseobacter* clade) and γ-*Proteobacteria*. Phylotypes affiliating with δ-*Proteobacteria* and *Flavobacteria/Sphingobacteria* of the *Bacteroidetes* phylum were detected occasionally in the AG-associated and SE-associated communities. During certain periods, one phylotype affiliating with *Actinobacteria* and one with β-*Proteobacteria* was detected in the FL-community. The prevalence of the rather stable composition of the communities over time, despite the strong hydrodynamic forcing and pronounced seasonal changes in the phytoplankton community, SPM and POC, indicated that the bacterial communities were adapted to the environmental conditions in this highly dynamic system. The band overlaps between the various communities, however, also reflected that exchange processes between the dissolved and particulate phase and the sediment occurred.

II.2 INTRODUCTION

Today it is well accepted that bacteria play an important role in the flux of energy and biogeochemical cycling of elements in aquatic environments. Until the late 1980ies, bacteria have been treated as a functional entity or best subdivided into functional guilds without considering the taxonomic composition (Pedrós-Alió 1989). Only during the last decade and the advent of appropriate molecular biological methods it became possible to analyze the composition of bacterial communities. Studies in various aquatic environments revealed that marine and freshwater bacterial communities exhibit different features but also that distinct communities exist in aquatic systems of similar environmental properties (Giovannoni & Rappé 2000, Zwart et al. 2002). Even though many reports are available on the composition of bacterial communities in various aquatic ecosystems fewer investigations have been carried out on long-term temporal and seasonal dynamics of the community composition. Such studies have been carried out in a few lakes (Höfle et al. 1999, Glöckner et al. 2000, Zwisler et al. 2003) and in estuarine and coastal ecosystems (Murray et al. 1998, Hollibaugh et al. 2000, Pinhassi & Hagström 2000, Eilers et al. 2001, Bouvier & Del Giorgio 2002, Cottrell & Kirchman 2003, Schauer et al. 2003, Selje & Simon 2003), shedding new light on the role of specific bacterial populations in a given ecosystem. In order to extend our knowledge on the role of aquatic bacterial communities it is important to investigate their composition also in other systems.

Tidal flats are one of the most productive coastal marine ecosystems and occur in tropical as well as temperate regions (Mauritania, Bangladesh, Australia, Korea, southern North Sea). The Wadden Sea is a tidal flat ecosystem with intense hydrodynamic forcing at the land-sea interface of the southern North Sea, highly affected by nutrient and organic matter input from land and the adjacent sea. Dynamics of nutrients, suspended matter, benthic and pelagic algal communities and primary production have been studied in various regions of the Wadden Sea (Admiraal et al. 1983, Van Leussen 1996, Behrends & Liebezeit 1999, Niesel & Günther 1999, Tillmann et al. 2000, Wolfstein et al. 2000). Bacterial production and turnover of organic matter have also been assessed, showing that the Wadden Sea is a net-heterotrophic system and emphasizing the significance of heterotrophic over autotrophic processes (Admiraal et al. 1985, Van Duyl & Kop 1988, Poremba et al. 1999a). Because of the shallow, turbid and highly dynamic nature of the Wadden Sea, free-living as well as aggregate-associated bacteria and those associated with the oxic sediment surface layer are important in the aerobic decomposition of organic matter. Despite the extensive work on microbial processes carried out in the Wadden Sea surprisingly little information is available on the composition of its bacterial communities. Böttcher et al. (2000) and Llobet-Brossa et al. (2002) examined the abundance of sulfate-reducing bacteria in sediments and only Llobet-Brossa et al. (1998) studied the composition of sediment-associated bacterial communities in the Wadden Sea, but only in winter, showing that bacteria of the *Cytophaga/Flavobacteria* cluster (now *Flavobacteria/Sphingobacteria* group of the *Bacteroidetes* phylum; Garrity et al. 2001) dominated at this season. No information, however, is available on the composition of free-living and aggregate-associated bacterial communities in the Wadden Sea.

The aim of our study was to investigate the composition of the free-living, aggregate-associated and sediment-associated bacterial communities in the Wadden Sea. We used denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments as one of the most widely used approaches in this context (Muyzer et al. 1993). Banding patterns were subjected to a cluster analysis and selected bands were sequenced for subsequent phylogenetic analysis.

II.3 MATERIALS AND METHODS

Sampling site and sampling. Samples were collected monthly between April 1999 and June 2000 in the East Frisian Wadden Sea, Germany, at a site protected from westerly winds and currents by a groyne (53° 43' 20" N, 07° 43' 20" E; Fig. 1). This site is representative for the composition of bacterial communities in the water column of this area, the tidal flat system of Spiekeroog Island, as shown by DGGE analyses of water samples of a horizontal survey (T. Brinkhoff and H.P. Grossart, unpubl. results). Sediment cores of the mixed sand/mud flat were taken with Plexiglass tubes (36 mm diameter) at low tide. Water temperature, salinity, pH and oxygen saturation were measured *in situ* with probes LF 196, pH 192 and OXI 196 (all WTW, Weilheim, Germany), respectively. Wind data were obtained from Deutscher Wetterdienst, Hamburg. Water samples were collected at high tide using black 10-l plastic jugs rinsed with sea water. For bacteria and phytoplankton cell counts subsamples were withdrawn from the jugs immediately, fixed with 2 % (bacteria) and 4 % (algae) Formalin. Samples were brought to the lab in a cooling box on ice and processed further. From the sediment core the top 2 mm layer was sliced and transferred into a 2 ml Eppendorf cap. To separate aggregate-associated and free-living bacteria water samples were size-fractionated by serial filtration of 100 to 250 ml through 5 µm and 0.2 µm Nuclepore filters (47 mm diameter). Filters and sediment samples were stored and kept frozen at –80 °C until DNA-extraction.

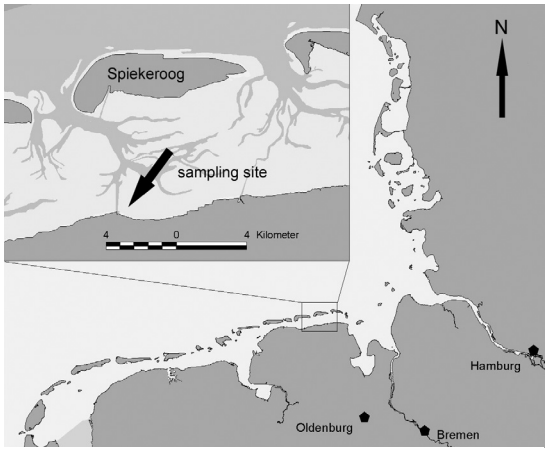

Fig. 1: Map of the study area in the German Wadden Sea, southern North Sea

Enumeration of bacteria and phytoplankton. Total bacteria in the water samples were stained by 4,6-diamidino phenyl indole (DAPI), counterstained by acridine–orange (Crump et al. 1998) and enumerated in duplicates by epifluorescence microscopy on black 0.2 µm Nuclepore filters (Porter & Feig 1980). Phytoplankton cells were counted by inverted microscopy (Utermöhl 1958). Depending on the sediment load, 3 to 10 replicates per sample were counted. Identification of phytoplankton species was done according to Drebes (1974). For estimating phytoplankton biomass cell numbers were multiplied by cell carbon. The latter was estimated from measured cell sizes of individual species converted to carbon according to empirical carbon/cell volume conversion factors from the Biologische Anstalt Helgoland (J. Berg unpubl. results).

Suspended matter, particulate carbon and chlorophyll. For suspended matter (SPM) and particulate carbon analysis 200 to 500 ml of seawater were filtered onto pre-weighed combusted (550 °C, 2 hours) Whatman GF/F filters immediately after return to the lab and kept frozen at –20 °C in the dark until further processing. Filters were not rinsed by deionized water for salt removal to avoid any loss of particulate organic material but dry weight (DW) was corrected for salt by a newly developed method, which subtracts the amount of salt retained in the filter according to the ambient salinity and the pore volume of the filter (Lunau et al. 2003). Dry weight of SPM was determined on pre-weighed GF/F filters after drying at 110 °C for 1 h. Total particulate carbon (PC) was determined by a coulometric method using a Coulomat 702-SO instrument (Ströhlein Instruments, Viersen, Germany) according to Babu et al. (1999). Samples were combusted at 1350 °C and titrated against $Ba(ClO_4)_2$. Particulate inorganic carbon (PIC) was also determined by a coulometric method (Coulometrics 5012, UIC Inc., Joliet, USA). Prior to combustion at 950 °C inorganic carbon of the samples was removed by treatment with 2 M perchloric acid. CO_2 produced was titrated against $Ba(ClO_4)_2$. Particulate organic carbon (POC) was determined as the difference between PC and PIC. For chlorophyll analysis 200 to 500 ml of seawater were filtered onto a Whatman GF/F filter immediately after return to the lab and kept frozen at –20 °C in the dark until further processing. Filters were extracted at 75 °C in 90 % ethanol and concentrations of chlorophyll *a* (Chl *a*) were determined by standard procedures (Parsons et al. 1984). Phaeopigment concentrations were measured after acidification.

DNA extraction. Bacterial genomic DNA of filtered water and sediment samples was isolated after bead beating, phenol-chloroform extraction, and isopropanol precipitation as described previously (Stahl et al. 1988, Mac Gregor et al. 1997) but slightly modified. Treatment by sodium-dodecyl-sulfate (SDS, 1.75 % of a 25 % solution) was applied instead of lysozyme, precipitation done at –20 °C and molecular grade water (Eppendorf, Hamburg, Germany) was used for resuspension at 4 °C over night. The DNA yield was 5-60 ng μl^{-1} as estimated by a DNA ladder (Low DNA Mass™ Ladder; Gibco, USA) .

PCR amplification of 16S rRNA gene fragments. Primers GM5F (341F) and 907R (Muyzer et al. 1998) and the RedTaq® Polymerase (1unit μl^{-1}; Sigma, Germany) were used to amplify 550-bp fragments of 16S rRNA genes. At the 5´-end of the GM5F primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added for DGGE analysis (Muyzer et al. 1993). A "touch-down" PCR (Don et al. 1991) was performed (67 °C – 55 °C, step-down 1 °C every second cycle) as described (Brinkhoff & Muyzer 1997). Altogether the PCR program included 26 cycles. Aliquots (4 µl) of the amplicons were analyzed in 2 % (w/v) agarose gels and stained with ethidium bromide (1 $\mu g\ ml^{-1}$) (Sambrook et al. 1989).
For reamplifying the fragments the primer GM5F was used without clamp, annealing temperature was 55 °C without touchdown.

DGGE analysis of PCR products. DGGE was performed as described (Brinkhoff & Muyzer 1997) with the D-Code system (Bio-Rad Laboratories, Hercules, USA). Briefly, 1 mm-thick, 6 % (w/v) polyacrylamide gels, 1 x TAE electrophoresis buffer (40 mM), Tris-HCl (pH 8.3), 20 mM acetic acid, 1 mM EDTA, 20 to 70 % denaturant, and an electrophoresis time of 20 h at a constant voltage of 100 V and a constant temperature of 60 °C were used. The gels were stained with SYBR Gold (Molecular Probes, Eugene, USA) and documented by a BioDoc Analyze Transilluminator (Biometra, Göttingen, Germany). Numbers of bands per lane were

determined on various DGGE images with different exposure times such that all bands were detected. Here we present images for the three communities with the highest numbers of visible bands in a given image. Analysis of the DGGE banding patterns was done by a cluster analysis with the software package GelCompar II, version 2.5 (Applied Maths, St-Martens-Latem, Belgium). We applied 5 to 20 % background subtraction depending on the signal-to-noise ratio of the gel. Patterns were compared curve-based using Pearson correlation as similarity coefficient and UPGMA (unpaired group method of analysis) to generate the dendrogram. We used the position tolerance optimization option of the software to fit the curves to the best possible matching. From the given cophenetic correlation values and the similarity values for the internal standards the most likely result was chosen. We used the curve-based approach instead of comparing single bands because Ferrari & Hollibaugh (1999) and Selje & Simon (2003) showed this analysis to be more robust. Twenty four bands of interest, i.e. persisting and dominant bands, and those occurring only seasonally or once, were excised from the gel by a scalpel, and the DNA was eluted into 50 µl molecular grade water (Eppendorf, Hamburg, Germany) by incubation at 4 °C overnight. The fragments were amplified using PCR conditions as described above. Purity of the bands and their migration behavior were checked by DGGE. For subsequent sequence analysis PCR products of DGGE bands were purified by using the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, USA).

Cloning. Sixteen DGGE bands which could not be directly sequenced (FL-2, -3, -5, -6, -7; AG-4, -5, -6, -7, -9; SE-1, -2, -3, -4, -5, -7) were first cloned using the pGEM®-T Vector System II (Promega, Madison, USA) according to the technical manual of the manufacturer. Clones with an insert were picked (ten for each band to be sequenced) with sterile toothpicks and directly transferred into a PCR–reaction mix. Amplicon length was checked on agarose gel after a PCR with primers pUC/M13 forward and pUC/M13 reverse (Messing 1983) without touchdown (annealing temperature of 48 °C). Finally, DGGE migration behavior of 5 clones was examined after PCR amplification with primers as described for DGGE analysis. Only clones with an insert of the correct length and with the same migration behavior as the original sample in a DGGE gel were sequenced, one of each band cloned.

Sequencing and phylogenetic analysis. DGGE bands and clones were sequenced using the DYEnamic direct cycle sequencing kit (Amersham Life Science, Little Chalfont, UK) and a Model 4200 Automated DNA Sequencer (LI-COR Inc., Lincoln, Nebraska, USA). Sequencing primers were GM5F (341F) and 907R (Muyzer et al. 1998) labeled with IRDye™800. Sequences without the primer sequence were compared to those in GenBank using the BLAST function of the NCBI server (http://www.ncbi.nlm.nih.gov). The sequences obtained in this study are available from GenBank under accession no. AY274227 to AY274250.

II.4 RESULTS

Physico-chemical parameters

The surface water temperature from June to September 1999 ranged from 17 to 22.5 °C and decreased thereafter until January 2000, reaching a minimum of 3 °C. Until May 2000 it rose again to 16 °C (Fig. 2A). pH and oxygen saturation remained

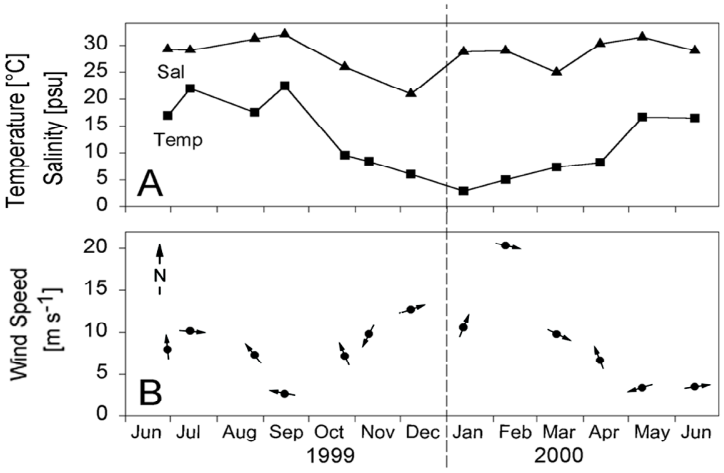

Fig. 2. Physico-chemical data in the German Wadden Sea between June 1999 and June 2000. A: water temperature (Temp), salinity (Sal); B: wind speed. The wind data (mean of twelve hours before sampling) is from Spiekeroog (island 8 km offshore the sampling site). The arrows indicate the wind direction, the dashed line marks the turn of the year.

rather constant throughout the investigation period, ranging between 7.5 and 7.9 and 86 and 95 % respectively. The salinity ranged from 21 to 32 psu (practical salinity units) with lowest values in December 1999 and March 2000 and highest values in September 1999 and May 2000 (Fig. 2A). A T/S plot identified two major water masses, one of the summer-fall period with high salinity and high temperatures, including June to September 1999 and May and June 2000, and another one of the late fall to spring period with low salinity and low temperatures, including October 1999 and January to April 2000 (data not shown). In November 1999 salinity was not measured. In December, rainfall was twice the long-term mean (140-160 mm month^{-1}) resulting in a high run off and thus a reduced salinity. Therefore, the December T/S value did not fit to either of the water masses.

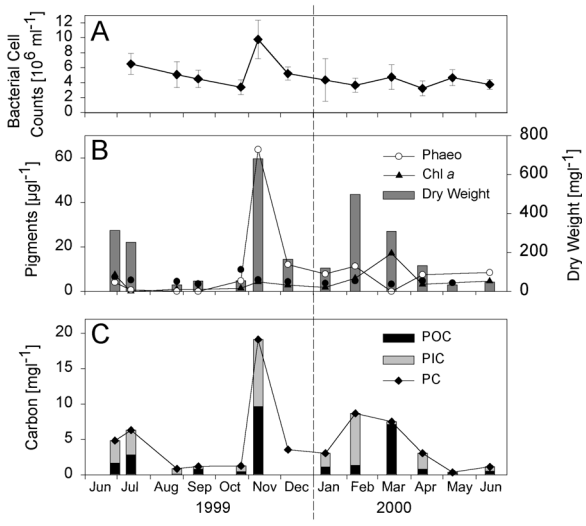

Fig. 3. A: Bacterial cell counts; B: concentrations of pigments (chlorophyll, phaeopigments) and suspended matter dry weight; C: particulate carbon (PC), particulate organic carbon (POC) and particulate inorganic carbon (PIC) in the German Wadden Sea between June 1999 and June 2000. The dashed line marks the turn of the year.

Wind speed varied greatly with lowest values in September 1999 and May 2000 (<5 ms^{-1}) and highest values in February 2000 (>20 ms^{-1}). Wind direction was quite variable but only in November 1999 blew the wind from NNE (Fig. 2B).

Suspended matter and particulate carbon

Suspended matter ranged between 66 and 700 mg DW l^{-1} with lowest values in August and October 1999 and May and June 2000, and highest values in November 1999 and February 2000 (Fig. 3B). Seasonal dynamics of PC, ranging between 0.4 and 19.1 mg C l^{-1}, basically followed those of SPM with lowest and highest values in the same months (Fig. 3C). POC concentrations ranged between 0.13 mg C l^{-1} in August 1999 and 9.7 mg C l^{-1} in November 1999 and comprised always <2.2 % of DW.

Chlorophyll *a*, phytoplankton, and bacterial cell numbers

Chl *a* concentrations ranged from <1 µg l^{-1} in July 1999 to 17.1 µg l^{-1} in March 2000 when a pronounced spring bloom occurred (Fig. 3B). Phaeopigment concentrations exhibited large variations with lowest values near the detection limit (0.2 µg l^{-1}) from July to September 1999 and in March 2000, and enhanced values between October 1999 and February 2000. The maximum of 63.8 µg l^{-1} occurred in November 1999.

Phytoplankton cell counts, determined since August 1999, ranged between 3.9×10^4 cells l^{-1} in August 1999 and 2.5×10^5 cells l^{-1} at the spring bloom in March 2000. Until January 2000 cell counts fluctuated around 5×10^4 cells l^{-1} (Fig. 4A). The enhanced cell number in February 2000 was presumably due to resuspension as indicated by the wind speed and direction (Fig. 2B). Diatoms always dominated the phytoplankton but small unidentified chlorophytes constituted ~4 to 34,8 % (Fig. 4B). Even though most of the phytoplankton algae were determined on the species level, we pooled them for clarity and present data on the genus and higher taxonomical level. Most of the diatoms were typical planktonic forms, e.g. *Chaetoceros* spp., *Guinardia* spp., *Nitzschia* spp., *Odontella* spp., *Rhizosolenia* spp., *Thalassiosira* spp. While *Thalassiosira* spp. were present in all samples other species

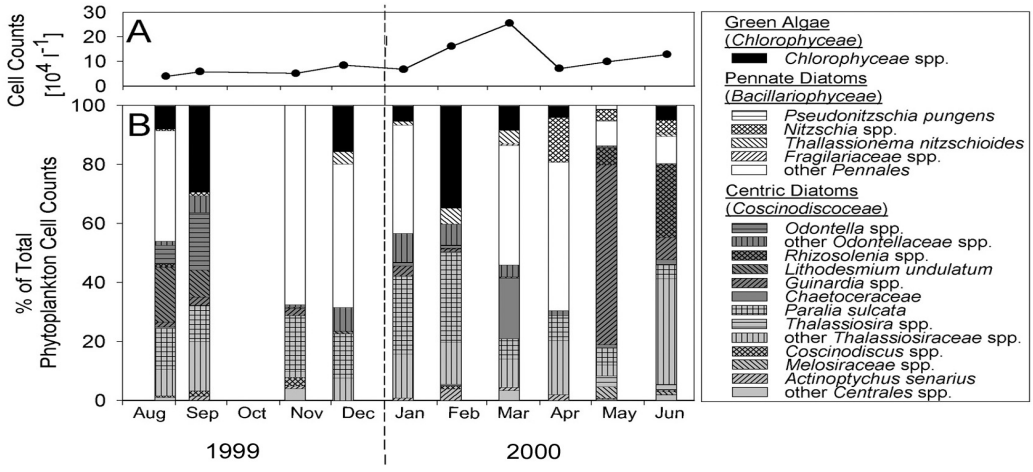

Fig. 4. A: Phytoplankton cell numbers. B: composition (% of total numbers) of the phytoplankton community in the German Wadden Sea between August 1999 and June 2000. The dashed line marks the turn of the year.

occurred with seasonal peaks: *Odontella* spp. in August and September 1999, *Chaetoceros* spp. during the spring bloom in March 2000, *Nitzschia* spp. from April to June 2000 and *Guinardia* spp. and *Rhizosolenia* spp. in May and June 2000. Benthic diatoms, in particular *Paralia sulcata*, constituted always substantial proportions. In August and September 1999, *Lithodesmium undulatum* and *P. sulcata* constituted 32.4 and 21.6 % of total algal cell counts, respectively, and the latter also 31 % in January and February 2000.

Phytoplankton biomass (PhytoC) ranged between 23.3 µg C l^{-1} in April 2000 and 234.5 µg C l^{-1} in November 1999 (data not shown) when the large diatom *Coscinodiscus wailesii* constituted high proportions despite relatively low cell numbers (Fig. 4B). The ratio of PhytoC/POC varied greatly. Lowest values of <3% occurred in January, March and April 2000, whereas in August and September 1999 and in May 2000 31, 63 and 83 % were recorded (data not shown).

Bacterial numbers continuously decreased from July to October 1999 from 6.5×10^6 to 3.4×10^6 cells ml^{-1} (Fig. 3A). After the absolute maximum in November 1999 with 9.7×10^6 cells ml^{-1} numbers remained fairly stable around 4×10^6 cells ml^{-1} without any significant response to POC or phytoplankton dynamics.

Correlation analysis

In order to identify potentially important variables controlling the seasonal dynamics of suspended matter, phytoplankton and bacterial abundance we performed a correlation analysis using the software STATISTICA, (StatSoft, Inc., Tulsa, USA). Data were log-transformed prior to analysis to fit a log-normal distribution. Wind speed was significantly correlated with most fractions of suspended matter such as SPM, PC, and POC (Table 1). Chl *a* was significantly correlated with SPM, PC, POC, and phytoplankton cell numbers. Bacterial numbers did not show any significant correlation for the entire data set. In summer (data of June - September 1999 and May and June 2000) they correlated significantly with phaeopigments, but only four data point are included in this analysis. When cold-water masses prevailed between October 1999 and April 2000, bacterial cell numbers were significantly correlated with POC.

Table 1. Correlation analysis of wind speed (WS), Chl *a*, and bacterial numbers (BN) with water temperature (Temp), suspended matter dry weight (DW), PC, POC, Chl *a*, phaeopigments (Phaeo), phytoplankton cell numbers (PhytoN) and phytoplankton carbon (PhytoC). Pearson product-moment correlation coefficients (r^2) and number of samples (in brackets) are given. Analysis of log-transformed data was done for the entire study period (June 1999 – June 2000), for the warmer month (June - September 1999 May and June 2000) and for the colder month (October 1999 - April 2000). Significant correlations ($p < 0.05$) are shaded grey, # = correlations with $r^2 < 0.45$; X = auto-correlation.

	WS		Temp		DW		PC		POC		Chl *a*		Phaeo		PhytoN	
June 1999 – June 2000																
WS	X		-0.51	(13)	0.66	(13)	0.68	(13)	0.67	(12)	0.54	(11)	#		0.49	(11)
Chl a	0.54	(11)	#		0.63	(11)	0.69	(11)	0.80	(10)	X		#		0.86	(11)
BN	#		#		0.51	(12)	0.46	(12)	0.51	(11)	#		#		#	
June – September 1999 and May - June 2000																
WS	X		#		0.72	(6)	0.78	(6)	0.84	(6)	0.66	(4)	0.52	(5)	#	
Chl a	0.66	(4)	-0.70	(4)	0.51	(4)	0.75	(4)	0.97	(4)	X		0.91	(4)	0.93	(3)
BN	0.84	(5)	0.57	(5)	0.78	(5)	0.64	(5)	0.53	(5)	#		0.94	(4)	#	
October 1999 - April 2000																
WS	X		#		0.47	(7)	#		#		#		#		0.60	(6)
Chl a	#		#		0.66	(7)	0.66	(7)	0.72	(6)	X		0.71	(7)	0.84	(6)
BN	#		#		0.63	(7)	0.73	(7)	0.84	(6)	#		#		#	

Bacterial community structure

The DGGE analysis showed distinct differences between the banding patterns of the free-living, aggregate-associated and sediment surface-associated bacterial communities (Fig. 5). Results for free-living bacteria from December 1999 and January 2000 are not available due to problems with DNA-extraction. Banding patterns of the aggregate-associated community exhibited the highest number of g1999 with a mean of 20±2. The numbers of bands per lane of the sediment-associated community was lowest, varying between 9 bands in January 2000 and 25 bands in March 2000 with a mean of 17±5. This number was significantly lower ($p=0.025$) than that of the aggregate-associated bacterial community, but not statistically different from that of the free-living bacterial community (ANOVA-test).

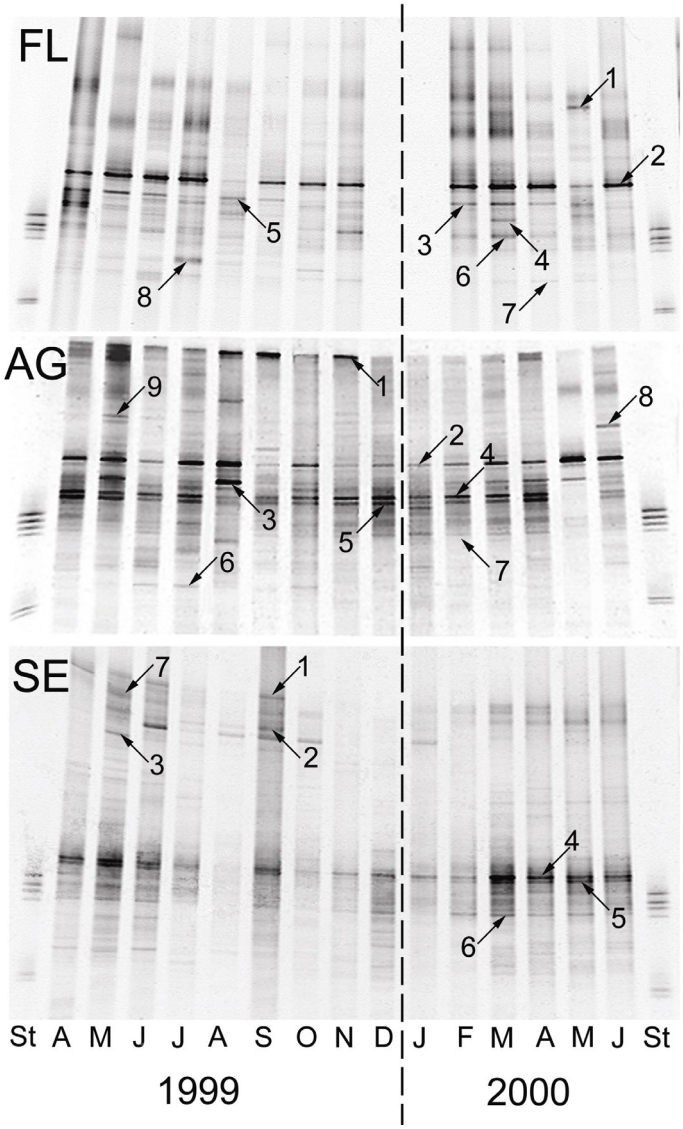

Fig. 5. Inverted images of DGGE banding patterns of PCR amplified 16S rRNA gene fragments of the free-living (FL), aggregate associated (AG) and sediment associated (SE) bacterial communities in the German Wadden Sea between April 1999 and June 2000. The arrows and numbers denote sequenced bands, the dashed line marks the turn of the year. St = Standard.

In all three fractions some bands persisted throughout the entire study period even though with varying intensity, such as bands FL-2 and FL-3 in the free-living bacterial fraction, bands AG-2, AG-4 and AG-5 in the aggregate-associated fraction, and bands SE-4, SE-5, and SE-6 in the sediment surface-associated fraction (Fig. 5). Others occurred only during certain periods or only once. Band FL-6 occurred only between November 1999 and April 2000, band FL-7 between March and May 2000, and band FL-1 only in May 2000. In the aggregate-associated bacterial fraction, band AG-6 was detected between May and July and in October 1999. Bands SE-1, SE-2, SE-3, and SE-7 of the sediment-associated bacterial fraction occurred only between May and September in 1999.

The cluster analysis substantiated that the three fractions constituted different communities (Fig. 6). The sediment-associated community formed a distinct cluster with similarities of >78 % (Pearson correlation) of all samples except of those from August and October 1999. Between March and June 2000 a stable community established as documented by a particularly narrow subcluster for this period. The aggregate-associated and free-living bacterial fractions exhibited different clusters but the subcluster of aggregate-associated bacteria from May 1999 and 2000 and June 2000 and August 1999 was more similar to that of free-living bacterial communities than to that of other aggregate-associated bacteria. In general, the aggregate-associated bacterial fraction was more similar to the sediment-associated than to the free-living fraction. Aggregate-associated bacteria from April 1999 and December 1999 to April 2000 formed a distinct subcluster, thus reflecting the cold-water masses identified by a T/S-plot (see above), but possibly also the fact that band AG-1, identified as a chloroplast sequence (Table 2), was absent during this time. Free-living bacteria from April and August 1999 and May 2000 formed a subcluster more similar to that of aggregate-associated bacteria than to that of other free-living bacteria. The latter formed a well-separated cluster with similarities of >78 %.

Conspicuous bands of all three fractions were excised and sequenced. Eight sequences affiliated with α-*Proteobacteria*, 1 with β-*Proteobacteria*, 6 with γ-*Proteobacteria*, 2 with δ-*Proteobacteria*, 3 with the *Flavobacteria/Sphingobacteria* group, 1 with *Actinobacteria*, and 5 sequences were identified as plastid-like, 3 of them occurring in the sediment surface and one in the free-living and the aggregate-associated community, each (Table 2).

Phylotypes of α-*Proteobacteria* were retrieved only from the fractions of free living and aggregate associated bacteria and constituted 4 of the 8 phylotypes from the free-living bacterial community, including the dominant phylotypes FL-2, FL-3, and FL-5 (Fig. 5). The dominant DGGE band of phylotype AG-2 of the aggregate-associated fraction affiliated also with α-*Proteobacteria* and was identical to FL-2. The sequence of band FL-6 affiliated with β-*Proteobacteria* and represented the only phylotype of this phylogenetic subgroup. Three of the 6 phylotypes affiliating with γ-*Proteobacteria* occurred in the aggregate-associated bacterial community and two of them represented prominent members (AG-4, AG-5). One phylotype from the free-living (FL-7) and 2 of the sediment-associated bacterial fraction affiliated with γ-*Proteobacteria* (SE-4, SE-5). Phylotype SE-5 shows a 99 % sequence similarity to phylotype AG-5. The 2 phylotypes affiliating with δ-*Proteobacteria* of the aggregate-associated (AG-7) and sediment-associated fractions (SE-6) exhibited a sequence similarity of 98 % to each other. Phylotypes affiliating with the *Flavobacteria/Sphingobacteria* group were detected only in the aggregate- and sediment-associated fractions such as in May 1999 and June 2000 (AG-8, AG-9, SE-7, Fig. 5, Table 2)

Table 2. Phylogenetic affiliation of sequences obtained from DGGE bands from samples of the German Wadden Sea taken between April 1999 and June 2000. Given are phylogenetic affiliation, band identification (ID), numbers of base pairs (bp) of the sequence, accession number, date of sampling (date) and sequence similarity to the closest phylotype and described species (type strain) as retrieved by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast). The type species are validly published in the International Journal of Systematic and Evolutionary Microbiology. ID of the bands refer to those indicated in Fig. 4. FL = free living; AG = aggregate associated; SE = sediment associated; ND = not denoted.

Group	Band ID, (bp) acc. number	date	Closest phylotype / closest species (acc. number)	Similarity [%]	Remarks on closest phylotype/relative
Free-living					
Eukarya	GWS-FL-1, (517) AY274227	May 00	uncultured bacterium clone RCP2-13 (AF523902)	97	environmental wetland sample
			algal chloroplast (AF514855)	97	chloroplast of *Haslea wawrikae*;
α-Proteobacteria	GWS-FL-2, (533) AY274228	Jun 00	Uncultured alpha proteobacterium clone DC11-80-2 (AY145625)	99	DGGE band, 10^{-6} dilution culture of the polyhaline section of the Weser estuary
			Ruegeria gelatinovorans (D88523)	94	environmental marine sample
	GWS-FL-3, (522) AY274229	Feb 00	Uncultured alpha proteobacterium clone DC5-80-8 (AY145594)	99	DGGE band, 10^{-5} dilution culture of the polyhaline section of the Weser estuary
			Ruegeria gelatinovorans (D88523)	96	environmental marine sample
	GWS-FL-4, (430) AY274230	Mar 00	*Brevundimonas vesicularis* (AY169433)	99	low temperature anaerobic enrichment of a Greenland ice core sample from 3042 m depth
			Brevundimonas vesicularis (AJ227780)	99	
	GWS-FL-5, (525) AY274231	Aug 99	Uncultured alpha proteobacterium isolate WM11-36 (AF497864)	98	DGGE band, polyhaline section of the Weser estuary, November 1999
			Sulfitobacter pontiacus (Y13155)	96	environmental Black Sea sample
β-Proteobacteria	GWS-FL-6, (223) AY274232	Nov 99	Uncultured beta proteobacterium clone DC11-51-11 (AY145622)	97	DGGE band, 10^{-5} dilution culture of the brackish section of the Weser estuary
			Leptothrix mobilis (X97071)	96	
γ-Proteobacteria	GWS-FL-7, (534) AY274233	Apr 00	*Halomonas* sp. ARD M36 (AB085657)	100	environmental terrestrial sample (Antarctica)
			Halomonas meridiana (AJ306891)	99	
Actinobacteria	GWS-FL-8, (526) AY274234	Jul 99	Uncultured actinobacterium clone SAa03 (AY124414)	99	environmental sample
			Beutenbergia cavernae (Y18378)	92	environmental cave sample
Aggregate-associated					
Eukarya	GWS-AG-1, (533) AY274235	Nov 99	plastid of *Skeletonema pseudocostatum* (X82155)	98	
α-Proteobacteria	GWS-AG-2, (539) AY274236	Feb 00	uncultured alpha-Proteobacterium clone DC11-80-2 (AY145625)	99	DGGE band, 10^{-6} dilution culture of the polyhaline section of the Weser estuary
			Ruegeria gelatinovorans (D88523)	94	environmental marine sample
	GWS-AG-3, (457) AY274237	Aug 99	*Rhodobacteraceae* bacterium TL (AY177716)	94	Isolate from the German Wadden Sea (10^{-5} dilution culture)
			Ruegeria gelatinovorans (D88523)	91	environmental marine sample

Table 2. cont.

Group	ID, (bp) , acc.no.	date	Closest phylotype / closest species (acc. number)	Sim. [%]	Remarks on closest phylotype/relative
γ-Proteobacteria	GWS-AG-4, (545) AY274238	Feb 00	uncultured gamma-Proteobacterium Sva0318 (AJ240989)	97	permanently cold marine sediment
			Alkalilimnicola halodurans (AJ404972)	90	sediments of soda-depositing lake
Cont. Tab. 2.					
	GWS-AG-5, (547) AY274239	Dec 99	Uncultured proteobacterium clone Bol92 (AY193138)	98	marine sediment
			Thiorhodovibrio winogradskyi (AJ006214)	91	cold lake sediment sample
	GWS-AG-6, (544) AY274240	Jul 99	*Nitrosococcus halophilus* (AF287298)	88	
			Nitrosococcus oceani (AF363287)	89	marine sample
δ-*Proteobacteria*	GWS-AG-7, (555) AY274241	Feb 00	uncultured delta-Proteobacterium clone D062 (AF367389)	99	envir. biofilm sample from tidal creek (southeastern US)
			Haliangium tepidum (AB062751)	91	envir. coastal marine sample, moderately halophilic
Bacteroidetes	GWS-AG-8, (539) (AY274242)	Jun 00	uncultured bacterium SB-42-DB (AJ319829)	96	satellite bacterium of *Dytilum brightwellii*
			Chryseobacterium meningosepticum (AF207075)	85	
	GWS-AG-9, 308 AY274243	May 99	uncultured *Cytophagales* QSSC9L-1 (AF170779)	93	Antarctic quartz stone sublithic communities
			Lewinella nigricans (AF039294)	92	marine species
Sediment-associated *Eukarya*	GWS-SE-1, (558) AY274244	Sept 99	plastid of *Skeletonema pseudocostatum* (X82155)	98	
	GWS-SE-2,	Sept 99	uncultured vent bacterium ML-2d (AF208994)	97	shallow submarine hydrothermal vent near Milos island
	(524) AY274245		chloroplast of *Haslea wawrikae* (AF514855)	97	
	GWS-SE-3, (529) AY274246	May 99	chloroplast of *Haslea nipkowii* (AF514850)	99	
γ-Proteobacteria	GWS-SE-4, (548) AY274250	Apr 00	Uncultured bacterium clone s105 (AY171392)	100	environmental marine sediment sample
			Thiorhodovibrio winogradskyi (AJ006214)	90	environmental lake sediment sample
	GWS-SE-5, (548) AY274247	May 00	uncultured proteobacterium clone Bol92 (AY193138)	98	environmental marine sample
			Alkalilimnicola halodurans (AJ404972)	91	sediments of soda-depositing lake
δ-*Proteobacteria*	GWS-SE-6, (515) AY274248	Mar 00	uncultured delta proteobacterium clone Hyd24-27 (AJ535224)	98	marine sediment above hydrate ridge
			Anaeromyxobacter dehalogenans (AF382396)	89	
Bacteroidetes	GWS-SE-7, (419) AY274249	May 99	*Flavobacteriaceae* bacterium T15 (AY177723)	100	German Wadden Sea (10^{-5} dilution culture)
			Tenacibaculum mesophilum (AB032501)	94	marine species

II.5 DISCUSSION

The strong hydrodynamic forcing, rapid restructuring of the composition and size structure of suspended aggregates and intense sedimentation and resuspension processes in shallow and turbid ecosystems like the Wadden Sea have been well studied (Wainright 1990 and 1997, Milligan 1995, Van Leussen 1996, Jago et al. 2002, McCandliss et al. 2002) but not yet how they affect the composition of bacterial communities in such environments. Our results show differences in the banding patterns and number of bands in the free-living, aggregate-associated and sediment-associated bacterial communities, resulting in separate clusters, which prevailed through most of the study period. Obviously, specific bacterial communities persisted in the three habitats, despite the strong hydrodynamic forcing and rapid restructuring of the suspended aggregates. Some phylotypes, however, were found in the free-living as well as in the aggregate-associated, or in the latter as well as in the sediment-associated community. These notions reflect that transitions and exchange processes occurred between the free-living and aggregate-associated and between the sediment-associated and the

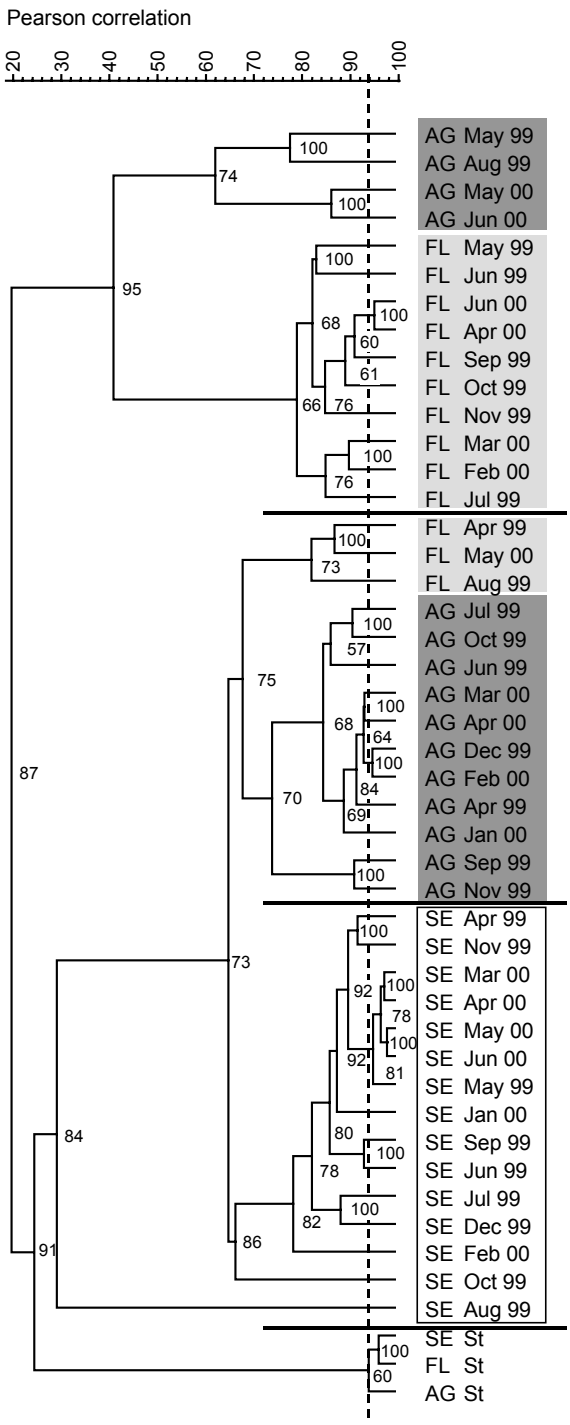

Fig. 6. Curve based cluster analysis (UPGMA = unpaired group method of analysis) of DGGE banding patterns of the free-living (FL), aggregate associated (AG) and sediment associated (SE) bacterial communities in the German Wadden Sea between April 1999 and June 2000. The Pearson coefficient was used to calculate levels of similarity between the patterns of three different DGGE gels. The same standard was used on all gels. Comparison of the standard from the different gels shows the gels being comparable from 93 % of the Pearson correlation (dashed line). The numbers in the branches indicate the calculated cophenetic correlations of each branching, clusters of the various fractions are boxed or shaded gray. FL = free living; AG = aggregate associated; SE = sediment associated, St = standard.

aggregate-associated communities. The fact, that some bands were present only in the free-living and aggregate-associated but not in the sediment-associated community indicates that the aggregates were not just a vehicle between the water column and the sediment surface but that they harbour a specific community with mediating features between the two other communities.

Suspended matter, chlorophyll, algae and bacterial numbers

The wind strength and direction were important in controlling dynamics of suspended matter (SPM, PC, POC), as shown by the correlation analysis and at events like in November 1999 and February 2000. At these times the highest concentrations of SPM and PC were recorded at the highest wind strength (Feb) and a particular situation with even higher resuspension, when the wind blew from NNE over a wide wind fetch (Nov) without any protection by the groyne. The peak in bacterial numbers at this time of low Chl *a* concentration and the extremely high value in phaeopigment concentration also point to a resuspension event. These findings of the significance of wind-induced vertical mixing for controlling SPM dynamics in the Wadden Sea are in line with previous observations (Van Leussen 1996, Joordens et al. 2001, McCandliss et al. 2002). Chlorophyll *a* and phaeopigment concentrations were not significantly correlated to wind strength, SPM or POC, which is not surprising because phytoplankton biomass constituted highly variable proportions of POC. This notion reflects that phytoplankton primary production is not the predominant source of organic matter, i.e. substrate for growth of heterotrophic bacteria, and that other sources contribute substantial and variable amounts.

Bacterial numbers did not show any significant correlation to any parameter for the entire study period, but were significantly correlated to POC during the winter period and to phaeopigments during the summer period, however, only on the basis of four data points. Similar findings of weak or non-existing correlations were reported from other studies, in particular when phytoplankton primary production was not the major substrate source (e.g. Findlay et al. 1991, Poremba et al. 1999c). Our bacterial numbers are well in the range of other studies in the Wadden Sea (Admiraal et al. 1985, Van Duyl & Kop 1988, Grossart et al. 2004). Poremba et al. (1999b) reported lower numbers from the North Frisian Wadden Sea. We want to note that we only assessed total bacterial numbers in the water column and did not differentiate between the free-living and aggregate-associated fraction. Relative proportions of both fractions in the Wadden Sea do vary seasonally with proportions of free-living bacteria of 70-85 % in May and equal proportions of both fractions in November (Simon et al. 2003). Bacterial numbers in Wadden Sea sediments are higher by roughly three orders of magnitude than in the water column (H. Stevens unpubl. data, Llobet-Brossa et al. 1998) and document the completely different substrate and growth conditions in these two compartments.

One of the major factors controlling bacterial growth dynamics is the input of organic matter. In shallow turbid systems like the Wadden Sea with strong hydrodynamic forcing and light limitation organic matter is supplied by primary production of phytoplankton but also by benthic microalgae (De Jonge & van Beusekom 1995, Heip et al. 1995, Tillmann et al. 2000, Jago et al. 2002, de Brower et al. 2003). We found a pronounced seasonal variability of the phytoplankton composition. During and after the spring bloom from March to June, when Chl *a* concentrations peaked and PhytoC constituted high proportions of POC, typical planktonic diatoms also found in the German Bight (*Guinardia* spp., *Chaetoceros*

spp., *Rhizosolenia* spp., *Thalassiosira* spp.) were most abundant, suggesting that organic matter input of phytoplankton origin was relatively high during this period. The major forms we identified were typical for the given season and are in line with previous studies from the same area (Niesel & Günther 1999). The relatively high proportions of benthic algae such as *Paralia sulcata, Lithodesmium undulatum* and other pennate diatoms in August and September 1999 and from November 1999 to February 2000 indicate that during these periods growth of benthic algae and resuspension was important in supplying organic matter to bacteria in the water column. The high phaeopigment concentrations during winter and in April and June 2000 suggest that decaying algae and not only supply of fresh organic matter by primary production was the major source of organic matter during these periods.

Bacterial communities

For the analysis of the structure of the bacterial communities we used DGGE of PCR-amplified 16S rRNA gene fragments and cluster analysis. These methods have been widely used but include limitations and biases such as selective PCR amplifications and detection of target sequences constituting only 1 % or more of the extractable rDNA (e.g. Muyzer et al. 1993, Murray et al. 1996, Cottrell & Kirchman 2000, Fromin et al. 2002). Detection of phylotypes of the *Flavobacteria/Sphingobacteria* group appeared to have been biased particularly. Even though we detected only three phylotypes of this group, AG-8, AG-9 and SE-7 (Fig. 5, Table 2), it has been shown by fluorescence *in situ* hybridisation (FISH) that this group constitutes substantial fractions of all three bacterial communities in the Wadden Sea (Llobet-Brossa et al. 1998, Simon et al. 2003). Further, the application of group-specific primers with DGGE analysis detected several and different phylotypes of this group in the free-living and aggregate-associated bacterial communities in the Wadden Sea (Rink et al. 2003). Hence, our results present a selected suite of phylotypes of the three bacterial communities, biased against the *Flavobacteria/Sphingobacteria* group. Because we applied general primers used in many other DGGE studies as well, our results are well comparable to those studies and nevertheless provide a valuable insight into the composition and temporal dynamics of the bacterial communities in the Wadden Sea.

Cluster analysis is a common method to evaluate DGGE-banding patterns (Hollibaugh et al. 2000, Juck et al. 2000, van der Gucht et al. 2001, Casamayor et al. 2002) but possible biases with respect to the method of analysis and the quality of the gel documentation can not be excluded. According to preliminary analyses and on the basis of other authors (Ferrari & Hollibaugh 1999, Selje & Simon 2003) we assume that the method we chose to evaluate the cluster analysis yields reliable results. The inclusion of the chloroplast sequences (FL-1, AG-1, SE-1, SE-2, SE-3) may have affected our cluster analysis to a certain extent such as the separation of the narrow subcluster of the aggregate-associated bacterial community from December 1999 to April 2000 because band AG-1 was absent during this time (Fig. 5).

The identification of the large clusters of the three bacterial communities by the cluster analysis, however, was not biased because the general banding patterns of these communities exhibited pronounced differences (Fig. 5). These clusters are separated well below the 93 % level of the Pearson correlation, beyond which branching is not significant (Fig. 6).

The sediment surface-associated bacterial community remained most stable over time indicating that this habitat was least affected by changing environmental and

biological conditions. Temporal changes in benthic algal communities, sedimentation and resuspension events obviously were not reflected in pronounced changes in the numbers of detected DGGE bands. Because of our approach of detecting rDNA and not rRNA by rt-PCR we can not rule out that the relative metabolic activity of the phylotypes changed seasonally. Prominent phylotypes detected throughout the study period were identified as two γ-*Proteobacteria* (SE-4, SE-5) and one δ-Proteobacterium (SE-6, Fig. 5, Table 2). Phylotype SE-6 exhibited a sequence similarity of 98 % to a phylotype on aggregates (AG-7) and to one detected above gas hydrates (SE-6, Knittel et al., direct submission to GenBank). δ-*Proteobacteria* have been often found in marine sediments and include sulfate-reducing bacteria and the strictly aerobic and chemoorganotrophic *Myxobacteria* (Llobet-Brossa et al. 1998 and 2002, Iizuka et al. 1998 and 2003, Ravenschlag et al. 1999, Wieringa et al. 2000, Rütters et al. 2002). According to the sequence similarities of the closest relatives our phylotypes belong to the *Myxobacteria*, which appear to be present consistently in the surface sediment and on aggregates. We also detected phylotypes of algal chloroplasts and only one sediment surface-associated phylotype of the *Flavobacteria/Sphingobacteria* group, which occurred in May 1999 (SE-7, Fig. 5, Table 2). It was identical to the sequence of an isolate from the German Wadden Sea obtained from a 10-5 dilution culture (Brinkhoff et al. 2004). The detection of only one phylotype of this group appears surprising because bacteria of this phylogenetic group were found to constitute up to 6 % of total bacteria in surface sediments of the Wadden Sea in December, applying FISH (Llobet-Brossa et al. 1998). We do not think that bacteria of this phylogenetic group, in fact, were of such low significance as our findings imply, but that methodological biases led to this result (see above).

The fact, that we did not detect any β-*Proteobacteria* among the prominent bands in the surface sediment is in line with low numbers of β-*Proteobacteria* reported by Llobet-Brossa et al. (1998).

The composition of the free-living bacterial community exhibited seasonal changes, which however, were not directly related to any environmental or biological variables we assessed. Certain phylotypes, however, occurred only when certain algae were present such as FL-4 during a *Chaetoceros* bloom in March 2000, suggesting that relationships between specific bacteria and algae exist. Tidal dynamics, however, have no effect on the composition of the free-living bacterial community, except that North Sea water may be introduced at high tide, resulting in lower bacterial numbers (Grossart et al. 2004, Simon et al. 2003). The most prominent sequences which occurred throughout or over most of the study period affiliated with the Roseobacter clade of α-*Proteobacteria* and had sequence similarities of 99 % to sequences retrieved from 10^{-5} and 10^{-6} dilution cultures of the polyhaline section of the Weser estuary, located east of the Wadden Sea (FL-2, FL-3, Table 2; Selje & Simon 2004), and to a phylotype from the polyhaline section of the Weser estuary (FL-5, Table 2; Selje & Simon 2003). As phylotypes FL-3 and FL-5 had nearly identical band positions we are unable to decide which one occurred at which time. In the North Sea and the Weser estuary, phylotypes of the Roseobacter clade were reported to be prominent members of the bacterioplankton throughout most of the year, constituting up to 9 % of total bacterial numbers (Eilers et al. 2000, Selje & Simon 2003, Selje et al. 2004). We identified only one γ-proteobacterial phylotype (FL-7) within the free-living bacterial community and only one phylotype affiliating with Actinobacteria (FL-8) and one with β-*Proteobacteria* (FL-6), which, however, occurred only during certain periods. β-*Proteobacteria* are known to be

prominent members of freshwater bacterial communities (Glöckner et al. 1999), and only few phylotypes of this group have been found in brackish and marine waters (Rappé et al. 2000, Selje & Simon 2003 and 2004). FL-6 showed a sequence similarity of 97 % to a phylotype of a 10^{-5} dilution culture from the brackish section of the Weser estuary (Selje & Simon 2004).

In the aggregate-associated bacterial community we detected the highest number of bands and pronounced seasonal changes. Interestingly, this community exhibited overlaps with the free-living and sediment surface-associated communities, indicating that exchange processes with and transitions to both other environments existed and/or that several bacteria were able to prosper in more than one of these habitats. One of the three most prominent phylotypes of the aggregate-associated community detected throughout the study period, AG-2, affiliated with the Roseobacter clade, was identical to a prominent member of the free-living bacterial community (FL-2) and closely related to phylotypes of adjacent water masses (see above). We do not think that this overlap was due to clogging of filters, trapping free-living bacteria also in the aggregate-associated fraction, because cells of this phylotype were also detected on aggregates by a specific FISH probe targeting this phylotype and close relatives (D. Dotschkal, unpubl. results). Phylotype AG-7 detected during most of the study period (September 1999 to April 2000, Fig. 5) and affiliated with δ-*Proteobacteria*, was 98 % similar to SE-6, which was detected consistently on the sediment surface (see above). Two prominent members of the aggregate-associated bacterial community affiliated with γ-*Proteobacteria* (AG-4, AG-5). AG-5 showed a 99 % sequence similarity with SE-5. Phylotypes of γ-*Proteobacteria* were only sporadically detected in the free-living bacterial community (see above), but consistently on the sediment surface. Applying FISH γ-*Proteobacteria* were found to be prominent members of the free-living, aggregate-associated and sediment-associated bacterial communities in the Wadden Sea (Simon et al. 2003, Llobet-Brossa et al. 1998) and the Weser and Elbe estuaries (Selje & Simon 2003, Simon et al. 2002). Possible reasons why we did not detect more sequences of γ-*Proteobacteria* include the fact that we only picked selected bands for sequencing and a high diversity of γ-proteobacterial phylotypes (see above). The detection of phylotypes of the *Flavobacteria/Sphingobacteria* group only at two occasions presumably underestimates the significance of this phylogenetic group on aggregates for reasons discussed above.

Conclusions

Our study complements investigations from other systems, which also showed that several bacteria and bacterial groups are basic constituents of the bacterial communities but that some members only occur during certain or colder periods (Höfle et al. 1999, Glöckner et al. 2000, Pinhassi & Hagström 2000, Eilers et al. 2001, Zwisler et al. 2003). Prominent members occurred over most of or during the entire study period indicating that the basic structure of each community prevailed despite the strong hydrodynamic forcing and intense exchange processes between the dissolved and particulate phase and the sediment, and with the adjacent land and sea. The composition of these communities was not directly related to seasonal dynamics in suspended matter and phytoplankton properties, even though some variations did occur seasonally. In the aggregate-associated community we detected the highest numbers of bands, and overlaps to the other communities existed, indicating its mediating role for bacterial processes in the water column and the sediment surface.

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III

CULTIVABLE BACTERIA FROM BULK WATER, AGGREGATES AND SURFACE SEDIMENTS OF A TIDAL FLAT ECOSYSTEM

Cultivable Bacteria from Bulk Water, Aggregates and Surface Sediments of a Tidal Flat Ecosystem

Running title: Cultivable Bacteria of a Tidal Flat Ecosystem

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III.1 ABSTRACT

In the present study the heterotrophic aerobic bacterial communities of bulk water, suspended aggregates, the oxic layer and the oxic/anoxic transition zone of sediment of a tidal flat ecosystem, the German Wadden Sea, was assessed. We used most-probable-number (MPN) dilution series to enumerate bacteria from the four habitats, able to grow on various substrates, and to obtain isolates from the cultures for subsequent phylogenetic analysis. Substrates were agar-agar, alginate, cellulose, chitin, dried and pestled *Fucus vesiculosus*, Marine Broth 2216, palmitate and starch. MPN counts of bulk water and aggregate samples ranged between 0.18×10^1 and 1.1×10^6 cells per ml, and those of the sediment surface and the transition zone between 0.8×10^1 and 5.1×10^7 cells per gram dry weight (gdw). Marine Broth and *Fucus vesiculosus* yielded highest values of all substrates tested. MPN counts with these substrates corresponded to 2.3 to 5.4 % of DAPI cell counts, and for the *F. vesiculosus* assay of the bulk water sample, even to 32 % of total DAPI cell counts. Phylogenetic analyses revealed that strains of seven phylogenetic classes were isolated, i.e. *Actinobacteria*, *Bacilli*, α- and γ-*Proteobacteria*, *Sphingobacteria*, *Flavobacteria* and *Planctomycetacia*. Between 23 and 44 different strains were isolated from the four habitats analyzed, resulting in a total number of 129 strains. The majority of different strains affiliated with α- (34.8 %) and γ-*Proteobacteria* (24.7 %) and with *Actinobacteria* (25.8 %). Only with agar-agar as substrate organisms of all seven classes were isolated. Within the different phylogenetic classes sequence similarities to described species of ≤90 % were found for six strains or sequence groups, respectively, which affiliate to the γ –*Proteobacteria,* the *Sphingobacteria*, and the *Planctomycetacia*, but, surprisingly, all these strains were obtained from very low MPN dilution steps. Transfer of our isolates for at least five times on substrate amended media was performed to demonstrate that they are not only enrichable, but truly culturable and able to grow on the substrates used for isolation.

III.2 INTRODUCTION

One important aim of microbiology is to isolate bacteria from a given environment to characterize its bacterial community and to identify important and typical members. This approach was first used for marine environments more than 50 years ago (51), and for a long time the main attempt to unveil the diversity of marine bacterial communities and physiological properties of single members (38, 42). Dilution culture techniques, but mainly plate counts with the addition of various substrates have been used to assess the abundance of bacteria growing under the given conditions or to isolate them. The advent of molecular tools to study the diversity of bacterial communities by culture-independent methods, however, revealed that classical methods often failed to isolate relevant bacteria (14). The discovery of this uncharacterized fraction initiated new efforts to enrich and isolate members of these communities and demonstrated the need to refine isolation procedures. Recent studies, in fact, showed that it is possible to isolate bacteria, which are prominent members of the ambient bacterial community or were previously only known from phylotypes (12, 16, 32, 35). Detailed physiological and genetic characterization of relevant members of the bacterial community is only possible with pure cultures, and is important since it helps to elucidate the potential role of these organisms in the transformation and decomposition of organic matter in the environment.

Coastal areas at the land-sea interface, particularly shallow zones with high tidal dynamics, are a sink of organic matter mainly from terrestrial and estuarine areas, but also from the open sea (1). Heterotrophic bacteria play an important role in organic matter decomposition in these systems (1). In the recent past several studies have examined the diversity of bacterial communities or subcommunities in such systems by culture-independent molecular methods in the water column (8, 33, 44) and in sediments (4, 23, 39). Few investigations, however, focused on studying the diversity of bacterial communities by culture-dependent methods. Selected heterotrophic bacteria from the water column in the German Bight of the North Sea were isolated by Eilers et al. (11, 12), and bacteria involved in the sulfur cycle in sediments were isolated by several authors (4, 6, 22, 27, 50). To our knowledge, no investigation has been carried out which aimed to examine the diversity of heterotrophic bacterial communities in coastal areas by culture-dependent methods, and in particular in shallow turbid systems.

Recently we showed by a culture-independent approach that distinct bacterial subcommunities occur in the bulk water, associated to suspended aggregates, and on the sediment surface of the Wadden Sea (44). This tidal flat ecosystem in the southern North Sea ranges from Den Helder, The Netherlands, to Esbjerg, Denmark. The aim of our present study was to characterize the heterotrophic aerobic bacterial communities in the bulk water, associated to suspended aggregates, and in the oxic layer, and the oxic/anoxic transition zone of the sediment of the Wadden Sea by culture-dependent methods. Our approach was to use most-probable-number (MPN) dilution series on various substrates to enumerate bacteria and to obtain isolates from the four habitats listed above for subsequent phylogenetic analysis.

III.3 MATERIALS AND METHODS

Sampling site and sampling. Samples were collected on 25 October 1999 in the East Frisian Wadden Sea, Germany (53° 42' 20" N, 07° 43' 11" E). Sediment cores were taken with Plexiglas tubes (36 mm diameter) four hours before high tide from a mud flat. Water samples were collected three hours before high tide using black 10-liter plastic jugs rinsed with seawater. Subsamples for bacterial cell counts were withdrawn from the jugs immediately and fixed with 2 % formalin. Samples were brought to the lab in a cooling box on ice for further processing. Water temperature, salinity, pH and oxygen saturation were measured in situ by probes LF 196, pH 192 and OXI 196 (all WTW, Weilheim, Germany), respectively. In the sediment the depth of oxygen penetration was determined by microsensor measurements (see below).

Slicing of sediment cores. Sediment cores used for MPN counts and analysis of Chl *a*, particulate organic carbon (POC), particulate inorganic carbon (PIC), particulate carbon (PC), bacterial organic carbon (BOC), dry weight (DW), and total cell counts were sliced by pushing the sediment up in the Plexiglas cylinder. The upper 2 mm (oxic sediment surface) and the following 2 mm (oxic/anoxic transition zone, determined by microsensor measurements) were scraped off with a sharp spatula and stirred in order to obtain an even distribution of cells before it was used for MPN assays. Subsamples used for cell counts were fixed as described (34), and subsamples used for analysis of the other parameters were directly frozen and stored at –80 °C until they were used.

Microsensor measurements. Microsensor measurements of oxygen concentration (37) of a sediment core were performed at 15 °C in a flow cell with aeration and circulation of filtered Wadden Sea water. The oxygen concentration in the flow cell was kept at the air saturation value. Measurements were obtained at a light intensity of 830 µE /m^2 /s. The microsensor, mounted on a micromanipulator, was positioned at the sediment surface by using a dissecting microscope. Profiles were recorded by penetrating the sediment in 100-µm steps with the micromanipulator. The oxygen electrode was calibrated as described previously (37).

Enumeration of bacteria. Bacterial numbers in the water samples were counted in duplicates by epifluorescence microscopy after staining with DAPI (4,6-diamidino phenyl indole) and counterstaining by acridine orange on black 0.2 µm Nuclepore filters (9, 31). Bacterial numbers of sediment samples were enumerated according to Gough and Stahl (15). Briefly, samples were diluted 5000-fold and enumerated in triplicates after DAPI-staining as described above.

Suspended matter, particulate carbon and chlorophyll *a*. Suspended matter and particulate carbon were analyzed as described by Stevens et al. (44). Briefly, 200 to 500 ml of seawater were filtered onto pre-weighed combusted (550 °C) Whatman GF/F filters immediately after return to the lab and kept frozen at –20 °C in the dark until further processing. Total particulate carbon (PC) and particulate inorganic carbon (PIC) were determined by coulometric methods. Particulate organic carbon (POC) was determined as the difference between PC and PIC. Chlorophyll *a* was determined after extraction in 75 °C 100 % ethanol by standard procedures (30).

Most Probable Number (MPN) counts. The MPN technique was applied as described before (48). Triplicate 10-fold dilution series were prepared in 20-ml test tubes for each substrate (see below) and each fraction, i.e. bulk water [BW], aggregates [AG], oxic sediment layer [SE] and the oxic/anoxic transition zone [TZ].

One ml of well-mixed unfiltered seawater and 1 g wet weight (gww) sediment was used for inoculation of 9 ml medium, respectively. For dry weight determination 1 g wet sediment of each zone was dried at 110 °C for one hour and weighed thereafter (= 0.455 g dry weight for the SE, and 0.395 g for the TZ). To separate the aggregates from the surrounding water 150 ml bulk water was centrifuged for 10 min at 3500 rpm, the supernatant was discarded and the pellet washed twice with autoclaved artificial seawater (ASW, see "media and supplements") and subsequently resuspended in 30 ml ASW. One ml of this solution was used for inoculation. Between every dilution step the samples were vigorously shaken on a vortex mixer. Incubation of the MPN cultures was done at 15 °C in the dark for eight weeks and bacterial growth was determined by changes in the OD (660 nm) and microscopically (after DAPI staining). The number of bacteria for each MPN series was determined with a Qbasic computer program (21). The upper and lower 95 % confidence limit was calculated after Cornish and Fischer (7).

Media and supplements. For the MPN dilution series and isolation of bacteria we modified the artificial seawater used by Atlas (3) and amended it with 0.1 % (w/v) source of organic carbon. The composition of the artificial seawater (in gram per liter) was: $NaCl_2$, 18.16; $MgCl_2$ x $6H_2O$, 11.4; KCl, 0.66; $CaCl_2$, 1.47; KBr, 0.09; KH_4Cl, 0.3; NH_4Cl, 0.38; $NaHCO_3$, 0.29. NH_4Cl and $NaHCO_3$, were autoclaved separately (in 20 ml), and added thereafter. One ml of an autoclaved trace elements solution (Na-EDTA, 5.2 g; FeSO x $7H_2O$, 2.1 g; H_3BO_3, 0.03 g; $MnCl_2$ x $4H_2O$, 0.1 g; $CoCl_2$ x $6H_2O$, 0.19 g; $NiCl_2$ x $6H_2O$, 0.024 g; $CuCl_2$ x $2H_2O$, 0.002 g; $ZnSO_4$ x $7H_2O$, 0.144 g; $NaMoO_4$ x $2H_2O$, 0.036 g per liter) and a selenium-wolfram solution (Na_2SeO_3 x $5H_2O$, 0.006 g; Na_2WO_4 x $2H_2O$, 0.008 g, NaOH, 0.4 g per liter), as well as 1 ml per liter of a 0.2 µm-filtered vitamin solution (in 100 ml: 4-aminobenzoe acid, 4 mg; D(+)-biotin, 1 mg; nicotine acid,10 mg; Ca-D(+)-panthotenate, 5 mg; pyridoxine x HCl, 15 mg; thiamine x 2HCl, 10 mg; vitamin B12, 5 mg) were added to the medium. Substrates were agar-agar, alginate, cellulose, chitin, dried and pestled *Fucus vesiculosus*, palmitate and starch. They were either added before autoclaving or autoclaved separately (alginate). *Fucus vesiculosus* was collected from the Wadden Sea coast at the sampling site where it grows copiously on solid surfaces. It was transported directly to the laboratory, dried (70 °C, 3 days), and pestled (particle diameter ~1 mm) prior to addition to the medium. The fatty acids were saponified before being added hot and under vigorous stirring. Chitin was produced from crab shells (Sigma, Germany) as follows: To 20 g crab shells 150 ml ice-cold sulfuric acid (50 %) was added and incubated at 4 °C over night. After filtration on glass fiber filters (Whatman type GF/C) 9 liters of Seralpur® water were added and stirred until the chitin precipitated. Subsequently the chitin was allowed to sink to the bottom and the supernatant was removed. The precipitate was washed in Seralpur® water 3 to 4 times, dried and pestled in a mortar to a whitish powder. The pH of all media was adjusted to 7.2 – 7.4. For agar plates 2 % (w/v) agar was added. Furthermore MPN-dilution series were performed with Marine Broth 2216 (Difco, Germany) as medium following the manufactures instructions.

Isolation of the organisms. Bacteria were isolated from several dilutions of the MPN series in which growth was detected using agar plates prepared with the media described above. Depending on the media used for the MPN counts, agar plates with modified ASW and supplemented with 0.1 % of the various carbon sources or with Marine Agar 2216 (Difco, Germany) were used for isolation. To obtain a broad range of different colonies 100 µl of a liquid culture was spatuled on an agar plate and 20 µl were transferred on a second agar plate and streaked out to single colonies. Colonies showing different morphological features were selected for isolation and

single colonies were transferred at least five times until considered as pure. Isolates were checked for purity by denaturing gradient electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments as described by Teske et al. (47). Isolates showing a single band on the denaturing gradient gel were sequenced thereafter. After isolation, pure cultures were further cultivated on plates with Marine Agar 2216 (Difco, Germany). For long-term storage the cultures were grown in Marine Broth 2216 and stored at –80 °C in 20% (vol/vol) glycerol. Strains isolated more than once were identified by identical or similar colony morphology on Marine Agar, the media and fraction from which they were isolated, the next related sequences according to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/), and by DGGE analysis. The final decision was made by a pairwise sequence alignment with the BioEdit sequence alignment editor version 5.0.1 (17). From strains that were found to be identical and isolated from the same fraction and with the same substrate only one is listed in Tab. 2.

DNA extraction. Bacterial genomic DNA of pure cultures was isolated after bead-beating, phenol-chloroform extraction, and isopropanol precipitation as described previously (25, 43), but modified such that lysozyme treatment was not applied. Precipitation was done at –20 °C and molecular grade water (Eppendorf, Germany) used for resuspension at 4 °C over night.

PCR amplification of 16S rRNA gene fragments. Primers GM3 (8F), GM4 (1498R) (29), and 907RM (28) were used to amplify ~1490-bp or 899-bp long fragments of the 16S rRNA genes of the isolates. PCR amplification was performed as described before (5). Aliquots (4 µl) of the amplicons were analyzed in 2 % (w/v) agarose gels and stained with ethidium bromide (1 µg ml^{-1}) (41). For subsequent sequence analysis PCR products were purified by using the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, USA).

Sequencing and phylogenetic analysis. PCR amplified 16S rRNA genes of the bacterial isolates were sequenced using the DYEnamic direct cycle sequencing kit (Amersham Life Science, Little Chalfont, UK) and a Model 4200 Automated DNA Sequencer (LI-COR Inc., Lincoln, Nebraska, USA). Sequencing primers were GM3 (8F) and GM4 (1498R) (29) and 907RM (28) labeled with IRDye^{TM}800. Sequences of at least 580 bp were determined and compared to those in GenBank using the BLAST function of the NCBI server (http://www.ncbi.nlm.nih.gov).

To avoid overestimation of the diversity of the isolated strains and to exclude possible sequencing errors, within every category we analyzed (substrate, habitat, phylogenetic class) strains were only treated as different and taken into account, when they showed less than 99 % sequence similarity. This led to different total numbers of isolates for the various analyses. All different strains obtained with a single substrate from a habitat are listed in Table 2. Table 3 shows all groups of strains which show sequence similarities of ≥ 99 %. Within group 14 (*Vibrio* sp.) sequence similarity between single sequences was found to be slightly lower than 99 %, however, every sequence showed similarities of ≥ 99 % to at least four of the other sequences of this group.

Phylogenetic analysis. The phylogenetic trees were constructed with the ARB software package (http://www.arb-home.de [24]). Only sequences with more than 1300 bp were considered for a backbone-tree constructed by the maximum likelihood method. Only type strains were used with the following exceptions because no 16S rRNA gene sequence of the type strain was available: *Phyllobacterium myrsinacearum*, D12789; *Hyphomicrobium vulgare*, Y14302; *Cyclobacterium marinum*, M26788. Not validly published species are marked with quotation.

Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA, which cause mistakes in tree topology. Shorter sequences were added afterwards by maximum parsimony using the same filter. For each of the trees sequences from organisms of other classes were used as outgroup.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from GenBank under accession no. AY332093 to AY332221.

III.4 RESULTS

Environmental parameters. The oxygen content of the bulk water was 93 % and the pH value was 7.9. All other determined physico-chemical parameters for the different habitats are listed in Table 1. These values represent a typical situation for fall in the Wadden Sea (44). Compared to the water temperature, temperature of the sediment surface and the transition zone was somewhat lower at low tide, probably due to an air temperature during sampling of ~8 °C. Bacterial cell counts were higher in the sediment samples by two orders of magnitude. Oxygen was found to penetrate into the sediment to a depth of 3 mm.

MPN counts of heterotrophic bacteria. MPN counts of bulk water and aggregate samples ranged between 0.18 x 10^1 and 1.1 x 10^6 cells per ml with generally lower values of aggregate samples (Fig. 1). MPN counts of samples of the sediment surface and the transition zone ranged between 0.8 x 10^1 and 5.1 x 10^7 cells per gdw without systematic differences between the two habitats (Fig. 1). Palmitate and cellulose yielded lowest values, but no MPN counts with these two substrates were determined in the transition zone. Marine Broth and *F. vesiculosus* yielded highest values of all substrates tested. MPN counts with these substrates correspond to 2.3 to 5.4 %, and for the *F. vesiculosus* assay of the bulk water sample even 32 % of total DAPI cell counts. DAPI cell numbers on aggregates were not determined since they are not very reliable and therefore no comparison with MPN counts could be made.

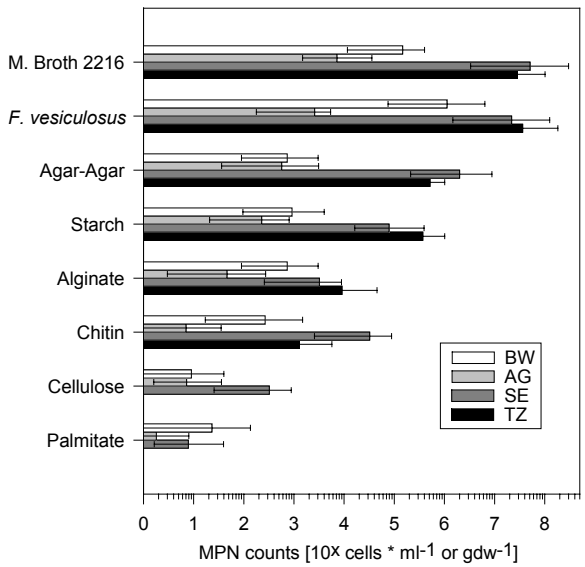


FIG. 1. MPN counts of heterotrophic bacteria from bulk water (BW), aggregates (AG), the oxic layer of the sediment (SE), and the oxic/anoxic transition zone (TZ) of the Wadden Sea in October 1999 with various substrates. Bars indicate 95 % confidence intervals. Cell numbers on aggregates refer to cells on aggregates of 1 ml bulk water. For the TZ no MPN series were performed with cellulose and palmitate.

Diversity and occurrence of isolated strains. Between 23 and 44 different strains were isolated with the various substrates from the four habitats analyzed (Table 2), resulting in a total number of 129 strains. Sequencing of the 16S rRNA genes of these strains revealed between 11 (on starch) and 27 (on chitin) different sequence types on the various substrates (Fig. 2A). The highest number of different strains was obtained from the sediment surface (SE) using chitin (10 isolates) and palmitate (9 isolates), and from the transition zone (TZ) using chitin as substrate (9 isolates). Lower numbers of strains were isolated from the bulk water (BW) and aggregate samples (AG) on most substrates except for BW using cellulose (8 isolates). From two out of 30 MPN series no isolates were obtained, i.e., from the aggregate fraction using alginate as medium and from the transition zone using starch. Overall 89 of the 129 strains showed less than 99 % sequence similarity among each other. Strains falling in groups with similarities of 99 % or higher are listed in Table 3.

Phylogenetic analyses revealed that strains of seven phylogenetic classes were isolated: *Actinobacteria*, *Bacilli*, α- and γ-*Proteobacteria*, *Sphingobacteria*, *Flavobacteria* and *Planctomycetacia* (Fig. 2B). Affiliations of all strains within the different classes are shown in phylogenetic trees (Fig. 5A-E), except for the *Planctomycetacia* for which only one sequence-type was obtained. The majority of different strains affiliated with α- (34.8 %) and γ-*Proteobacteria* (24.7 %) and with *Actinobacteria* (25.8 %). Only with agar-agar as substrate organisms of all seven classes were isolated. With all other substrates bacteria of five (chitin and *F. vesiculosus*) or fewer classes were obtained (least with alginate, starch, and palmitate with n=3). Isolates affiliating to the *Actinobacteria* and α-*Proteobacteria* were obtained with all substrates. γ-*Proteobacteria* were obtained with all substrates except with alginate. The highest percentages of *Actinobacteria* were found with alginate (50 %) and starch (45.5 %), of α-*Proteobacteria* with palmitate (43.8 %) and alginate (40.0 %) (Fig. 2B), and of γ-*Proteobacteria* with chitin and MB 2216 (40.9 and 33.3 %, resp.). Isolates affiliating to other phylogenetic classes were obtained with four or less of the substrates.

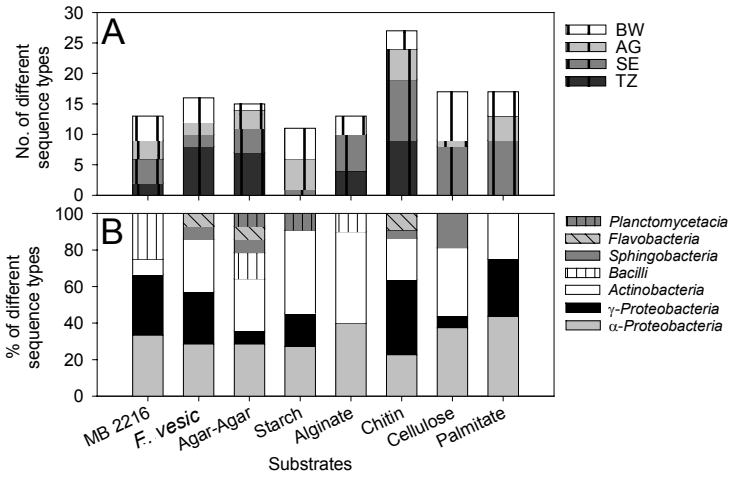


FIG. 2. Number and phylogenetic affiliation of different sequence types obtained from the Wadden Sea in October 1999 by MPN-series with various substrates. (A) Number of different sequence types isolated from the different habitats: bulk water (BW), aggregates (AG), the oxic layer of the sediment (SE), and the oxic/anoxic transition zone (TZ). (B) Percentage of the different sequence types affiliating with various phylogenetic classes. For the TZ no MPN series were performed with cellulose and palmitate.

The highest number of different sequence types was derived from the sediment surface (n=39), comprising organisms of all seven phylogenetic classes (Fig. 3). From the other habitats bacteria affiliating with five (BW and TZ) or four (AG) classes were obtained with lowest number of different sequence-types obtained from

the AG fraction (n=17). The fraction of *Actinobacteria* ranged between 22.2 (TZ) and 41.7 % (BW) and fractions of α- and γ-*Proteobacteria* between 29.2 (AG) and 35.9 % (SE), and between 16.7 (BW) and 29.4% (AG, TZ), respectively. The highest percentage of isolates affiliating to the *Bacilli* was obtained from the TZ (11.1 %). Bacteria affiliating with the *Sphingobacteria* were isolated from the more oxygenated habitats BW, AG and SE with highest percentage in the BW (8.3 %). Isolates affiliating with *Flavobacteria* were exclusively found in the sediment fractions (SE and TZ). One sequence-type (two isolates) affiliating with the *Planctomycetacia* was found in the SE fraction (2.6 %, Fig. 3) on agar and starch as substrates (Table 2). Organisms of the classes *Bacilli*, *Flavobacteria* and *Planctomycetacia*, which were isolated from the BW and/or the SE, were not obtained from AG.

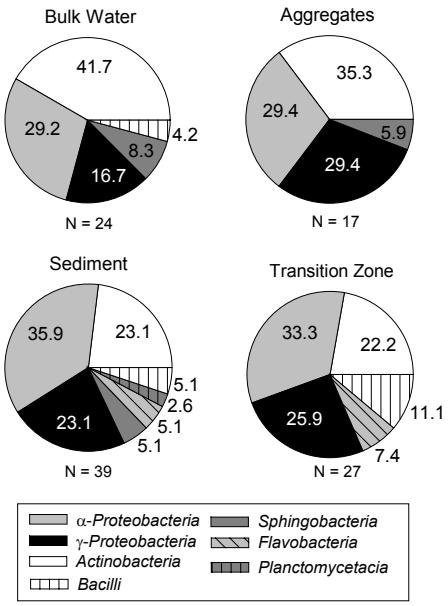


FIG. 3. Percentages of the different phylogenetic classes obtained from bulk water, aggregates, the oxic layer, and oxic/anoxic transition zone of the sediment of the German Wadden Sea in October 1999. "N" is the number of sequence types obtained from the different habitats.

Comparative sequence analyses of the 109 different sequence-types from the four habitats showed that 53 sequence-types (48.6 %) had a sequence similarity of ≥97 % to validly described species, 48 sequence types (44.0 %) showed similarities from <97 to >90 %, and eight sequence types (7.3 %) had a similarity of ≤90 %. Three sequences of the latter category obtained from three different habitats, however, were highly related and fall into the *Cyclobacterium* sp. group shown in Table 3. The proportions of similarity differ for the various habitats (Fig. 4A).

Within the different phylogenetic classes sequence similarities to a described species of ≤90 % were found for six strains or sequence groups, respectively. They affiliate to γ –*Proteobacteria* (strains GWS-SE-H242b, and GWS-BW-H144), to *Sphingobacteria* (strains GWS-BW-H159 [group 18, Table 3], GWS-SE-H150, and GWS-BW-H154) and to *Planctomycetacia* (strain GWS-SE-H106 [group 10, Table 3]), and all were obtained from MPN dilution steps 10^{-1} and 10^{-2} (Table 2). Strain GWS-SE-H242b was isolated with palmitate, strains GWS-BW-H144, GWS-BW-H159, GWS-SE-H150, and GWS-BW-H154 with cellulose, and GWS-SE-H106 with agar. The ratio of sequence types with ≥97 % sequence similarity vs. <97 to >90 % is for the SE fraction about 1 to 1, and for the TZ about 1.3 to 1. For the BW samples this ratio is much higher (2.5 to 1), and for the AG fraction much lower (0.5 to 1With respect to phylogenetic classes the ratio is 1 to 1 for *Bacilli* and *Flavobacteria*, but much higher for *Actinobacteria* and γ-*Proteobacteria* (1.8 to 1, and 1.4 to 1, respectively), and lower for α-*Proteobacteria* (about 0.6 to 1). For the *Sphingobacteria* and *Planctomycetacia* all obtained isolates exhibited sequence similarities of ≤90 % (Fig. 4B).

The majority of different sequence-types was isolated exclusively from one habitat, i.e. 47 % of isolates from BW were exclusively obtained from this habitat, 48 % from AG, 68 % from SE and 70 % from TZ. Some sequence-types were found in two habitats (groups 2, 5, 6, 7, 9, and 15, Table 3), only six sequence-types were found in three habitats (groups 1, 8, 11, 13, 14, and 16), while none was found in all four habitats. The three largest groups (group 8, 13, and 14) include 7 and 8 strains, respectively. Strains of group 8 affiliate with the genus *Nocardioides*, those of group 13 with *Mesorhizobium*, and of group 14 with *Vibrio*. Strains of group 14 were obtained with four different media, those of group 8 and 13 with five different media each. With one exception strains of group 8 were isolated from MPN dilutions of 10^{-4} or higher.

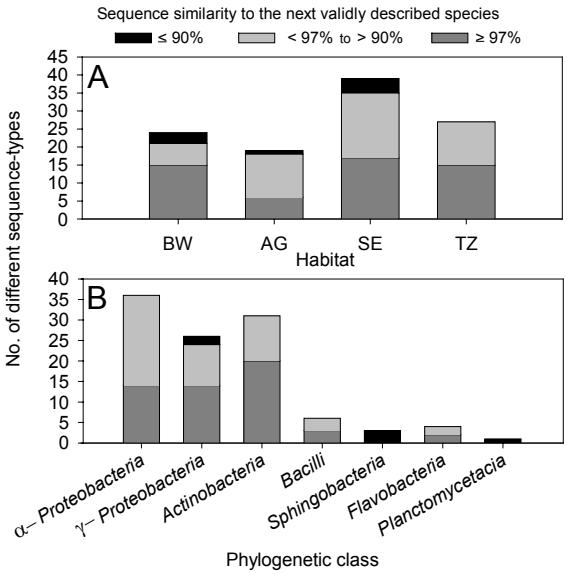


FIG. 4. Numbers of different sequence-types isolated from the German Wadden Sea in October 1999 and classified by sequence similarity to the next validly described species, (A) for sequence types obtained from bulk water (BW), aggregates (AG), the oxic layer of the sediment (SE), and oxic/anoxic transition zone (TZ), (B) for sequence types falling within various phylogenetic classes.

TABLE 2. Phylogenetic affiliation based on a 16S rRNA comparison of bacteria obtained from bulk water, aggregates, the oxic sediment layer and the adjacent tansition zone from oxic to anoxic of the Wadden Sea in october 1999. Bacteria were isolated from MPN series with various substrates. Given are habitate and substrate, isolate Identification (ID), MPN dilution step the isolate was obtained from, phylogenetic affiliation, closest phylotype and described species (validly published type strain) and sequence similarity as retrieved by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast).

Habitat Substrate (no. of isolates)	Strain ID	MPN (10^x)	Phylogenetic class	Closest phylotype / closest described relative (accession no.)	Seq. sim. [%]
Bulk water (32)					
Agar-Agar (1)	GWS-BW-H99	-5	*Actinobacteria*	uncult. Actinobacterium (AB074621) / *Nocardioides luteus* (AF005007)	97; 95
Alginate (3)	GWS-BW-H125	-4	*Actinobacteria*	Bacterium CAGY6 (AF538745) / *Rhodococcus fascians* (X79186)	99; 98
	GWS-BW-H126	-2	*Actinobacteria*	Agricultural soil bacterium isolate SI-12 (AJ252579) / *Arthrobacter pascens* (X80740)	99; 99
	GWS-BW-H127	-1	*Actinobacteria*	*Pseudonocardiaceae* bacterium T4 (AY177725) / *Pseudonocardia alni* (AJ252823)	98; 98
Cellulose (8)	GWS-BW-H157	-2	*Actinobacteria*	*Arthrobacter oxidans* (AJ243423) / *Arthrobacter pascens* (X80740)	99; 99
	GWS-BW-H158	-2	*Actinobacteria*	*Kocuria rosea* (Y11330) / *Kocuria polaris* (AJ278868)	99; 98
	GWS-BW-H159	-1	*Sphingobacteria*	*Cyclobacterium* sp. V4.MS.32 (AJ244689) / *Flexibacter tractuosus* (AB078072)	98; 86
	GWS-BW-H154	-1	*Sphingobacteria*	Marine bacterium HP28 (AY241563) / *Flexibacter elegans* (AB078048)	97; 89
	GWS-BW-H153	-1	*Actinobacteria*	*Micrococcus luteus* strain IFO 16250 (AB023371) / *Micrococcus luteus* (AJ536198)	100; 99
	GWS-BW-H152	-1	*α-Proteobacteria*	Manganese-oxidizing bacterium SI85-9A1 (U53824) / *Mesorhizobium tianshanense* (AF041447)	99; 94
	GWS-BW-H144	-1	*γ-Proteobacteria*	*Pseudomonas* sp. PE1 (AB052968) / *Aeromonas hydrophila* (X60416)	98; 90
	GWS-BW-H145	-1	*Actinobacteria*	*Microbacterium phyllosphaerae* (AJ277840)	99
Chitin (3)	GWS-BW-H198	-5	*Actinobacteria*	uncult. *Nocardioides* sp. clone GCPF41 (AY129809) / *Nocardioides luteus* (AF005007)	96; 94
	GWS-BW-H165	-3	*γ-Proteobacteria*	*Vibrio* sp. V4.BP.07 (AJ244774) / *Vibrio splendidus* (X74724)	97; 97
	GWS-BW-H164	-2	*Sphingobacteria*	*Cyclobacterium* sp. V4.MS.32 (AJ244689) / *Flexibacter tractuosus* (AB078072)	98; 86
F. vesic. (4)	GWS-BW-H202	-5	*Actinobacteria*	*Rhodococcus tukisamuensis* (AB067734) / *Rhodococcus koreensis* (AF124343)	98; 98
	GWS-BW-H203	-5	*α-Proteobacteria*	*Brevundimonas diminuta* strain DSM 1635 (X87274) / *Brevundimonas diminuta* (M59064)	98; 98
	GWS-BW-H204	-2	*Sphingobacteria*	*Cyclobacterium* sp. V4.MS.32 (AJ244689) / *Flexibacter tractuosus* (AB078072)	98; 86
	GWS-BW-H213	-2	*Actinobacteria*	*Micrococcus luteus* (AB023371) / *Micrococcus luteus* (AJ536198)	99; 99
MB 2216 (4)	GWS-BW-H231	-7	*Actinobacteria*	*Micrococcus luteus* strain Ballarat (AJ409096) / *Micrococcus luteus* (AJ536198)	99; 99
	GWS-BW-H222	-6	*Bacilli*	*Bacillus licheniformis* strain GXN151 (AY291582) / *Bacillus licheniformis* (X68416)	99; 99
	GWS-BW-H225	-2	*α-Proteobacteria*	*Mesorhizobium* sp. TUT1018 (AB098586) / *Defluvibacter lusatiae* (AJ132378)	98; 93
	GWS-BW-H226	-1	*α-Proteobacteria*	unidentified bacterium (Y10913) / *Stappia stellulata* (D88525)	98; 97
Palmitate (4)	GWS-BW-H238	-2	*α-Proteobacteria*	*Mesorhizobium* sp. WG (AF156710) / *Mesorhizobium tianshanense* (AF041447)	98; 97
	GWS-BW-H239	-2	*α-Proteobacteria*	uncult. alpha-Proteobacterium, clone BIfdii32 (AJ318134) / *Devosia riboflavina* (D49423)	98; 95
	GWS-BW-H252	-2	*Actinobacteria*	*Nocardioides* sp. NCFB3005 (X76178) / *Aeromicrobium fastidiosum* (AF005022)	97; 97
	GWS-BW-H240	-1	*γ-Proteobacteria*	*Halomonas* sp. ARD M34 (AB085656) / *Halomonas meridiana* (AJ306891)	97; 96
Starch (5)	GWS-BW-H257	-4	*α-Proteobacteria*	*Phyllobacteriaceae* bacterium TK (AY177715) / *Mesorhizobium amorphae* (AF041442)	100; 97
	GWS-BW-H259	-4	*Actinobacteria*	uncult. *Nocardioides* sp. clone GCPF40 (AY129808) / *Nocardioides luteus* (AF005007)	98; 95
	GWS-BW-H260	-4	*Actinobacteria*	*Kocuria rosea* (Y11330) / *Kocuria polaris* (AJ278868)	99; 98
	GWS-BW-H262	-2	*α-Proteobacteria*	Marine arctic deep-sea bacterium FF8b (AJ557867) / *Erythrobacter citreus* (AF118020)	98; 98
	GWS-BW-H264	-2	*γ-Proteobacteria*	*Pseudomonas* sp. K458 (AF489288) / *Pseudomonas pseudoalcaligenes* (Z76666)	99; 98
Aggregates (23)					
Agar-Agar (3)	GWS-AG-H107	-2	*Actinobacteria*	*Pseudonocardia alni* (AJ252823)	99
	GWS-AG-H109	-1	*Sphingobacteria*	*Cyclobacterium* sp. V4.MS.32 (AJ244689) / *Flexibacter tractuosus* (AB078072)	96; 85
	GWS-AG-H110	-1	*α-Proteobacteria*	*Phyllobacteriaceae* bacterium TK (AY177715) / *Mesorhizobium amorphae* (AF041442)	100; 97
Alginate (0)					
Cellulose (1)	GWS-AG-H302	-2	*α-Proteobacteria*	*Rhodobacter sphaeroides* (X53855)	96
Chitin (5)	GWS-AG-H191	-4	*Actinobacteria*	uncult. *Nocardioides* sp. clone GCPF41 (AY129809) / *Nocardioides luteus* (AF005007)	97; 95
	GWS-AG-H192	-3	*Actinobacteria*	*Cellulomonas fermentans* (X79458)	94
	GWS-AG-H197	-3	*Actinobacteria*	*Microbacterium* sp. V4.BP.11 (AJ244677) / *Microbacterium terrae* (AB004720)	98; 97
	GWS-AG-H177	-3	*γ-Proteobacteria*	*Marinobacter* sp. Splume2.1814c (AF212211) / *Marinobacter aquaeolei* (AJ000726)	98; 94
	GWS-AG-H178	-2	*γ-Proteobacteria*	*Marinobacter* sp. NCE312 (AF295032) / *Marinobacter hydrocarbonoclasticus* (AB021372)	99; 95
F. vesic. (2)	GWS-AG-H206	-6	*Actinobacteria*	Uncult. *Nocardioides* sp. clone GCPF41 (AY129809) / *Nocardioides luteus* (AF005007)	97; 95
	GWS-AG-H210	-2	*α-Proteobacteria*	*Phyllobacteriaceae* bacterium TK (AY177715) / *Mesorhizobium amorphae* (AF041442)	100; 96
MB 2216 (3)	GWS-AG-H221	-6	*α-Proteobacteria*	*Roseobacter* sp. PRLIST06 (Y15343) / *Sulfitobacter mediterraneus* (Y17387)	97; 96
	GWS-AG-H224	-2	*γ-Proteobacteria*	*Alteromonadaceae* bacterium T1 (AY177717) / *Shewanella gelidimarina* (U85907)	99; 95
	GWS-AG-H228	-2	*γ-Proteobacteria*	*Halomonas* sp. TNB I20 (AB085649) / *Halomonas boliviensis* (AY245449)	98; 98
Palmitate (4)	GWS-AG-H249	-2	*α-Proteobacteria*	*Phyllobacteriaceae* bacterium TK (AY177715) / *Mesorhizobium amorphae* (AF041442)	99; 96
	GWS-AG-H250-19	-1	*Actinobacteria*	*Nocardioides* sp. strain 30-71 (X86618) / *Nocardioides luteus* (AF005007)	95; 95
	GWS-AG-H250-20	-1	*γ-Proteobacteria*	Gamma proteobacterium UMB20C (AF505745) / *Pseudoalteromonas porphyrae* (AF475096)	99; 99
	GWS-AG-H251	-1	*α-Proteobacteria*	*Sagittula stellata* E-37 (U58356) / *Antarctobacter heliothermus* (Y11552)	99; 95
Starch (5)	GWS-AG-H271	-5	*Actinobacteria*	Uncult. *Nocardioides* sp. clone GCPF41 (AY129809) / *Nocardioides luteus* (AF005007)	97; 94
	GWS-AG-H266	-4	*Actinobacteria*	*Nocardioides* sp. V4.BE.17 (AJ244657) / *Pimelobacter simplex* (Z78212)	98; 94
	GWS-AG-H267	-4	*α-Proteobacteria*	uncult. marine eubacterium HstpL2 (AF159654) / *Paracoccus carotinifaciens* (AB006899)	97; 95
	GWS-AG-H268b	-1	*Actinobacteria*	Marine bacterium P_wp0234 (AY188942) / *Plantibacter flavus* (AJ310417)	100; 96
	GWS-AG-H269	-1	*γ-Proteobacteria*	*Vibrio* sp. CJ11052 (AF500207) / *Vibrio alginolyticus* (X74690)	98; 98
Sediment, oxic layer (44)					
Agar-Agar (4)	GWS-SE-H117	-7	*Bacilli*	*"Bacillus baekryungensis"* (AF541965) / *Bacillus licheniformis* (X68416)	99; 93
	GWS-SE-H100	-6	*Actinobacteria*	*Microbacterium* sp. VKM Ac-2050 (AB042084) / *Microbacterium fololiorum* (AJ249780)	99; 99
	GWS-SE-H106	-1	*Planctomycetacia*	*Pirellula* sp. 7*97* (AF453520) / *Planctomyces maris* (AJ231184)	99; 86
	GWS-SE-H103	-1	*α-Proteobacteria*	*Mesorhizobium* sp. WG (AF156710) / *Mesorhizobium tianshanense* (AF041447)	97; 97
Alginate (6)	GWS-SE-H300	-5	*Actinobacteria*	*Microbacterium* sp. LB030 K / *Plantibacter flavus* (AJ310417)	99; 99
	GWS-SE-H128	-4	*Actinobacteria*	Agricultural soil bacterium isolate SI-12 (AJ252579) / *Arthrobacter pascens* (X80740)	99; 99
	GWS-SE-H136	-4	*Bacilli*	*Bacillus* sp. Fa25 (AY131220) / *Bacillus weihenstephanensis* (AB021199)	99; 99
	GWS-SE-H129	-3	*α-Proteobacteria*	Manganese-oxidizing bacterium SI85-9A1 (U53824) / *Mesorhizobium tianshanense* (AF041447)	99; 94

TABLE 2. continued

Habitat Substrate (no. of isolates)	Strain ID	MPN (10^n)	Phylogenetic class	Closest phylotype / closest described relative (accession no.)	Seq. sim. [%]
Alginate (cont.)	GWS-SE-H130	-2	*α-Proteobacteria*	*Ancylobacter* sp. AS1.1761(AY056830) / *Ancylobacter aquaticus* (M62790)	99; 97
	GWS-SE-H131	-2	*α-Proteobacteria*	*Paracoccus* sp. isolate PRLIST3 (Y15324) / *Paracoccus carotinifaciens* (AB006899)	99, 98
Cellulose (8)	GWS-SE-H151	-4	*α-Proteobacteria*	Uncultured bacterium HOClCi74 (AY328623) / *Sphingobium chlorophenolica* (X87161)	99; 96
	GWS-SE-H155	-3	*α-Proteobacteria*	alpha proteobacterium Llg-6 (AJ439365) / *Mesorhizobium loti* (X67229)	99; 96
	GWS-SE-H76	-2	*α-Proteobacteria*	Unidentified bacterium oxSCC-25 (AJ387870) / *Sphingobium yanoikuyae* (D16145)	99; 99
	GWS-SE-H146	-2	*α-Proteobacteria*	*Stappia* sp. M8 (AY307927) / *Ochrobactrum grignonense* (AJ242581)	99; 93
	GWS-SE-H160	-2	*Sphingobacteria*	*Cyclobacterium* sp. V4.MS.32 (AJ244689) / *Flexibacter tractuosus* (AB078072)	98; 87
	GWS-SE-H161	-2	*Actinobacteria*	Psychrophilic marine bacterium PS32 (AF200218) / *Arthrobacter nicotianae* (X80739)	99; 98
	GWS-SE-H149	-2	*Actinobacteria*	Gram-positive bacterium str. 12-8 (AB008510) / *Plantibacter flavus* (AJ310417)	99; 97
	GWS-SE-H150	-1	*Sphingobacteria*	Bacterium IFAM-3359 (X90702) / *Reichenbachia agariperforans* (AB058919)	98; 87
Chitin (10)	GWS-SE-H169	-5	*γ-Proteobacteria*	Marine bacterium ATAM173a_36 (AF359543) / *Pseudoalteromonas haloplanktis* (AF214729)	99; 98
	GWS-SE-H183	-5	*Flavobacteria*	*Zobellia uliginosa* (M62799)	97
	GWS-SE-H184	-5	*α-Proteobacteria*	*Agrobacterium tumefaciens* MAFF301001(AB102735) / *Agrobacterium tumefaciens* (D14500)	98; 98
	GWS-SE-H196	-5	*γ-Proteobacteria*	*Vibrio* sp. LMG 19999 (AJ316194) / *Vibrio furnisii* (X74704)	97; 96
	GWS-SE-H170	-4	*Actinobacteria*	uncult. *Nocardioides* sp. clone GCPF41 (AY129809) / *Nocardioides luteus* (AF005007)	97; 94
	GWS-SE-H173	-2	*γ-Proteobacteria*	*Marinobacter* sp. NCE312 (AF295032) / *Marinobacter aquaeolei* (AJ000726)	99; 96
	GWS-SE-H171	-2	*Flavobacteria*	uncult. bacterium clone 4-Org1-38 (AF143825) / *Vladibacter vitellinus* (AB071382)	99; 94
	GWS-SE-H175	-1	*Actinobacteria*	*Rhodococcus* sp. MBIC01430 (AB088667) / *Rhodococcus erythropolis* (X80618)	99; 96
	GWS-SE-H176	-1	*α-Proteobacteria*	alpha-Proteobacterium MBIC3923 (AB016848) / *Mesorhizobium loti* (X67229)	98; 96
	GWS-SE-H303	-1	*γ-Proteobacteria*	*Vibrio furnisii* (X74704)	91
F. vesic. (2)	GWS-SE-H250x	-6	*α-Proteobacteria*	*Bacterium* K2-11 (AY345438) / *Roseivivax halotolerans* (D85831)	96; 95
	GWS-SE-H217	-2	*γ-Proteobacteria*	*Vibrio alginolyticus* CIP 70.65 (X74691) / *Vibrio alginolyticus* (X74690)	98; 98
MB 2216 (4)	GWS-SE-H229	-8	*α-Proteobacteria*	*Mesorhizobium* sp. WG (AF156710) / *Mesorhizobium tianshanense* (AF041447)	97; 97
	GWS-SE-H230	-8	*γ-Proteobacteria*	*Vibrio parahaemolyticus* clone Vp 16 (AF388387) / *Vibrio alginolyticus* (X74690)	99; 98
	GWS-SE-H223	-2	*γ-Proteobacteria*	*Vibrio* sp. NLEP97-1598 (AF410778) / *Vibrio pelagius* (X74722)	99; 98
	GWS-SE-H233	-2	*γ-Proteobacteria*	*Halomonas venusta* (AJ306894)	96
Palmitate (9)	GWS-SE-H241	-2	*α-Proteobacteria*	Antarctic bacterium R-9221 (AJ441010) / *Sphingopyxis witflariensis* (AJ416410)	100; 95
	GWS-SE-H242a	-2	*Actinobacteria*	*Microbacterium* sp. OS-6 (AJ296094) / *Microbacterium arborescens* (X77443)	99; 96
	GWS-SE-H242b	-2	*γ-Proteobacteria*	Gamma proteobacterium GMD16F03 (AY162108) / *Oceanospirillum maris hiroshimense* (AB006762)	97; 89
	GWS-SE-H243	-2	*Actinobacteria*	*Frigoribacter* sp. 801 (Y18807) / *Frigoribacterium faeni* (Y18807)	98; 98
	GWS-SE-H244	-2	*α-Proteobacteria*	alpha-Proteobacterium MBIC3923 (AB016848) / *Mesorhizobium loti* (X67229)	98; 96
	GWS-SE-H246	-2	*γ-Proteobacteria*	*Halomonas venusta* (AJ306894)	99
	GWS-SE-H247	-2	*α-Proteobacteria*	unidentified bacterium clone K2-S-15 (AY344375) / *Erythrobacter longus* (M96744)	97; 97
	GWS-SE-H253	-1	*α-Proteobacteria*	alpha-Proteobacterium MBIC3865 (AB015896) / *Stappia aggregata* (D88520)	97; 95
	GWS-SE-H254	-1	*γ-Proteobacteria*	*Microbulbifer salipaludis* SM-1 (AF479688) / *Pseudomonas elongata* (AF500006)	97; 96
Starch (1)	GWS-SE-H270	-1	*Planctomycetacia*	*Pirellula* sp. strain 1 (BX294149) / *Planctomyces maris* (AJ231184)	99; 86
Transition zone (sediment,oxic/anoxic boundary) (30)					
Agar-Agar (7)	GWS-TZ-H111	-7	*Actinobacteria*	*Microbacterium* sp. VKM Ac-2050 (AB042084) / *Microbacterium foliorum* (AJ249780)	100; 99
	GWS-TZ-H118	-5	*Actinobacteria*	*Cellulomonas* sp. IFO16243 (AB023364) / *Promicromonospora sukumoe* (AB056130)	96; 94
	GWS-TZ-H119	-5	*α-Proteobacteria*	uncult. marine eubacterium HstpL2 (AF159654) / *Paracoccus aminophilus* (AY014176)	94; 91
	GWS-TZ-H112	-2	*α-Proteobacteria*	*Paracoccus zeaxanthinifaciens* R-1506 (AF461159) / *Paracoccus seriniphilus* (AJ428275)	98; 98
	GWS-TZ-H113	-2	*Flavobacteria*	marine bacterium HP34 (AY241567) / *Arenibacter latericius* (AF052742)	99; 96
	GWS-TZ-H114	-2	*Bacilli*	*Bacillus* sp. HT-1 (AF463535) / *Bacillus firmus* (X60616)	96; 95
	GWS-TZ-H115	-1	*γ-Proteobacteria*	*Glaciecola gelidimarina* LMG 21855 (AJ548479) / *Glaciecola pallidula* (U85854)	99; 93
Alginate (4)	GWS-TZ-H134	-4	*α-Proteobacteria*	*Fulvimarina litoralis* strain HTCC2156 (AY178863) / *Mesorhizobium tianshanense* (AF041447)	99; 94
	GWS-TZ-H135	-1	*Actinobacteria*	*Rhodococcus fascians* KM6 (AJ011329) / *Rhodococcus fascians*(X79186)	100; 100
	GWS-TZ-H138	-1	*α-Proteobacteria*	alpha-Proteobacterium PII_GH1.2.A1 (AY162056) / *Ochrobactrum grignonense* (AJ242581)	99; 98
	GWS-TZ-H139	-1	*Actinobacteria*	*Microbacterium testaceum* SE034 (AF474327) / *Microbacterium testaceum* (X77445)	97; 97
Chitin (9)	GWS-TZ-H187	-8	*α-Proteobacteria*	*Agrobacterium tumefaciens* MAFF 301001 (AB102735) / *Agrobacterium tumefaciens* (D14500)	99; 99
	GWS-TZ-H188	-5	*γ-Proteobacteria*	*Vibrio rumoiensis* (AB013297)	99
	GWS-TZ-H190	-5	*α-Proteobacteria*	*Roseobacter* sp. OCh114 (M59063) / *Roseobacter litoralis* (X78312)	95; 95
	GWS-TZ-H304	-4	*γ-Proteobacteria*	*Vibrio* sp. NAP-4 (AF064637) / *Vibrio alginolyticus* (X74690)	99; 98
	GWS-TZ-H179	-2	*γ-Proteobacteria*	Saltmarsh clone LCP-79 (AF286035) / *Pseudomonas pseudoalcaligenes* (Z76666)	99; 98
	GWS-TZ-H180	-1	*α-Proteobacteria*	uncult. alpha-Proteobacterium clone BIfdii32 (AJ318134) / *Devosia riboflavina* (D49423)	98; 96
	GWS-TZ-H181	-1	*α-Proteobacteria*	*Phyllobacteriaceae* bacterium NL21 (AF534573) / *Pseudaminobacter salicylatoxidans* (AF072542)	97; 94
	GWS-TZ-H182	-1	*γ-Proteobacteria*	*Vibrio alginolyticus* CIP70.65 (X74691) / *Vibrio parahaemolyticus* (X74720)	99; 98
	GWS-TZ-H309	-6	*Actinobacteria*	*Rhodococcus tukisamuensis* (AB067734) / *Rhodococcus koreensis* (AF124343)	98; 98
F. vesic. (8)	GWS-TZ-H200	-2	*γ-Proteobacteria*	*Vibrio parahaemolyticus* clone Vp 27 (AF388389) / *Vibrio alginolyticus* (X74690)	98; 98
	GWS-TZ-H207	-2	*γ-Proteobacteria*	*Halomonas* sp. KMM 3550 (AF316143) / *Halomonas marina* (AJ306890)	99; 99
	GWS-TZ-H305	-2	*Actinobacteria*	*Microbacterium esteraromaticum* (Y17231)	95
	GWS-TZ-H306	-2	*γ-Proteobacteria*	*Halovibrio* sp. isolate PRLIST03 (Y15340) / *Halomonas venusta* (AJ306894)	98; 98
	GWS-TZ-H209	-2	*γ-Proteobacteria*	*Pseudomonas* sp. Hsa.28 (AY259121) / *Pseudomonas anguilliseptica* (X99540)	98; 97
	GWS-TZ-H201	-1	*Flavobacteria*	Marine gliding bacterium UWA-1 (AB039966) / *Tenacibaculum mesophilum* (AB032501)	98; 97
	GWS-TZ-H212	-1	*γ-Proteobacteria*	*Vibrio pelagius* (X74722)	99
	GWS-TZ-H218	-1	*α-Proteobacteria*	Uncult. alpha-Proteobacterium SIMOCL-S30-58 (AY149734) / *Rhodobium orientis* (D30792)	96; 93
MB 2216 (2)	GWS-TZ-H236	-8	*Bacilli*	*Bacillus* sp. Fa25 (AY131220) / *Bacillus weihenstephanensis* (AB021199)	99; 99
	GWS-TZ-H232	-1	*Bacilli*	*Planococcus rifitiensis* M8 (AJ493659) / *Planococcus kocurii* (X62173)	99; 95
Starch (0)					

TABLE 3: Groups of similar strains obtained from different habitats and/or with different substrates. Groups are defined by a similarity of the 16S rRNA gene sequences of ≥ 99%. Designation of the habitat is included in the strain name (BW = Bulk Water, AG = Aggregates, SE = Sediment surface, TZ = Transition Zone). Substrates were AGA = Agar-Agar, ALG = Alginate, CEL = Cellulose, CHI = Chitin, FUV = *Fucus vesiculosus*, PAL = palmic acid, STA = starch, MB = Marine Broth 2216.

Strain	MPN dilution (10^X)	Substrate
Actinobacteria		
Group 1 (*Microbacterium* sp.)		
GWS-BW-H145	-1	CEL
GWS-SE-H100	-6	AGA
GWS-TZ-H111	-7	AGA
Group 2 (*Arthrobacter* sp.)		
GWS-BW-H126	-2	ALG
GWS-BW-H157	-2	CEL
GWS-SE-H128	-4	ALG
Group 3 (*Micrococcus* sp.)		
GWS-BW-H153	-1	CEL
GWS-BW-H213	-2	FUV
GWS-BW-H231	-7	MB
Group 4 (*Kocuria* sp.)		
GWS-BW-H158	-2	CEL
GWS-BW-H260	-4	STA
Group 5 (*Rhodococcus* sp.)		
GWS-BW-H125	-4	ALG
GWS-TZ-H135	-1	ALG
Group 6 (*Rhodococcus* sp.)		
GWS-BW-H202	-5	FUV
GWS-TZ-H309	-6	CHI
Group 7 (*Pseudonocardia* sp.)		
GWS-BW-H127	-1	ALG
GWS-AG-H107	-2	AGA
Group 8 (*Nocardioides* sp.)		
GWS-BW-H99	-5	AGA
GWS-BW-H198	-5	CHI
GWS-AG-H191	-4	CHI
GWS-AG-H206	-6	FUV
GWS-AG-H250-19	-1	PAL
GWS-AG-H271	-5	STA
GWS-SE-H170	-4	CHI
Bacilli		
Group 9 (*Bacillus* sp.)		
GWS-SE-136	-4	ALG
GWS-TZ-H236	-8	MB
α-Proteobacteria		
Group 11 (*Aurantimonas* sp.)		
GWS-BW-H152	-1	CEL
GWS-SE-H129	-3	ALG
GWS-TZ-H134	-4	ALG
Group 12 (unknown *Rhizobiales* sp.)		
GWS-SE-H176	-1	CHI
GWS-SE-H244	-2	PAL
Group 13 (*Mesorhizobium* sp.)		
GWS-BW-H238	-2	PAL
GWS-BW-H257	-4	STA
GWS-AG-H110	-1	AGA
GWS-AG-H210	-2	FUV
GWS-AG-H249	-2	PAL
GWS-SE-H103	-1	AGA
GWS-SE-H229	-8	MB
γ-Proteobacteria		
Group 14 (*Vibrio* sp.)		
GWS-AG-H269	-1	STA
GWS-SE-H217	-2	FUV
GWS-SE-H223	-2	MB
GWS-SE-H230	-8	MB
GWS-TZ-H182	-1	CHI
GWS-TZ-H200	-2	FUV
GWS-TZ-H212	-1	FUV
GWS-TZ-H304	-4	CHI
Group 15 (*Marinobacter* sp.)		
GWS-AG-H177	-3	CHI
GWS-AG-H178	-2	CHI
GWS-SE-H173	-2	CHI
Sphingobacteria		
Group 16 (*Cyclobacterium* sp.)		
GWS-BW-H159	-1	CEL
GWS-BW-H164	-2	CHI
GWS-BW-H204	-2	FUV
GWS-AG-H109	-1	AGA
GWS-SE-H160	-2	CEL
Planctomycetacia		
Group 10 (unclassified *Planctom.*)		
GWS-SE-H106	-1	AGA
GWS-SE-H270	-1	STA

III.5 DISCUSSION

Our cultivation approach using MPN dilution series with eight different media gave insight into the substrate preferences of the culturable heterotrophic aerobic bacterial communities of four different habitats of the Wadden Sea. Highest MPN numbers for all four habitats were obtained with MB 2216 and *F. vesiculosus*, both quite diverse substrates, including biopolymers consisting of sugars, amino acids, and fatty acids. Lower MPN numbers were obtained with single polymers as substrates. This indicates that the more specialized organisms present in these cultures were less abundant in the environmental samples than those able to grow with the complex media. Whether the latter grow on single sugars or amino acids present in the media, or if just the combination of different substrates allows these organisms to grow remains to be tested. Agar-agar and starch as substrates yielded higher cell counts than chitin, although the latter is one of the most abundant biopolymers, especially in marine habitats (46), and contains N-acetylglucosamine as additional nitrogen source. MB 2216 medium has been used before in many studies, and hundreds marine bacteria have been isolated with this medium. Degradation of *Fucus* spp. or of fucoidan obtained from *Fucus* spp. by bacteria of different phylogenetic lineages has been described before (18, 40), however, it was surprising to us to obtain similar efficiencies with *F. vesiculosus* as with MB 2216 (Figs. 1 and 2). This shows that this algae is a suitable substrate to enrich and isolate bacteria at least from the Wadden Sea, where it grows copiously along the coast line.

Bacteria of seven different phylogenetic classes could be isolated. With alginate and palmitate organisms from only three of these were obtained. Agar-agar was the only substrate which allowed us to isolate bacteria from all seven classes (Fig. 2B), including one strain of the rarely isolated *Planctomycetacia* (Table 2). Thus agar-agar seems to be better suitable for isolation of bacteria of different phylogenetic groups than the other substrates used in our study.

Even though we observed growth in very high dilutions and obtained MPN numbers up to 32 % of total cell counts (for BW with *F. vesiculosus* as substrate), we could not isolate bacteria from all of these cultures, especially not from the highest dilution in which growth was still detected. Either these organisms were not able to grow on plates or ceased growing after a few transfers. Kaeberlein et al. (19) observed that the majority of microorganisms from the sediment surface could only undergo a limited number of divisions on a petri dish, and Eilers et al. (12) described that during isolation of bacteria from environmental samples colonies did not grow after reinoculation in liquid medium and termed these "not transferable". We suggest to differentiate clearly between enrichable and culturable organisms, and define that a bacterial species is enrichable when it shows detectable growth in a culture or on a plate, and culturable when it can also be transferred unlimited without loosing the ability to grow and reproduce. Therefore it is not mandatory that the organism can be grown in pure culture. Transfer of our isolates for at least five times on substrate amended media was performed to demonstrate that they are culturable and able to grow on the substrates used for isolation.

Determination of bacterial numbers by MPN counts is always a relatively rough estimation of the abundance. In all fractions the obtained bacterial numbers might be affected by uneven dilution of larger particles (aggregates or sediment). We are also aware of the fact that one- or two time isolation of a single strain from a high dilution step from one MPN assay is only a hint of its high abundance. Furthermore,

especially in the TZ some dominant bacteria might be growing anaerobically, e.g. δ-*Proteobacteria* (22, 23), and were thus not detected with our MPN approach. However, the MPN method is still the only possibility to determine the number of enrichable bacteria from an environment and a first step to isolate the culturable organisms.

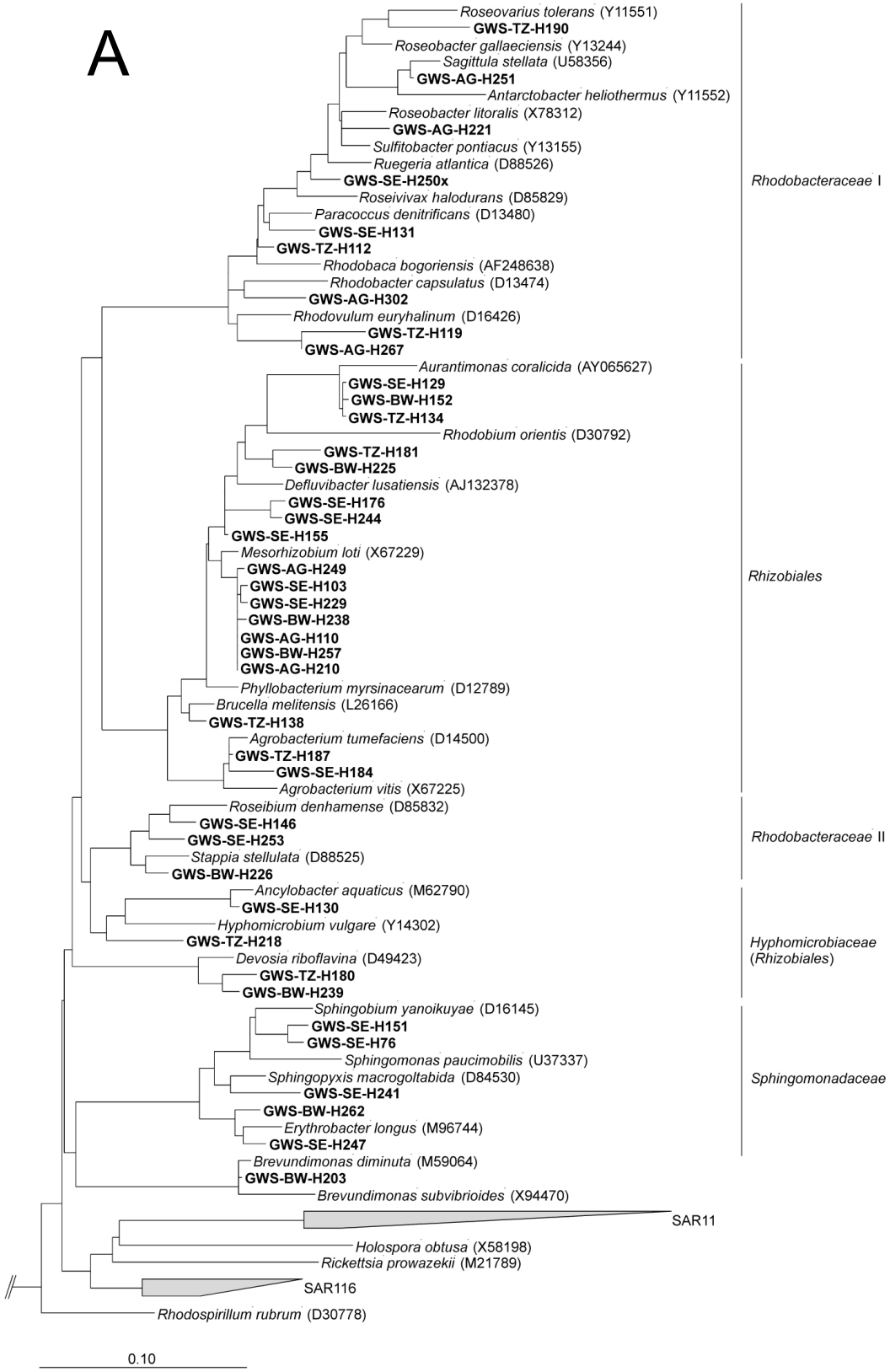
Most organisms which we obtained in our study affiliated with α- and γ-*Proteobacteria* and with *Actinobacteria*. Sequence types of these groups constituted about 85 % of all sequence types isolated from the four habitats (Fig. 3). While high numbers of α- and γ-*Proteobacteria* were previously detected in and isolated from the North Sea (10, 11), the high number of *Actinobacteria*, but also of *Bacilli* is unusual for a marine habitat and might partially be due to a cultivation bias. Isolation of many Gram positive strains from high dilutions, however, indicates a high abundance of these organisms (Table 2). This is especially applicable for the seven strains of sequence group 8 (Table 3) affiliating with the genus *Nocardioides*, because six of these strains were isolated from higher MPN dilutions (10^{-4} or higher). Bruns et al. (2003) recently described a new marine *Aeromicrobium* species, *A. marinum*, which was obtained from the German Wadden Sea and shown to be abundant in the water column. One of our strains, also obtained from the BW (GWS-BW-H252) is even closely related to *A. marinum* (Fig. 5C). It seems possible that high abundances of Gram positive bacteria are a special feature of the Wadden Sea, and might partially be due to its behavior as a melting pot and interface between marine and terrestrial environments and resuspension of sediment by the strong tidal currents.

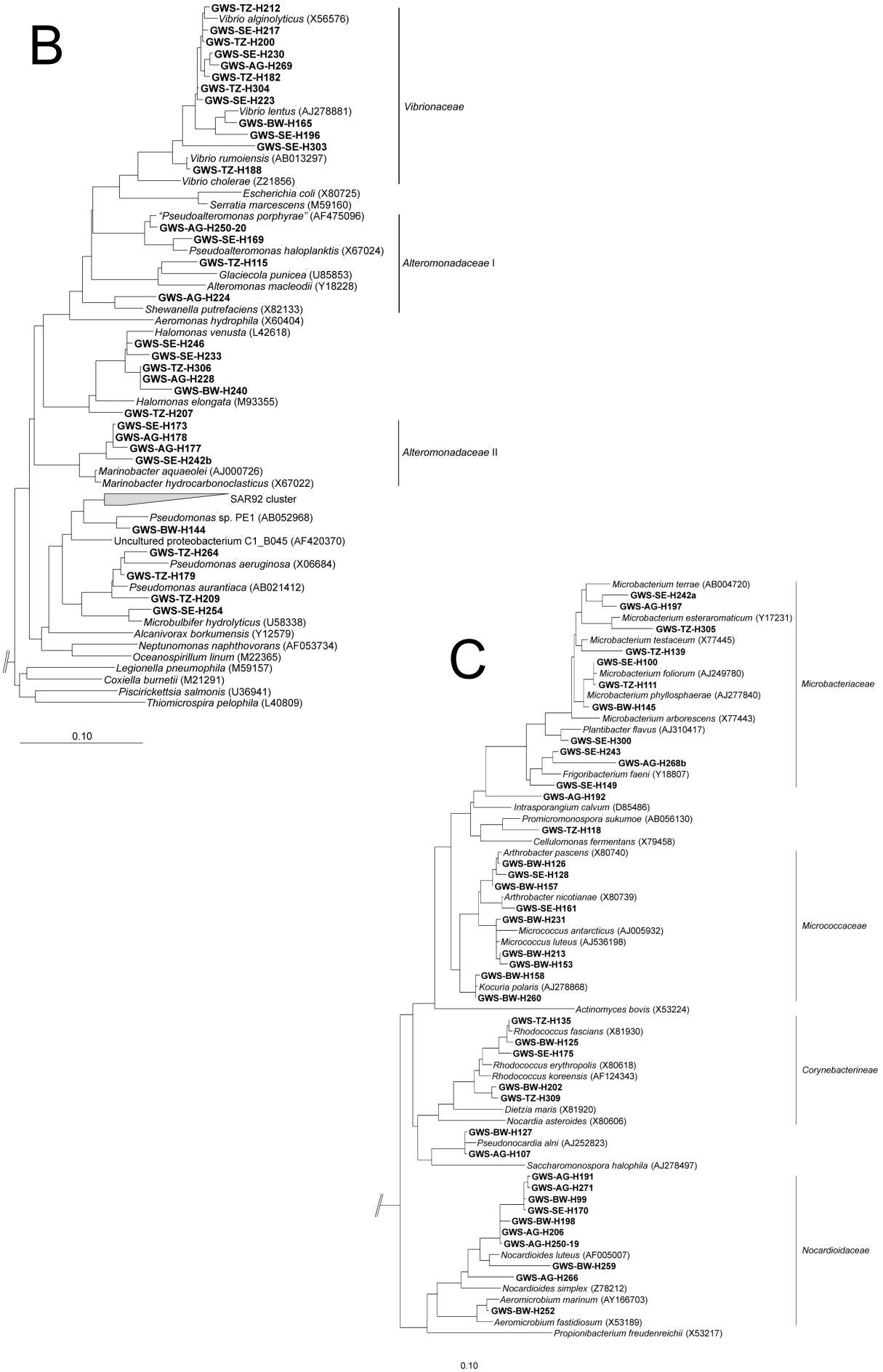
The analysis of the pelagic bacterial community of the North Sea by fluorescence *in situ* hybridization demonstrated that organisms belonging to the phylum *Bacteroidetes* constitute up to 30 % of total cell counts in this habitat (11).

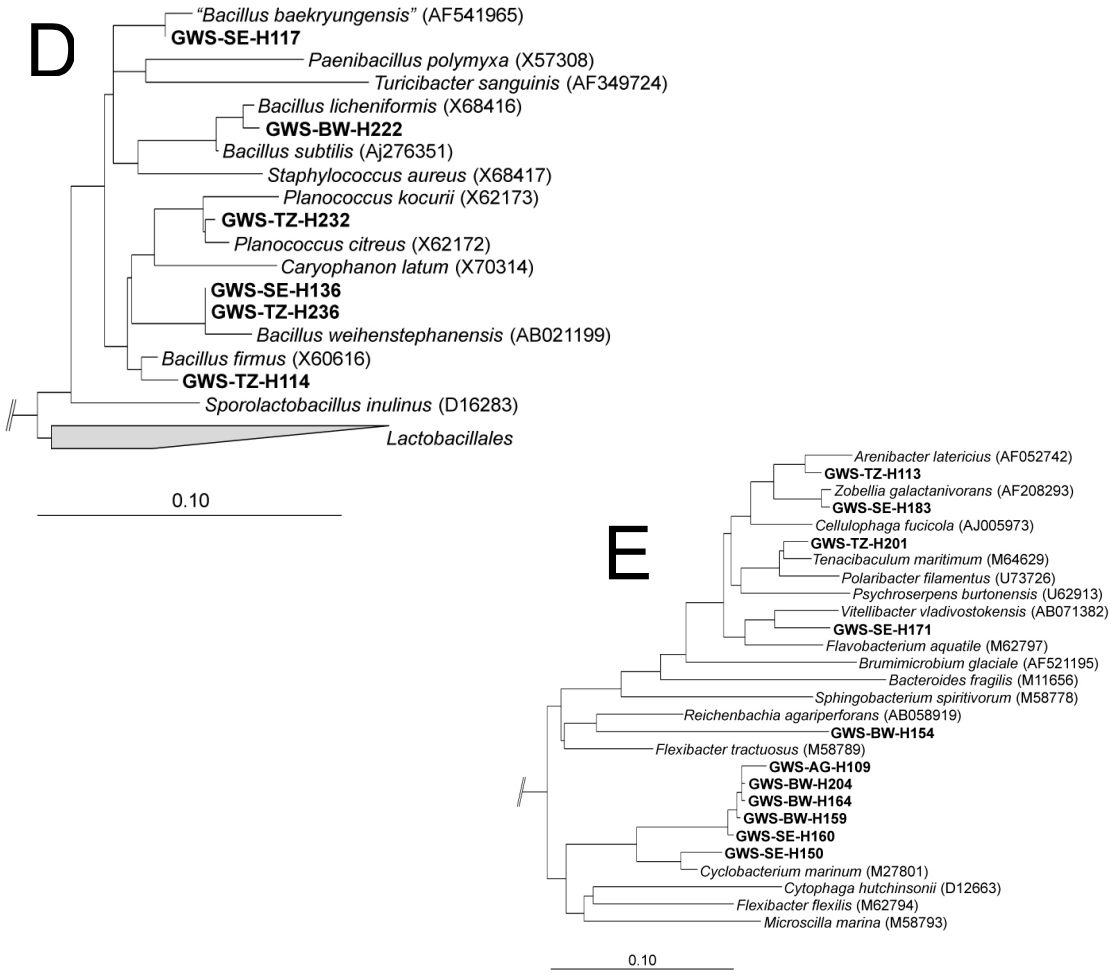
On the following pages:

FIG. 5. Maximum-likelihood trees based on 16S rRNA gene sequence analysis showing the phylogeny of the isolated strains affiliated with (A) α- *Proteobacteria*, (B) γ-*Proteobacteria*, (C) *Actinobacteria*, (D) *Bacilli*, and (E) *Sphingobacteria* and *Flavobacteria*. For tree construction only sequences >1300 bp from validly described species were used to calculate backbone trees. The only exceptions are *Pseudoalteromonas porphyrae*, and *Bacillus baekryungensis*, which were not validly described, but are a close relatives to two of our isolates, and *Phyllobacterium myrsinacearum*, *Hyphomicrobium vulgare*, and *Cyclobacterium marinum*, for which no 16S rRNA gene sequences of the type strains were available and where sequences of other strains were used. Not validly published names are marked with quotation

Shorter sequences of the new isolates were added later with the maximum parsimony method. For each tree selected sequences from other phylogenetic classes were used to root the tree. The bars indicate 10 % sequence divergence.


A
Roseovarius tolerans (Y11551)
GWS-TZ-H190
Roseobacter gallaeciensis (Y13244)
Sagittula stellata (U58356)
GWS-AG-H251
Antarctobacter heliothermus (Y11552)
Roseobacter litoralis (X78312)
GWS-AG-H221
Sulfitobacter pontiacus (Y13155)
Ruegeria atlantica (D88526)
GWS-SE-H250x
Roseivivax halodurans (D85829)
Paracoccus denitrificans (D13480)
GWS-SE-H131
GWS-TZ-H112
Rhodobaca bogoriensis (AF248638)
Rhodobacter capsulatus (D13474)
GWS-AG-H302
Rhodovulum euryhalinum (D16426)
GWS-TZ-H119
GWS-AG-H267
Rhodobacteraceae I
Aurantimonas coralicida (AY065627)
GWS-SE-H129
GWS-BW-H152
GWS-TZ-H134
Rhodobium orientis (D30792)
GWS-TZ-H181
GWS-BW-H225
Defluvibacter lusatiensis (AJ132378)
GWS-SE-H176
GWS-SE-H244
GWS-SE-H155
Mesorhizobium loti (X67229)
GWS-AG-H249
GWS-SE-H103
GWS-SE-H229
GWS-BW-H238
GWS-AG-H110
GWS-BW-H257
GWS-AG-H210
Phyllobacterium myrsinacearum (D12789)
Brucella melitensis (L26166)
GWS-TZ-H138
Agrobacterium tumefaciens (D14500)
GWS-TZ-H187
GWS-SE-H184
Agrobacterium vitis (X67225)
Rhizobiales
Roseibium denhamense (D85832)
GWS-SE-H146
GWS-SE-H253
Stappia stellulata (D88525)
GWS-BW-H226
Rhodobacteraceae II
Ancylobacter aquaticus (M62790)
GWS-SE-H130
Hyphomicrobium vulgare (Y14302)
GWS-TZ-H218
Devosia riboflavina (D49423)
GWS-TZ-H180
GWS-BW-H239
Hyphomicrobiaceae (Rhizobiales)
Sphingobium yanoikuyae (D16145)
GWS-SE-H151
GWS-SE-H76
Sphingomonas paucimobilis (U37337)
Sphingopyxis macrogoltabida (D84530)
GWS-SE-H241
GWS-BW-H262
Erythrobacter longus (M96744)
GWS-SE-H247
Sphingomonadaceae
Brevundimonas diminuta (M59064)
GWS-BW-H203
Brevundimonas subvibrioides (X94470)
SAR11
Holospora obtusa (X58198)
Rickettsia prowazekii (M21789)
SAR116
Rhodospirillum rubrum (D30778)
0.10


B
GWS-TZ-H212
Vibrio alginolyticus (X56576)
GWS-SE-H217
GWS-TZ-H200
GWS-SE-H230
GWS-AG-H269
GWS-TZ-H182
GWS-TZ-H304
GWS-SE-H223
Vibrio lentus (AJ278881)
GWS-BW-H165
GWS-SE-H196
GWS-SE-H303
Vibrio rumoiensis (AB013297)
GWS-TZ-H188
Vibrio cholerae (Z21856)
Escherichia coli (X80725)
Serratia marcescens (M59160)
"Pseudoalteromonas porphyrae" (AF475096)
GWS-AG-H250-20
GWS-SE-H169
Pseudoalteromonas haloplanktis (X67024)
GWS-TZ-H115
Glaciecola punicea (U85853)
Alteromonas macleodii (Y18228)
GWS-AG-H224
Shewanella putrefaciens (X82133)
Aeromonas hydrophila (X60404)
Halomonas venusta (L42618)
GWS-SE-H246
GWS-SE-H233
GWS-TZ-H306
GWS-AG-H228
GWS-BW-H240
Halomonas elongata (M93355)
GWS-TZ-H207
GWS-SE-H173
GWS-AG-H178
GWS-AG-H177
GWS-SE-H242b
Marinobacter aquaeolei (AJ000726)
Marinobacter hydrocarbonoclasticus (X67022)
SAR92 cluster
Pseudomonas sp. PE1 (AB052968)
GWS-BW-H144
Uncultured proteobacterium C1_B045 (AF420370)
GWS-TZ-H264
Pseudomonas aeruginosa (X06684)
GWS-TZ-H179
Pseudomonas aurantiaca (AB021412)
GWS-TZ-H209
GWS-SE-H254
Microbulbifer hydrolyticus (U58338)
Alcanivorax borkumensis (Y12579)
Neptunomonas naphthovorans (AF053734)
Oceanospirillum linum (M22365)
Legionella pneumophila (M59157)
Coxiella burnetii (M21291)
Piscirickettsia salmonis (U36941)
Thiomicrospira pelophila (L40809)
0.10
Vibrionaceae
Alteromonadaceae I
Alteromonadaceae II
C
Microbacterium terrae (AB004720)
GWS-SE-H242a
GWS-AG-H197
Microbacterium esteraromaticum (Y17231)
GWS-TZ-H305
Microbacterium testaceum (X77445)
GWS-TZ-H139
GWS-SE-H100
Microbacterium foliorum (AJ249780)
GWS-TZ-H111
Microbacterium phyllosphaerae (AJ277840)
GWS-BW-H145
Microbacterium arborescens (X77443)
Plantibacter flavus (AJ310417)
GWS-SE-H300
GWS-SE-H243
GWS-AG-H268b
Frigoribacterium faeni (Y18807)
GWS-SE-H149
GWS-AG-H192
Intrasporangium calvum (D85486)
Promicromonospora sukumoe (AB056130)
GWS-TZ-H118
Cellulomonas fermentans (X79458)
Arthrobacter pascens (X80740)
GWS-BW-H126
GWS-SE-H128
GWS-BW-H157
Arthrobacter nicotianae (X80739)
GWS-SE-H161
GWS-BW-H231
Micrococcus antarcticus (AJ005932)
Micrococcus luteus (AJ536198)
GWS-BW-H213
GWS-BW-H153
GWS-BW-H158
Kocuria polaris (AJ278868)
GWS-BW-H260
Actinomyces bovis (X53224)
GWS-TZ-H135
Rhodococcus fascians (X81930)
GWS-BW-H125
GWS-SE-H175
Rhodococcus erythropolis (X80618)
Rhodococcus koreensis (AF124343)
GWS-BW-H202
GWS-TZ-H309
Dietzia maris (X81920)
Nocardia asteroides (X80606)
GWS-BW-H127
Pseudonocardia alni (AJ252823)
GWS-AG-H107
Saccharomonospora halophila (AJ278497)
GWS-AG-H191
GWS-AG-H271
GWS-BW-H99
GWS-SE-H170
GWS-BW-H198
GWS-AG-H206
GWS-AG-H250-19
Nocardioides luteus (AF005007)
GWS-BW-H259
GWS-AG-H266
Nocardioides simplex (Z78212)
Aeromicrobium marinum (AY166703)
GWS-BW-H252
Aeromicrobium fastidiosum (X53189)
Propionibacterium freudenreichii (X53217)
0.10
Microbacteriaceae
Micrococcaceae
Corynebacterineae
Nocardioidaceae



Strong seasonal fluctuations of this group with relatively low numbers detected in fall and winter were also reported (12). We discriminate between the different classes of the *Bacteroidetes* (Fig. 2 to 4), as is the usual case for the *Proteobacteria*, but even the sum of the percentages of the different sequence types of the *Flavobacteria* and *Sphingobacteria* only results in percentages between 5.9 and 10.2 % (Fig. 3). Therefore the *Bacteroidetes* are probably underrepresented in our study. This is surprising since bacteria within this phylum are known to be chemoorganotrophic and especially proficient in degrading various polymers such as cellulose, chitin, and pectin (20, 36). Overall, we isolated 11 strains affiliated with the *Bacteroidetes* (Fig. 5E), and five of these strains are even highly related and fall in one sequence group (Table 3). Cellulose, chitin and other polymers were used in our study, and nine strains were isolated with polymers, as compared with two obtained with the complex media MB 2216 and *F. vesiculosus* (Table 2), supporting the notion that these organisms are proficient in degrading polymers. But our results also show that bacteria of other groups are obviously also able to use polymers very efficiently. For example strains of the genus *Vibrio* are known to produce several chitinases (46) and seven of 27 *Vibrio* strains we isolated with chitin.

We observed no pronounced preferential isolation of γ-*Proteobacteria*. This so called "culture induced gamma-shift" was often described when nutrient-enriched media were used for isolation (10, 13, 26, 45, 49). Since most of the γ-*Proteobacteria* (84 %) were isolated from low dilutions (10^{-3} or lower, Table 2) this

might partially be due to the dilution series applied, which also allowed enrichment of abundant but slowly growing bacteria, present in higher dilutions of the MPN series. Generally, γ-*Proteobacteria*, e.g. species of the *Vibrionaceae* are easy to isolate because they grow well on the ordinary peptone-type media (The Prokaryotes, release 3.14, 2003; http://141.150.157.117:8080/prokPUB/index.htm). On the other hand, some substrates, i.e. alginate, agar-agar, and cellulose, seemed not to be favourable for γ-*Proteobacteria* because they yielded no or only low percentages of organisms of this group.

Strains of four sequence groups affiliating with four different phylogenetic classes were isolated several times with four or five different substrates and from three different habitats. The largest group comprises eight strains (group 14, Table 3), which are affiliated with the genus *Vibrio* (Fig. 5B). Seven strains affiliated with the genus *Mesorhizobium* (group 13, Table 3; Fig. 5A), and five with *Cyclobacterium* (sequence group 16, Table 3; Fig 5E). Finally seven strains affiliated with the genus *Nocardioides* (group 8, Table 3; Fig. 5C). Strains of the latter group were obtained from higher dilutions (see above), while strains of the first three groups were mainly isolated from low dilutions indicating lower abundances. Stevens et al. (44) showed that distinct differences between the bacterial communities of bulk water, aggregates and the sediment surface of the Wadden Sea exist. From the isolation of a single strain from one habitat it is not possible to decide whether this organism prefers this environment. However, for the four sequence groups at least tendencies can be derived from the repeated isolation of specific habitats. While seven of the eight *Vibrio* strains of group 14 were isolated from the sediment (SE or TZ), most of the strains of the other groups were isolated from the water column (BW or AG). Isolation of strains of the four sequence groups with four or five different substrates indicates at least a better growth of the organisms with these substrates in the MPN cultures compared to other bacteria obtained during this study. Whether they have a higher metabolic variety remains to be tested.

Culture-dependent approaches are generally not suitable to identify all relevant species of an environment, as found by the observed discrepancies to culture-independent methods [for review see Amann et al. (2), and Giovannoni & Rappé (14)]. To detect, however, which bacteria are currently culturable and available for detailed characterization, one has to try to isolate the organisms from the environment. After identification of abundant organisms by molecular biological methods and realizing the differences to the so far cultured strains, these bacteria might also be isolated, but more systematically, as shown for the SAR11 clade (32). Therefore, in future studies we want to identify abundant bacteria in the Wadden Sea by culture independent techniques for comparison with our isolates and subsequently apply new directed isolation approaches.

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IV

DIVERSITY AND ABUNDANCE OF GRAM-POSITIVE BACTERIA IN A TIDAL FLAT ECOSYSTEM

Diversity and Abundance of Gram-Positive Bacteria in a Tidal Flat Ecosystem

Running title: Gram-Positive Bacteria in Tidal Flats

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IV.1 ABSTRACT

This study presents the phylogenetic affiliation of available sequences of gram-positive bacteria from the Wadden Sea, a tidal flat ecosystem in the German Bight of the North Sea. Sequences were derived from 16S rRNA genes from bacterial isolates and a clone library. The analysis includes gram-positive bacteria from the bulk water (BW), suspended aggregates (AG), the oxic sediment surface (SE), and the oxic/anoxic transition zone (TZ) of the sediment. Bacterial isolates were obtained from various steps of dilution series enriched with biopolymers such as agar, alginate, casein, cellulose, chitin, laminarin, palmitate, starch, stearate, dried and pestiled *Fucus vesiculosus* and Marine Broth. Seventeen of 63 isolates of a BW sample from May 1999, and 45 of 129 isolates from water and sediment samples taken in October 1999 affiliated to gram-positive bacteria. Most strains were isolated with alginate, agar, starch, and MB, and only 4 strains with casein, stearin and laminarin. Twenty-eight gram-positive isolates were derived from dilution steps 10^{-4} and higher. Five of 48 different clones of the 16S rRNA gene clone library affiliated to gram-positive bacteria. A total of 87 16S rRNA gene sequences from oxic habitats of the German Wadden Sea were phylogenetically analyzed. Forty one of 51 different sequence types affiliated to *Actinobacteria*, covering eight families of the order *Actinomycetales*, and 10 sequence types to *Firmicutes*. Nine of the latter affiliate to the order *Bacillales*, 6 to the family *Bacillaceae*, 3 to the *Planococcaceae*. Some of the sequences are most closely related to sequences from marine environments, but more than 50 % to other environments such as freshwater and soil. These relationships appear to reflect the melting pot signature of the Wadden Sea exposed to terrestrial as well as marine influences. The high potential to degrade polymeric substances suggest that gram-positive bacteria are important in the turnover and decomposition of organic matter in this ecosystem.

IV.2 INTRODUCTION

Traditionally, gram-positive (gram+) bacteria were thought to be of little significance in aquatic systems and their occurrence was predominantly attributed to soils (e.g. 28, 50). Recent studies in freshwater habitats, however, showed that they are more abundant than previously thought and appear to play a hitherto unknown role (16, 22, 57, 67). It has been known for more than 50 years that gram+ bacteria also exist in marine environments, but they were thought not to be indigenous but introduced from terrestrial habitats (9, 26, 36, 66). Since the mid-1990s, studies using culture-independent but also refined culture-dependent methods indicated that gram+ bacteria revealed an unexpected high diversity in marine bacterioplankton communities (15, 45, 46, 60) as well as in marine sediments and a surprisingly high abundance (26, 35, 36, 64). Phylogenetic analyses of the phylotypes and isolates obtained in these studies suggested that, in fact, indigenous marine gram+ bacteria exist. Interestingly, some of the gram+ bacteria obtained from marine habitats fall into distinct "marine" clusters, only distantly related to clusters comprising also gram+ bacteria from freshwater and soil. The Marine *Actinobacteria* Clade, (46), which includes the "BD1-5 cluster" (15), a deeply branching cluster within the *Actinobacteria* (high GC gram+ bacteria), comprises exclusively marine bacterioplankton phylotypes. The MAR 1 cluster (35) consists of *Actinobacteria* isolates from tropical and subtropical marine sediments, but is branching not as deeply as the Marine *Actinobacteria* Clade.

Quantitative studies on marine gram+ bacteria are still scarce. A biomarker study on the basis of the composition of phospholipid ester-linked fatty acids (PLFA) indicated that gram+ bacteria are major components of bacterial communities in sediments of a eutrophic bay (43). *Actinobacteria* constituted up to 5 % of total bacteria in shallow marine sediments and <1.4 % in an Arctic deep-sea sediment as determined by dot blot and fluorescence *in situ* hybridization (30, 36, 48). Hence, it appears possible that in shallow marine systems with high resuspension and exchange processes between the bulk water and the sediment gram+ bacteria constitute significant components of the bacterial community, not only in sediments but also in the overlaying bulk water. However, the abundance and diversity of gram+ bacteria in these systems has not yet been comprehensively investigated.

The Wadden Sea is a shallow and nutrient-rich tidally affected coastal ecosystem of the southern North Sea stretching from the Netherlands (Den Helder) to Denmark (Esbjerg). Due to the pronounced tidal dynamics and inputs of organic and inorganic nutrients from land, freshwaters, and the North Sea it can be considered as a melting pot in which microbial processes are of major significance (11, 40). In the recent past extensive work on microbial processes and on the composition of bacterial communities in the bulk water, on suspended aggregates and in the sediment of the Wadden Sea was initiated (e.g. 4, 30, 51, 58). Gram+ bacteria were found to constitute between 1 and 5.7 % of total DAPI cell counts in the sediment (30), and one actinobacterium isolate was reported to constitute up to 1 % of total bacteria in the water column (8). One the other hand, gram+ bacteria constituted only one of 24 phylotypes of bacterial communities in this habitat as analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments (58). Another study, applying a culture dependent approach with various isolation procedures including dilution series, yielded 44 gram+ isolates out of a total of 129

and often from high dilution steps (58). The latter results from the Wadden Sea are in contrast to those from studies of the open North Sea in which only very few of the obtained isolates affiliated to gram+ bacteria (12, 63).

In this study we present the phylogenetic affiliation of available gram+ bacteria from the Wadden Sea, mainly derived from 16S rRNA genes from bacterial isolates and a clone library. Bacterial isolates were obtained from various steps of dilution series enriched with a variety of biopolymers. Gram+ bacteria, in particular *Actinobacteria*, have been shown to be capable to degrade various complex polymeric substances such as cellulose and lignin (17, 23, 25). Our results show that most gram+ isolates were derived from enrichments with alginate, agar, starch and MB. Further, the gram+ bacterial community included *Firmicutes* and *Actinobacteria* and was highly divers within the latter group. Next relatives originate from marine as well as from terrestrial and freshwater habitats, reflecting the melting-pot signature of the Wadden Sea.

IV.3 MATERIALS AND METHODS

Sampling. Samples were collected on 27 May and 25 October 1999 in the East Frisian Wadden Sea, Germany (53° 37′ N, 07° 08′ E and 53° 42′ N, 07° 43′ E, respectively). Water samples were taken at high tide with pre-rinsed 10 l-plastic jugs. Sediment cores from an intertidal mixed sand/mud flat were taken only in October with Plexiglas tubes (36 mm diameter) at low tide. Samples were brought to the lab on ice in cooling boxes and processed further within 2 h.

Isolation of bacteria. For the October samples we applied the MPN (most probable number) technique (62) with different substrates and subsequent isolation of bacteria (59). For the May samples dilution series were used for isolation of bacteria. Therefore, 1 ml of bulk water was used as inoculum for 10-fold dilution series. Mineral media amended with various substrates and MB 2216 (Difco, Germany) were prepared as described previously (59). The following substrates were added (0.1 %): agar, alginate, casein, cellulose, chitin, laminarin, dried and pestiled Fucus vesiculosus (a brown algae growing copiously along the coast line of the Wadden Sea), palmitate, starch, and stearate. Growth was checked by turbidity and microscopically. Bacteria were isolated from various dilution steps on agar plates containing the same media as in the MPN assays amended with 1.5 % agar. For further cultivation Marine Agar 2216 (Difco, Germany) was used. Additionally, three isolates were obtained from 1 l-rolling tanks filled with natural seawater and amended with 0.1 % agar and alginate, respectively and incubated for 100 days at 15 °C in the dark. Single colonies were transferred at least five times until considered as pure. The purity of the isolated strains was examined by DGGE analysis according to Brinkhoff and Muyzer (6). Isolates from the same habitat and from assays with the same substrate with sequence similarities of ≥99 % (as determined by a similarity matrix calculated with ARB [31]) were considered identical and only one sequence, either from the highest dilution step or from the highest number of sequenced bp, was used in this study and submitted to GenBank.

Clone library construction. 250 ml of a water sample taken on 25 October was filtered onto a 0.2 µm Nuclepore filter (47 mm diameter). The filter was immediately frozen at –80 °C until DNA-extraction. Bacterial genomic DNA of the sample was isolated after bead beating, phenol-chloroform extraction, and isopropanol precipitation as described previously (32, 56), but slightly modified. Lysozyme treatment was not applied, precipitation done at –20 °C and molecular grade water (Eppendorf, Hamburg, Germany) was used for resuspension at 4 °C over night. PCR amplification of almost complete 16S rRNA gene fragments was performed as previously described (6) with primers GM3F (8F) and GM4R (1492R) (38). Amplification was done in triplicates and the products were pooled prior to cloning. For cloning the pGEM®-T Vector System II (Promega, Madison, USA) was used according to the technical manual of the manufacturer. Clones were picked randomly and sequenced until 50 different clones were obtained. Sequencing of the clones was performed as described (58). Clone sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project II (33).

Sequencing and phylogenetic analysis. PCR amplification of 16S rRNA gene fragments of bacterial isolates and subsequent sequencing was performed as described (6). Sequences were compared with similar sequences of reference

organisms by BLAST search (http://www.ncbi.nlm.nih.gov/blast [1]). Phylogenetic analysis was performed with the ARB software package (http://www.arb-home.de (31). For tree calculation, only sequences with more than 1300 bp were considered using maximum-likelihood analysis. Shorter sequences were added later to the final tree using the maximum parsimony option of the ARB program. Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA, which cause mistakes in tree topology (31). To analyze the phylogeny of gram+ bacteria of the Wadden Sea sequences of gram+ isolates obtained in May 1999 (this study) in October 1999 (59), from a clone library (this study), and from previous studies of the water column of the Wadden Sea using DGGE analysis or cultivation-based methods were considered (5, 8, 19, 53, 58). Sequences obtained from isolates and clones in this study are available from GenBank under accession no. AY370612 to AY370628, and AY370629 to AY370633, respectively.

IV.4 RESULTS

Clone library. Altogether 65 clones were sequenced to obtain 50 different clones. Two of them were identified as chimera. The majority of the remaining clones affiliated to γ- and δ-*Proteobacteria* and about 10 % to gram+ bacteria (Fig. 1.).

Isolation. Seventeen of 63 bulk water isolates (27 %) obtained from the dilution cultures of the May sample were gram+ strains. Isolates affiliating to α- and γ-*Proteobacteria* constituted proportions of 44.4 % and 20.6 %, respectively, and those affiliating to *Bacteroidetes* 7.9 %. In October, the majority of strains affiliated to α- (31.0 %) and γ-*Proteobacteria* (24.0 %) and to *Actinobacteria* (30.2 %), (60). Including the *Firmicutes* isolates (n=6) 45 of the 129 October strains (34.9 %) were gram+ bacteria, of which 35.6 % originated from the bulk water, 20 % from aggregates and the transition zone and 24.4 % from the oxic layer of the sediment. For more details on the isolates obtained in October see Stevens et al. (59). In the other studies included in this analysis only the bulk water (8, 53) or aggregates (19) were investigated.

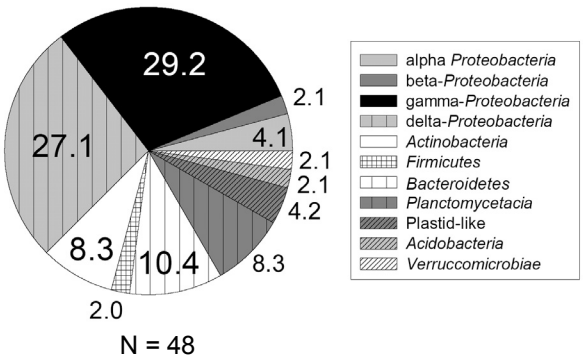


FIG. 1. Percentages of 16S rRNA gene sequences obtained from the clone library of a bulk water sample from the Wadden Sea affiliating with different classes of bacteria and plastids.

Gram+ isolates were obtained with all substrates used. The only isolate obtained with laminarin also affiliated to gram+ bacteria. The highest MPN dilution steps, which yielded isolates, was 10^{-8}. These isolates were derived from bulk water in May with casein and from the sediment

transition zone in October with MB as substrate (59) and affiliated to *Actinobacteria* and *Firmicutes*, respectively. Overall most gram+ isolates obtained in May and October were isolated with alginate (n=11), followed by agar (n=9), MB and starch (n=8). Only few isolates were retrieved from the dilution cultures amended with casein (n=2) and palmitate (n=4). From our isolation approaches in May and October bacteria from all dilution steps were isolated.
Dilution steps 10^{-1}, 10^{-2} and 10^{-4} yielded the highest numbers of gram+ isolates, i.e. 18, 11, and 12, respectively (Fig. 2). From the higher dilution steps fewer isolates were obtained, 7 from 10^{-5}, 4 from 10^{-6}, 3 from 10^{-7} and 2 from 10^{-8}.

Phylogenetic affiliation. A total of 87 gram+ 16S rRNA gene sequences was phylogenetically analyzed (Figs. 3A and B), 81 from isolates, five from the clone library and one from a DGGE band. To avoid an overestimation of diversity, sequences with similarities of ≥99 % were merged into 15 "sequence-groups" (I – XV) resulting in a total of 51 different sequence types (Tab. 1, Fig. 3). Sixty-tree sequences from isolates, four from clones and one from a DGGE band (phylotype GWS-FL-8) affiliated to *Actinobacteria*. The sequences mainly fall into seven families of the order *Actinomycetales*, the *Microbacteriaceae*, *Micrococcaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Nocardioidaceae*, *Promicromonosporaceae*, *Pseudonocardiaceae*, and the *Sanguibacteraceae*. Most sequences group with the *Microbacteriaceae* (18 isolates, 1 clone), with the *Micrococcaceae* (16 isolates) and with the *Nocardioidaceae* (15 isolates).
Because of their low sequence similarity to the next relative, *Streptomyces cinnabarum* and candidatus "*Microthrix parvicella*", clone K39 and DGGE band FL-8 could only be classified on the class-level (Table 1). Clone K46 and isolates GP-5 and GP-6 belong to the order *Actinobacteria* with next related species of the subclass *Frankinae*.

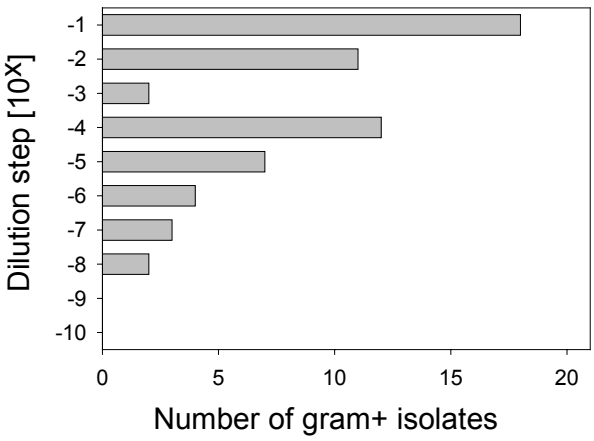


FIG. 2. Numbers of gram+ strains obtained in May and October 1999 from various dilution steps of dilution series of bulk water samples (May and October), of suspended aggregates (October), the sediment surface (October), and the oxic-anoxic transition zone in the sediment (October).

TABLE 1. Clones and isolates obtained from the German Wadden Sea affiliating with gram+ bacteria as well as their closest relatives determined by BLAST analysis (http://www-ncbi.nlm.nih.gov/blast/). Sequences with a similarity ≥99 % were grouped (sequence graoups I-XV), the given information pertains the longest obtained sequence of a sequence group. Given are phylogenetic affiliation, the sequence / isolate ID, and sequence group where applicable, summarized data on sequence or isolate, closest relative according to BLAST analysis, similarity of the 16S rRNA gene (%) and information concerning the closest relative. Remarks on the isolate give the MPN dilution step (dil [10^x]), habitats, substrates, and isolation dates. CAS=casein, CEL=cellulose, CHI=chitin, FUV=*Fucus vesiculosus*, LAM=laminarin, PAL=palmic acid, STA=starch, MB=Marine Broth 2216, MB*=Marine Broth prepared with natural seawater, SW=autoclaved seawater amended with trace elements and vitamins, BW=bulk water, AG=aggregates, SE=sediment, oxic layer, TZ= sediment oxic-anoxic transition zone, tank=rolling tank.

Phylum / *family*	Sequence / isolate ID (representative of sequence group)	Remarks on isolate / summary of sequence group (dil. [10^x], habitat, substrate, date)	Closest relative (acc. number)	[%]	Habitat or environmental features of closest relative
Actinobacteria					
Microbacteriaceae	GWS-SE-H242a	-2, SE, PAL, Oct 99	*Microbacterium* sp. OS-6 (AJ296094)	99	coastal marsh (Galicia, Spain)
	GWS-AG-H197	-3, AG, CHI, Oct 99	*Microbacterium* sp. V4.BP.11 (AJ244677)	98	marine bacterioplankton (Mediterranea)
	GWS-TZ-H305	-2, TZ, FUV, Oct 99	*Microbacterium esteraromaticum* (Y17231)	95	soil
	GWS-TZ-H139	-1, ALG, TZ, Oct 99	*Microbacterium testaceum* SE034 (AF474327)	97	endophytic, agronomic crop
	GWS-BW-H60M (Sequence-group I)	-8, -7, -6, -1; BW, SE, TZ, AGA, CAS, CEL, STA; May, Oct 99	*Microbacterium* sp. VKM Ac-2050 (AB042084)	99	plant nematode
	GWS-SE-H300	-5, ALG, SE, Oct 99	*Microbacterium* sp. LB030 (AF474326)	99	endophytic, prairie plant
	GWS-SE-H149	-2, CEL, SE, Oct 99	Gram+ bacterium strain 12-8 (AB008510)	99	copiotrophic, urban soil
	GWS-BWrt-H97M (Sequence-group II)	-1, rt; BW, AG, AGA, CAS, MB*, STA; May, Oct 99	Marine bacterium P_wp0234 (AY188942)	98	deep sea sediment/degrading PAH
	GWS-SE-H243	-2, SE, PAL, Oct 99	*Frigoribacterium faeni* (Y18807)	98	psychrophilic, non-marine
	Clone GWS- K13	From clone-library, BW, Oct 99	Actinobacterium MWH-Dar4 (AJ565416)	98	0.2 µm filtered freshwater
Sanguibacteraceae	GWS-AG-H192	-3; AG, CHI, Oct 99	*Cellulomonas fermentans* (X79458)	94	municipal dumping site
Promicromonosporaceae	GWS-TZ-H118	-5, TZ, AGA, Oct 99	*Cellulomonas* sp. IFO16243 (AB023364)	96	no information available
Micrococcaceae	GWS-BW-H45M (Sequence-group III)	-5, -4, -1; BW, ALG, CEL, MB; May 99	*Arthrobacter nicotianae* SB42 (AJ315492)	97	starter culture (cheese)
	GWS-SE-H161	-2, SE, CEL, Oct 99	Bacterium PS32 (AF200218)	99	psychrophilic, marine
	GWS-BW-H126 (Sequence-group IV)	-4, -2; BW, SE; ALG, CEL, MB*; Oct 99	Bacterium isolate SI-12 (AJ252579)	99	agricultural soil
	GWS-BW-H15M (Sequence-group V)	-7, -2, -1; BW; CEL, FUV, MB; May, Oct 99	*Micrococcus luteus* strain Ballarat (AJ409096)	99	activated sludge
	HP42	From aggregates	*Micrococcus* sp. V4.MO.20 (AJ244665)	98	marine bacterioplankton (Mediterranea)
	GWS-BWrt-H158 (Sequence-group VI)	-4, -2, rt; BW; CEL, STA; May, Oct 99	*Kocuria rosea* (Y11330)	99	soil and water
Mycobacteriaceae	GWS-BW-H82M	-1, BW, MB, May 99	*Mycobacterium* sp. IP20010961 (AY163341)	99	water supplies
	GWS-BW-H50M	-1, BW, STA, May 99	*Mycobacterium* sp. TH-2003 (AY266138)	98	associated with sepsis

TABLE 1. continued

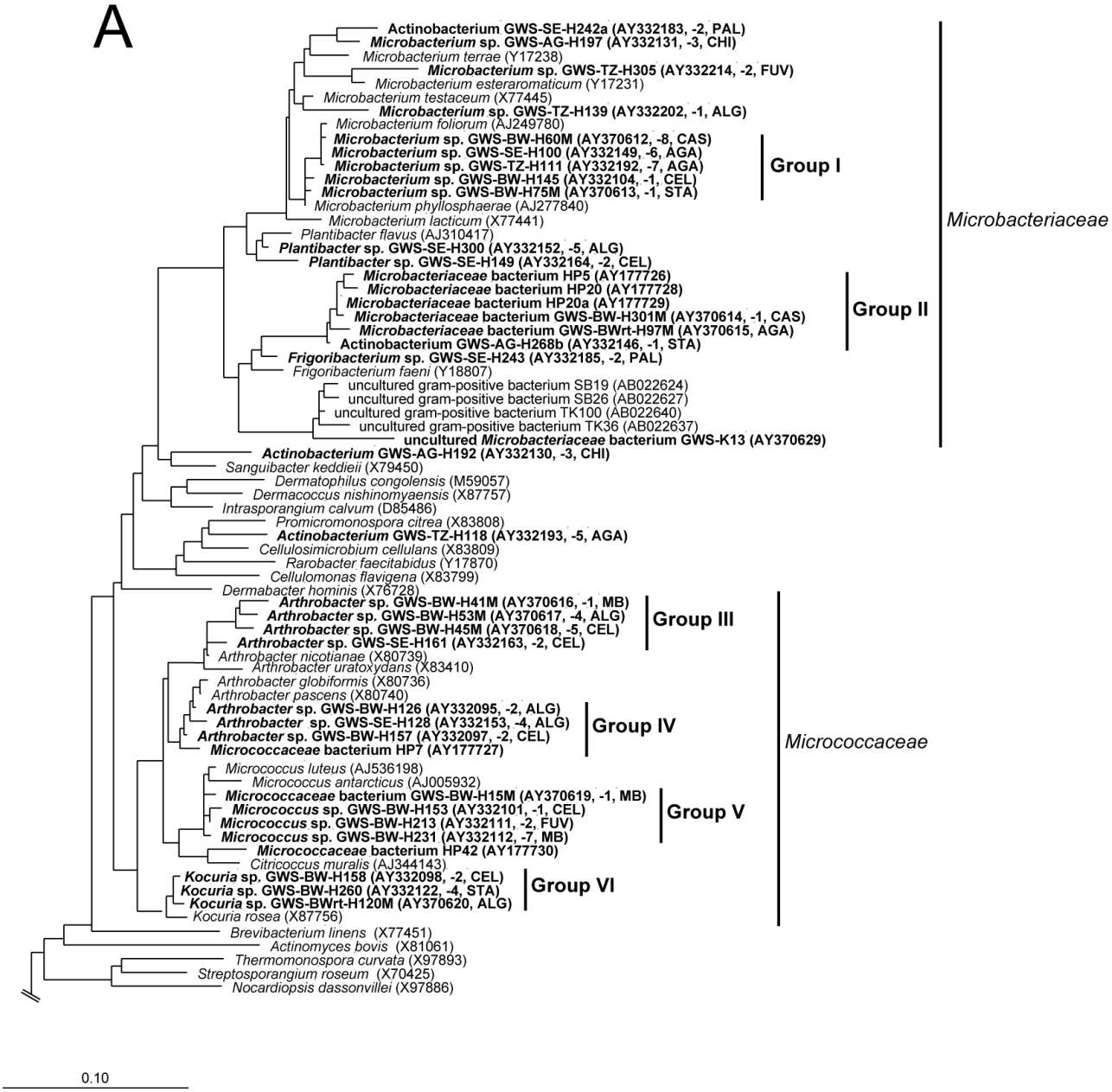
Phylum / *family*	Sequence / isolate ID (representative of sequence group)	Remarks on isolate / summary of sequence group (dil. [10^x], habitat, substrate, date)	Closest relative (acc. number)	[%]	Habitat or environmental features of closest relative
Nocardiaceae	GWS-BWrt-H95M	rt, AGA, May 99	*Rhodococcus* sp. UFZ-B520 (AF235011)	98	aquifer / degrading chlorobenzene
	GWS-TZ-H135 (Sequence-group VII)	-4, -1; BW, TZ; ALG; Oct 99	*Rhodococcus fascians* KM6 (AJ011329)	100	humus (spruce stands)
	GWS-SE-H175	-1, SE, CHI, Oct 99	*Rhodococcus* sp. MBIC01430 (AB088667)	99	no infomation available
	GWS-TZ-H309 (Sequence-group VIII)	-6, -5, BW, TZ, FUV, Oct 99	*Rhodococcus tukisamuensis* (AB067734)	98	depolymerizing, from soil
Pseudonocardiaceae	*Pseudonocardiaceae* bacterium T4	-1, BW, MB, Oct 99	*Pseudonocardia alni* IMSNU 20049 (AJ252823)	99	root nodules of alders
	GWS-BW-H127 (Sequence-group IX)	-2,-1; AG, BW, AGA, ALG, Oct 99	*Pseudonocardia alni* IMSNU 20049 (AJ252823)	99	root nodules of alders
Nocardioidaceae	GWS-BW-H99 (Sequence-group X)	-6, -5, -4, -1; AG, BW, SE; AGA, CHI, FUV, PAL, STA; Oct 99	Uncult. actinobacterium (AB074621)	97	aposymbiotic pea aphids
	GWS-BW-H259	-4, BW, STA, Oct 99	Uncult. *Nocardioides* sp. GCPF40 (AY129808)	98	nutrient-limited cave
	GWS-BW-H311M	-1, BW, LAM, May 99	*Nocardioides* sp. MWH-CaK6 (AJ565419)	99	0.2 µm filtered freshwater
	GWS-AG-H266	-4, AG, STA, Oct 99	*Nocardioides* sp. V4.BE.17 (AJ244657)	97	marine bacterioplankton (Mediterranea)
	GP-1	-4, estuary: mar., Aug 99	*Nocardioides* OS4 (U61298)	98	oil shale column (oxic zone)
	Aeromicrobium marinum	-8, BW, MB, Oct 99	*Aeromicrobium fastidiosum* (Z7820)	97	herbage
	GWS-BW-H252	-2, BW, PAL, Oct 99	*Nocardioides* sp. NCFB3005 (X76178)	97	No information available
	GWS-BW-H89M	-4, BW, ALG, May 99	*Nocardioides* sp. 2.20 (AJ299233)	98	freshwater biofilm
	GWS-BW-H84M	-4 BW, STA, May 99	*Nocardioides jensenii* KCTC 9134 (AF005006)	97	soil
uncertain actinomycetes	GP-5	-7, estuary: brack., Aug 99	Unident. bacterium strain rJ7 (AB021325)	97	activated sludge (0.5 g phenol)
	GP-6	-6, estuary: brack., Aug 99	Unident. bacterium strain rJ7 (AB021325)	96	activated sludge (0.5 g phenol)
	Clone GWS-K46	From clone library, BW, Oct 99	Unident. bacterium strain rJ7 (AB021325)	96	activated sludge (0.5 g phenol)
	Clone GWS-K39	From clone library, BW, Oct 99	Uncultured bacterium AT425_EubY10 (AY053479)	90	Gulf of Mexico gas hydrates
	Clone GWS-K11	From clone library, BW, Oct 99	Unidentified bacterium clone K2-30-12 (AY344421)	98	Hawaiian archipelago
	GWS-FL-8	DGGE band May-Aug 99	Uncultured actinobacterium clone SAa03 (AY124414)	99	marine sediment
Firmicutes					
Bacillaceae	GWS-SE-H117 (Sequence-group XI)	-7; AG, SE; AGA, MB*; Oct 99	"*Bacillus baekryungensis*" (AF541965)	99	seawater (Korea)
	HP 8	From aggregates, MB*	*Bacillus* sp. KMM3737 (AY228462)	99	seawater (Korea)
	GWS-BW-H68M (Sequence-group XII)	-1; BW; MB*, STA; May 99	*Bacillus pumilus* OM-F6 (AB020208)	98	No information available
	HP 10	From aggregates, MB*	Bacterium KA64 (AY345445)	95	Hawaiian archipelago
	GWS-BW-H220M (Sequence-group XIII)	-6, -1; BW, MB; May, Oct 99	*Bacillus licheniformis* Mo1 (AF372616)	99	GTN degrading
	GWS-TZ-H114	-2, TZ, AGA, Oct 99	*Bacillus* sp. HT-1 (AF463535)	96	hamster feces
Planococcaceae	GP14	-5, estuary: marine,	"*Planococcus*	99	No information

Twelve of the 51 gram+ sequence types obtained from the Wadden Sea, exhibit a closest relative of marine origin, 33 of other environments, such as freshwater, soil, a plant, or endophytic habitats. For 6 next related sequences no information on the source of isolation could be obtained (Table 1). Our strains affiliating with the *Micrococcaceae* were mainly isolated from BW, isolates affiliating with the *Nocardioidaceae* from BW and AG. For all other sequences and isolates no relationship exists between phylogeny and habitat or substrate from which they were obtained. Eight of our strains, 10 from other studies and one clone (K48, not shown in Fig. 3B) affiliate to the phylum *Firmicutes*. Fifteen of the isolates were merged into five sequence groups. All isolates affiliate to the order *Bacillales* and, except sequence group XIV (GP-13, GP-14) and isolate GWS-TZ-H232, fall within the family *Bacillaceae*. Sequence group XIV affiliates to the *Planococcaceae*. The clone K48 affiliates within the class *Clostridia* to the "*Peptostreptococcaceae*" (family name not validly published), with the next relative *Fusibacter paucivorans* (Tab. 1). Isolates affiliating to the *Firmicutes* were never obtained from aggregates, but from the sediment layers and the bulk water. They were obtained from assays performed with MB, agar, alginate, and stearine.

IV.5 DISCUSSION

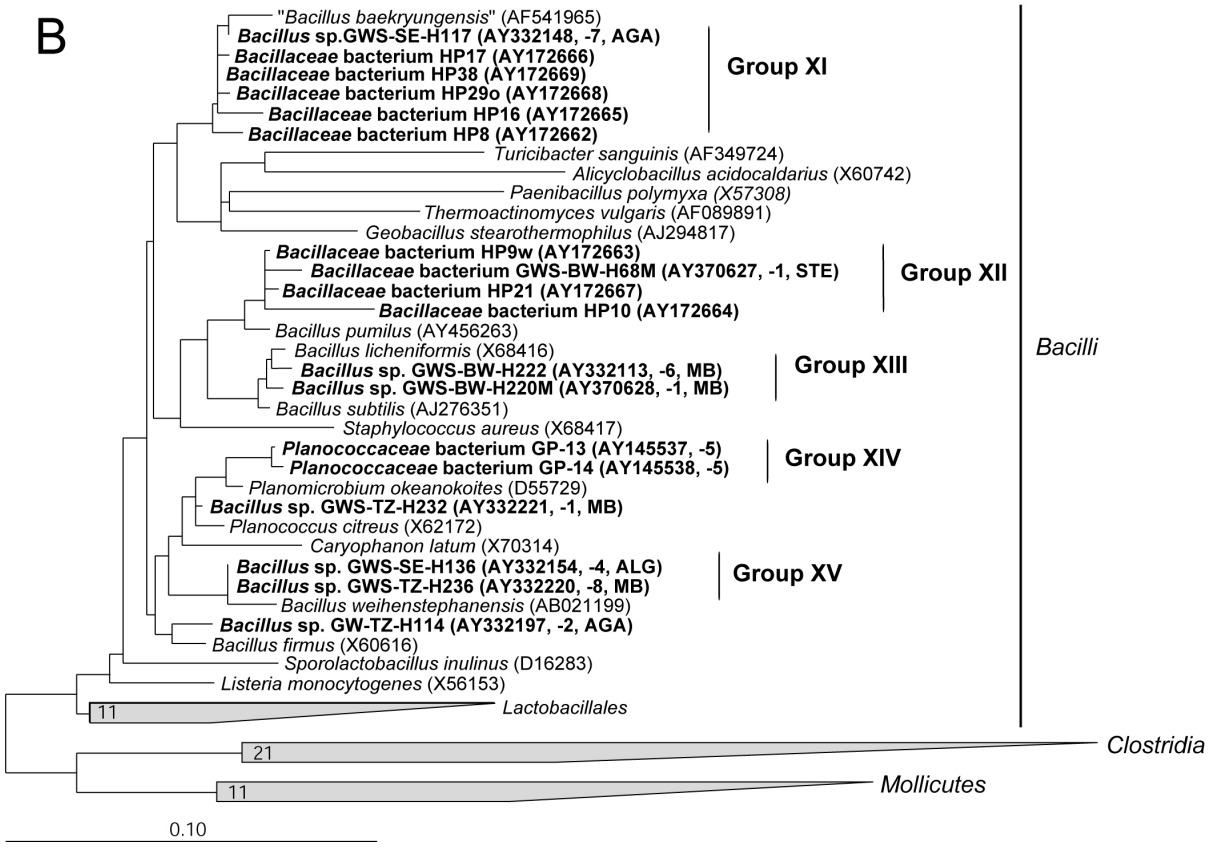
Gram+ bacteria comprised 25 and 34 % of all isolates we retrieved in May and October 1999 (59) respectively, and the majority affiliated to *Actinobacteria*, indicating that the latter constituted a substantial fraction of the cultivable bacterial community in the bulk water as well as in sediments of the Wadden Sea. Because the gram+ bacteria were obtained with various substrates we assume that the frequent isolation of these organisms was not due to a cultivation bias. Even though most of the isolates were retrieved from low dilution steps, several isolates from various habitats were obtained from 10^{-6} to 10^{-8} dilutions, suggesting that some of them are significant constituents of the bacterial community in this ecosystem. This is particularly true for sequence groups I and X.Three of the strains within group I were obtained from 10^{-6} or higher, and 6 of the 7 strains in group X were obtained from 10^{-4} or higher (Fig. 1). We did not examine the bacterial diversity in the dilution steps, e.g. by DGGE, and therefore do not know how many phylotypes were present. A study in the Weser estuary, adjacent to the Wadden Sea, which analyzed the ambient bacterial community in the Baltic Sea (7).

On this and following two pages:
FIG. 3. Maximum likelyhood trees of all gram+ isolates and clones obtained from the Wadden Sea (bold) showing the affiliation within the *Actinobacteria* (A) and the *Firmicutes* (B). Sequences <1300 bp were added with maximum parsimony. The scale bars indicate 10 % sequence divergence. The Marine Actinobacteria Clade was adopted from Rappé et al. (46) and the MAR 1 cluster from Mincer et al. (35). Isolates from May are marked with an "M" at the end of the name. If available, dilution step and substrate were added to the accession number (CAS = casein, CEL = cellulose; CHI = chitin, FUV = *Fucus vesiculosus*; LAM = laminarin, PAL = palmitate; STA = starch, MB = Marine Broth 2216, MB* = Marine Broth 2216 prepared with natural sea water (19), SW = autoclaved natural seawater amended with trace elements and vitamins (53). The sub-habitat can be derived from the name of the May and October isolates (BW = bulk water, AG = aggregates, SE = sediment surface, TZ = oxic /anoxic transition zone of the sediment). Numbers on branches with pooled sequences indicate the number of sequences used to calculate the cluster.



The small sample volumes with a transfer volume in the µl range presumably lead to a much higher transfer variability in the dilution steps than larger volumes such as 1 ml for the 10 ml sample volumes as applied in our study. Based on our results from the dilution series and MPN statistics and on published bacterial numbers in the bulk water and sediments (30, 58) we assume that the gram+ bacteria constitute, as an upper limit, 5-10 % of total bacterial numbers in the bulk water and the sediment transition zone. Our estimates of the percentages of gram+ bacteria are in the same range as those obtained by fluorescence *in-situ* hybridization (FISH) with probes specific for *Actinobacteria* in the upper 4 cm of Wadden Sea sediments, <1 to 3.6 % (30) and also as those for the genus *Streptomyces* detected in a shallow marine sediment by dot blot hybridization with a genus-specific probe (2.0 to 5.1 % of total extracted rRNA, [36]). Studies based on PLFA indicate that gram+ bacteria are important components of the bacterial communities in eutrophic bays in Japan (42-44). Interestingly, one new *Aeromicrobium* species, *A. marinum*, recently isolated from the Wadden Sea, was shown to constitute ~1 % of total bacteria in the bulk water of this habitat (8). Hence, some gram+ bacteria appear to be important components of the bacterial community in the Wadden Sea, whose biogeochemical significance needs be elucidated.

Several other recent studies also isolated *Actinobacteria* from various marine environments, but they constituted lower fractions of all isolates as compared with our results. In studies from various regions including the German Bight of the North Sea, the Baltic Sea and the Oregon coast of the Pacific, which examined the diversity of isolates from bacterioplankton samples and enrichments with various substrates, between <1 and 15 % of all isolates affiliated to *Actinobacteria* (12, 21, 60, 63). In tropical and subtropical marine sediments, Mincer et al. (35) isolated over 200 marine *Actinomycetes* affiliating to the newly defined cluster MAR 1. Jensen and Fenical (26) isolated >180 gram+ bacteria from tropical sediments of which the great majority affiliated to *Firmicutes*. However, no information is given in these studies



on the total number of isolates retrieved. Our results indicated that in near-shore environments such as the Wadden Sea they constitute a substantial fraction of this community, possibly because of exchange processes with the sediment.

The proportion of gram+ clones in our clone library of a bulk water sample (10 %) is in the same range as that found by other studies in coastal and open ocean environments (15, 46, 60) but lower than that reported from sediments from bays in Japan, where 20 and 35 % of all clones affiliated to gram+ bacteria (18, 64). In line with the culture-dependent approaches these data indicate that gram+ bacteria appear to constitute substantial proportions of bacterial communities in coastal ecosystems and in particular in sediments.

Our phylogenetic analysis of sequences from the clone library and the isolates shows a surprisingly high diversity of the gram+ bacteria. The diversity within *Actinobacteria* we detected was greater than that described in other studies using either culture-dependent (26, 35, 60) or culture–independent approaches (15, 18, 46, 60, 64). This may be due to the various isolation procedures we applied, such as dilution series and different substrates, but may also reflect the specific signature of the Wadden Sea ecosystem as a melting pot with marine as well as terrestrial impacts (see below). None of our sequences, however, affiliates to the Marine *Actinobacteria* Clade (46) or the MAR 1 cluster (35), consisting exclusively of marine members. About 65 % of the *Actinobacteria* sequence types we detected are related most closely to isolates and phylotypes from freshwater and terrestrial environments, and only 23.5 % are affiliated to marine gram+ bacteria. In contrast, most clones and isolates retrieved from marine environments in other studies are only distantly related to terrestrial isolates (47, 64) or were proven to be obligatory marine as tested by their requirements for seawater and sodium (26, 35). In line with our results, the recently described *Aeromicrobium marinum* (see above), the first described marine species within the family *Nocardioidaceae*, clusters with terrestrial isolates but exhibits a requirement for seawater typical of marine bacteria (8). We did not test the seawater or sodium requirements of our isolates, but they grew well (transferred at least 7 times) on marine media (~ 30 ‰ salinity). Because they were isolated from a marine habitat on marine media, the isolates are literally defined as marine, like other authors proposed (13, 65). The fact that at least the isolates we retrieved from higher dilution steps (see above) obviously prosper in the Wadden Sea, may reflect that this shallow marine ecosystem with limited water exchange to the German Bight of the North Sea, due to its protection by a chain of islands (11), is more exposed to terrestrial impacts than other coastal areas. This notion may further explain that quite a few of our isolates cluster together with those from soil and freshwater habitats but does not rule out that truly marine ones are among them, as demonstrated by the *Aeromicrobium* isolate (8). In fact, because of its particular signature the Wadden Sea exhibits a great variety of habitats, which may explain the surprisingly high diversity of *Actinobacteria*.

The fact that we successfully enriched and isolated *Actinobacteria* from different habitats of the Wadden Sea with various biopolymers shows that these strains are capable of degrading a variety of polymeric substances. Some of these substances are typical for coastal marine environments such as *F. vesiculosus*, cellulose, starch, chitin, and laminarin. *Actinobacteria* are well known to be capable to degrade various polymeric substances such as cellulose and lignin, but also rubber and polyester (17, 23, 25, 29, 41). Their hydrolytic potential appears comparable or even greater than that of the *Sphingobacteria* and *Flavobacteria* group of the *Bacteroidetes* phylum (formerly *Cytophaga-Flavobacteria-Bacteroides* group; 27,

49). It may explain why *Actinobacteria* prosper in the bulk water and sediment of the Wadden Sea and other coastal environments which are characterized by high concentrations of various biopolymers (24). The fact that several of our strains, clustering closely together or belonging to the same sequence group, were isolated from different habitats and with different media further suggests that they are able to degrade various biopolymers and area adapted to various environments.

In studies applying DGGE with eubacterial primers gram+ bacteria were rarely detected in the Wadden Sea (52, 58), which appears surprising on the basis of our findings and may be due to methodological biases. The cells of gram+ bacteria are more difficult to lyse than those of gram-negative bacteria (39) and the DNA extraction procedure we applied is optimized for the latter. However, most of our isolates tested on DGGE exhibited distinct bands. Another reason for not detecting 16S rRNA gene fragments of gram+ bacteria by DGGE may be the fact that each gram+ strain or phylotype constituted <1 % of the extracted DNA, which is the detection limit of the DGGE method with eubacterial primers for a target sequence (37). The application of primer systems specific for this phylogenetic group appears more appropriate to analyze the diversity of gram+ bacteria by DGGE (55).

In contrast to sequences affiliating to *Actinobacteria*, sequences from the Wadden Sea affiliating to *Firmicutes* were much less divers. Sequences of all bacterial isolates of this group clustered within the class *Bacilli*. The only clone obtained from the *Firmicutes* belongs to the class *Clostridia*. These results are in line with most other reports (16, 26, 57). Since the early 1970s various *Bacilli* were isolated from marine habitats (e.g., 2, 3, 10, 20, 54), but only very few marine isolates affiliated to the classes *Lactobacillales*, *Clostridia,* and *Mollicutes* (14, 18, 34, 61). Hence, *Bacilli* seem to be the most abundant marine *Firmicutes*. Further, the available reports suggest that *Firmicutes* in marine coastal environments are of lower significance than *Actinobacteria*.

Our analysis of the community of gram+ bacteria in the Wadden Sea showed that it was surprisingly divers, mainly consisting of various groups of *Actinobacteria*, and to a much lesser extent of *Firmicutes*. Phylotypes and isolates mainly clustered closely together with sequences of terrestrial origin even though our isolates appear to exhibit truly marine features. On the basis of our dilution cultures and of other studies we estimate that gram+ bacteria constitute 5 to 10 % of total bacteria in the water column and sediment of the Wadden Sea. Hence, they appear to be prominent members of the bacterial communities in the Wadden Sea and, presumably because of their high potential to degrade various biopolymers, are important in the turnover and decomposition of organic matter in this ecosystem.

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V

PHYLOGENY OF *PROTEOBACTERIA* AND *BACTEROIDETES* FROM OXIC HABITATS OF A TIDAL FLAT ECOSYSTEM

Phylogeny of *Proteobacteria* and *Bacteroidetes* from Oxic Habitats of a Tidal Flat Ecosystem

Running title: Phylogeny of Bacteria from a Tidal Flat

Key words: *Proteobacteria*, *Bacteroidetes*, phylogeny, Wadden Sea, polymers

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V.1 SUMMARY

Bacteria of the phyla *Proteobacteria* and *Bacteroidetes* are known to be the most prominent heterotrophic organisms in marine surface waters. In order to investigate the occurrence of these phyla in a coastal environment, the tidal flat ecosystem German Wadden Sea, we analysed a clone library of PCR amplified and sequenced 16S rRNA gene fragments and isolated 46 new strains affiliated with these phyla from the water column with various polymers and complex media as substrates. The phylogenetic affiliation of these strains was analysed on the basis of sequenced 16S rRNA gene fragments. Subsequently a comprehensive phylogenetic analysis of *Proteobacteria* and *Bacteroidetes* including available sequences from oxic habitats of earlier studies of this ecosystem was performed. Sequences of the earlier studies were derived from isolation approaches and from denaturing gradient gel electrophoresis (DGGE) analyses of environmental samples and high dilution steps of MPN (most probable number) cultures. The majority of the 265 sequences included in this analysis affiliated with α-*Proteobacteria* (45.3%), γ-*Proteobacteria* (31.7%), and *Bacteroidetes* (16.2%). Almost seven percent belong to the δ-*Proteobacteria* and several of these clones affiliated with the *Myxococcales*, a group comprising obligate aerobic organisms. Within the α- and γ-*Proteobacteria* specific clusters were identified including isolates from high dilution steps of dilution cultures and/or clones from the clone library or DGGE gels, implying a high abundance of some of these organisms. Within the γ-*Proteobacteria* a new cluster is proposed, which consists of marine surface-attached organisms. This SAMMIC (Surface Attached Marine MICrobes) cluster comprises predominantly phylotypes and exhibits a global distribution with a particular emphasis on polar, subpolar and temperate regions. Overall the analysis indicates that *Proteobacteria* and *Bacteroidetes* of the Wadden Sea have a surprisingly high diversity, presumably a result of the signature of this ecosystem as a melting pot at the land-sea interface and comprising a great habitat variety.

V.2 INTRODUCTION

During the last years, in most studies, which analyzed the structure of marine bacterial communities, culture-independent methods were used and only relatively few studies are available which included culture-dependent approaches (e.g., Gray and Herwig, 1996; Suzuki *et al.*, 1997; Eilers *et al.*, 2000; Selje and Simon, 2003, 2004). Culture-independent methods such as the construction of clone libraries (e.g., Giovannoni *et al.*, 1990; Cottrell and Kirchman, 2000a), denaturing gradient gel electrophoresis of PCR amplified 16S rRNA gene fragments (DGGE, Muyzer *et al.*, 1998), and fluorescence in situ hybridization (FISH; Amann *et al.*, 1995) aim to assess the diversity of and quantify selected phylotypes or phylogenetic groups in a given habitat. These attractive methods, however, may be biased by selective PCR amplification from mixed templates (e.g., Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Cottrell and Kirchman, 2000a; Ishii and Fukui, 2001) and unspecific probe hybridization (Kirchman *et al.*, 2003). Classic cultivation approaches, preferentially using solid media, have shown that only a minor fraction, i.e. < 1% of total bacterial numbers, can be cultivated (Staley and Konopka, 1985). Refined cultivation approaches recently succeeded in isolating many new and abundant bacteria including ones which affiliate to clusters of so far uncultured phylotypes (Bruns *et al.*, 2002; Connon and Giovannoni, 2002; Rappé *et al.*, 2002; Bruns *et al.*, 2003; Hahn *et al.*, 2003; Selje and Simon, 2004). Only cultivation allows a comprehensive physiological characterization of relevant members of bacterial communities and, therefore, is an important complement of culture-independent studies of these communities in aquatic and other environments.

Proteobacteria and *Bacteroidetes* were frequently found to be the dominant bacterial phyla in marine ecosystems (e.g., Llobet-Brossa *et al.*, 1998; Glöckner *et al.*, 1999; Simon *et al.*, 1999; Eilers *et al*., 2000; Hagström *et al.*, 2000; Cottrell and Kirchman, 2000a, 2000b). While β-*Proteobacteria* are usually predominant in lakes and rivers (Pernthaler *et al.*, 1998; Glöckner *et al*., 1999; Böckelmann *et al.*, 2000; Schweitzer *et al.*, 2001), α- and γ-*Proteobacteria* were often found in high abundances in marine systems (e.g., Glöckner *et al*., 1999; Eilers *et al*., 2000; Cottrell and Kirchman, 2000a, 2000b).

Tidal flat systems occur at the land-sea interface in tropical and temperate regions and are one of the most productive coastal marine ecosystems (Alongi, 1998). The Wadden Sea is such a shallow, nutrient-rich, and tidally affected coastal ecosystem located in the southern North Sea and stretches from the Netherlands along the German coast to Denmark. Microbial processes are of major significance in this system (Dittmann, 1999; Poremba *et al.*, 1999), but only scarce information exists on the structure of its bacterial communities in particular in the water column. Some information based on culture independent approaches exists on sediment-associated bacterial communities, e.g. on the sulfate reducing subcommunity (Llobet-Brossa *et al.*, 1998; Böttcher *et al.*, 2000; Rütters *et al.*, 2002), and a few isolates from selected physiological groups from sediments are also available (Brinkhoff and Muyzer, 1997; Llobet-Brossa *et al.*, 2002; Mussmann *et al.*, 2003). Recently, bacterial communities in the water column, freely suspended, and associated to suspended aggregates were studied by culture-independent (DGGE, FISH) and culture-dependent approaches in the polyhaline section of the Weser estuary, which is part of the Wadden Sea (Selje and Simon, 2003, 2004). In previous

publications we extended these studies to another, non-estuarine Wadden Sea location by also including the sediment surface-associated bacterial community, applying the DGGE approach (Stevens *et al.*, 2004a), and dilution series with various biopolymers and complex media as substrates to obtain a wide array of isolates (Stevens *et al.*, 2004b). The aim of the present study was to comprehensively assess the diversity of *Proteobacteria* and *Bacteroidetes* of the free-living, aggregate-, and oxic sediment-associated bacterial communities in the Wadden Sea. Therefore, we compiled available information on the occurrence of these groups in the Wadden Sea and analyzed the phylogenetic affiliation of their 16S rRNA genes. To increase the basis for this analysis we extended the available information twofold: I) Further bacteria from another location and from various dilution steps of dilution series amended with biopolymers and other organic substrates were isolated and their 16S rRNA genes sequenced; II) a clone library was constructed from PCR amplified 16S rRNA gene fragments of a bulk water sample and the clones were sequenced. These new sequences together with all currently available sequence information on 16S rRNA genes of α-, γ- and δ-*Proteobacteria* and *Bacteroidetes* from oxic habitats of the Wadden Sea enabled us to perform a comprehensive phylogenetic analysis. The results indicate that *Proteobacteria* and *Bacteroidetes* of the Wadden Sea exhibit a surprisingly high diversity, presumably an effect of the signature of this ecosystem as a melting pot at the land-sea interface and comprising a great habitat variety

V.3 RESULTS

Isolation of bacteria

From the water sample taken in May 1999 (sampling site A, Fig. 1) we isolated 63 different strains. Sequencing of the 16S rRNA genes and subsequent BLAST analysis showed that 28 of these strains belong to α-, 13 to γ-*Proteobacteria*, and five to *Bacteroidetes*. Seventeen isolates belong to the Gram-positive bacteria. The highest proportions of different strains were obtained with agar, alginate and casein (14.3% each), the lowest with laminarin and stearic acid (3.2% each). With cellulose and marine broth 2216 11.1% (each) of all different strains were obtained, 9.5% with *F. vesiculosus*, and 6.4% with chitin, palmitate, and starch, respectively. Information concerning the substrate of the isolation procedure, dilution steps of a dilution series, or isolation from a rolling tank (marked by "rt"), or a batch culture (marked by "ba") are given in the phylogenetic trees (Fig. 3A-D). Isolates from May are marked by an "M" at the end of their identification number

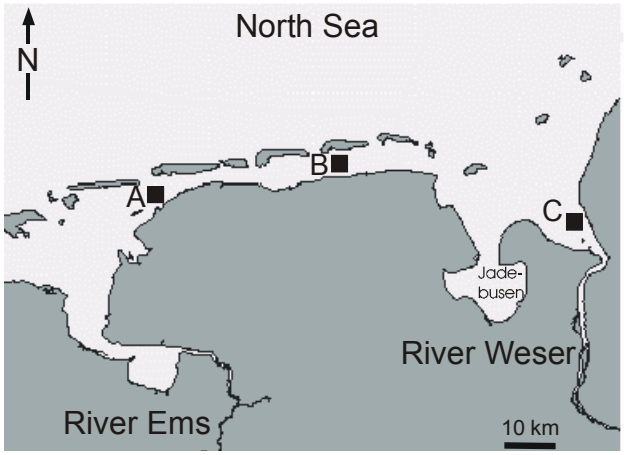


Fig. 1. Map of the study area and sampling sites (A, B, C) in the German Wadden Sea, southern North Sea. Isolates of this study were obtained from position A, the sample for the clone library from position B. Samples of most other studies were taken at position B and C.

Clone library

From 65 sequenced clones, 50 different clones were obtained, of which two were identified as chimera. The majority of the other sequences affiliate to γ- and δ-*Proteobacteria* (14 and 13, respectively). Lower numbers were found for *Bacteroidetes* (5), *Planctomycetes* (4), *Actinobacteria* (4), α-*Proteobacteria* (2), *Firmicutes* (1), β-*Proteobacteria* (1), *Acidobacteria* (1), and *Verrucomicrobia* (1). In addition, two of the obtained clones affiliate to plastid-like sequences. Identical sequences were found only for clones affiliating with γ- and δ-*Proteobacteria* (9 and 5, respectively), and for one plastid-like sequence..

Phylogenetic analysis

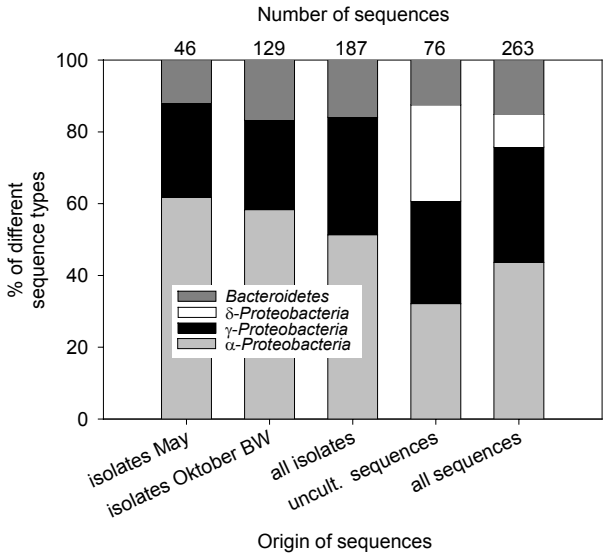


Fig. 2. Percentages of sequences of isolates and clones obtained from oxic habitats of the Wadden Sea and affiliating to α-, γ-, δ-*Proteobacteria* and *Bacteriodetes*. Data of this study (Isolates May), a previous study (Isolates October; Stevens *et al*. 2004b), all isolates, all uncultured sequences and all sequences are shown. BW=bulk water.

A total of 265 sequences from this and 11 other studies were considered (Brinkhoff and Muyzer, 1997; Bruns and Berthe-Corti, 1999; Bruns *et al.*, 2001; Llobet-Brossa *et al*., 2002; Mussmann *et al*., 2003; Selje and Simon, 2003; Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Selje and Simon, 2004; Stevens *et al*., 2004a; Stevens *et al*., 2004b). Of the 265 sequences 45.3% affiliate to α-, 31.7% to γ-, 6.8% to δ-*Proteobacteria*, and 16.2% to *Bacteroidetes*. The sequences were obtained from 188 isolates and 77 clones (Fig. 2). The clones were derived from the clone library (44.1%), and from DGGE analyses of environmental samples (35.1%), and dilution cultures (20.8%). While no strain of the δ-*Proteobacteria* was isolated, 90 isolates (47.9%) affiliate with α-*Proteobacteria*, 62 (33.0%) with γ-*Proteobacteria*, and 36 (19.1%) with *Bacteroidetes*. Overall, sequences affiliate with 15 different orders of *Proteobacteria*, i.e. with five orders of α-*Proteobacteria*, six orders of γ-*Proteobacteria*, and four orders of δ-*Proteobacteria* (Fig. 3A-C). Further, sequences affiliate with all three orders of *Bacteroidetes*, however, only two sequences from the clone library fall within the *Bacteroidales* (Fig. 3D). Some sequences could not be assigned to an existing order, because some taxonomic units, e.g., the SAR116 and SAR86 clusters, are only classified on the class level.

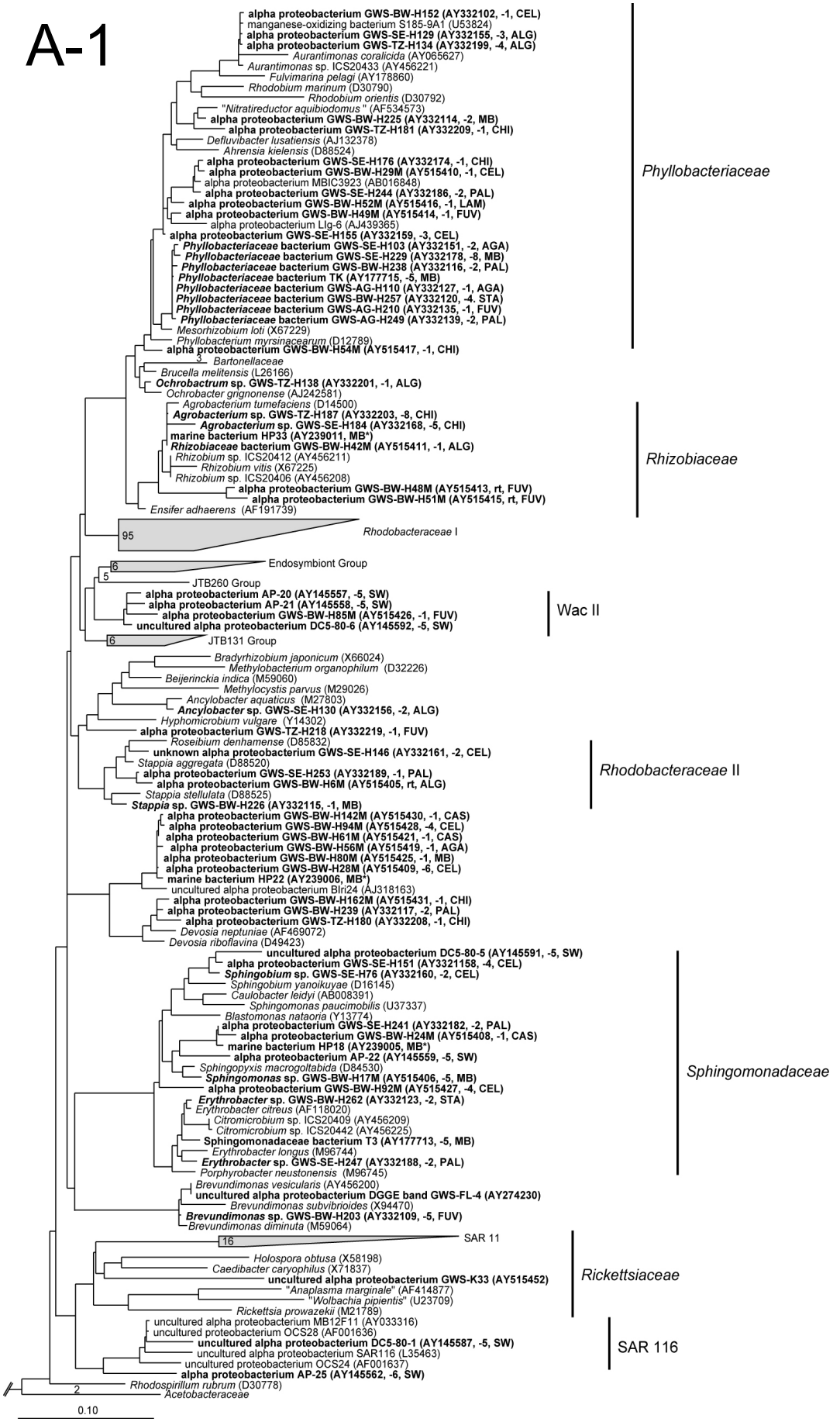
α-Proteobacteria

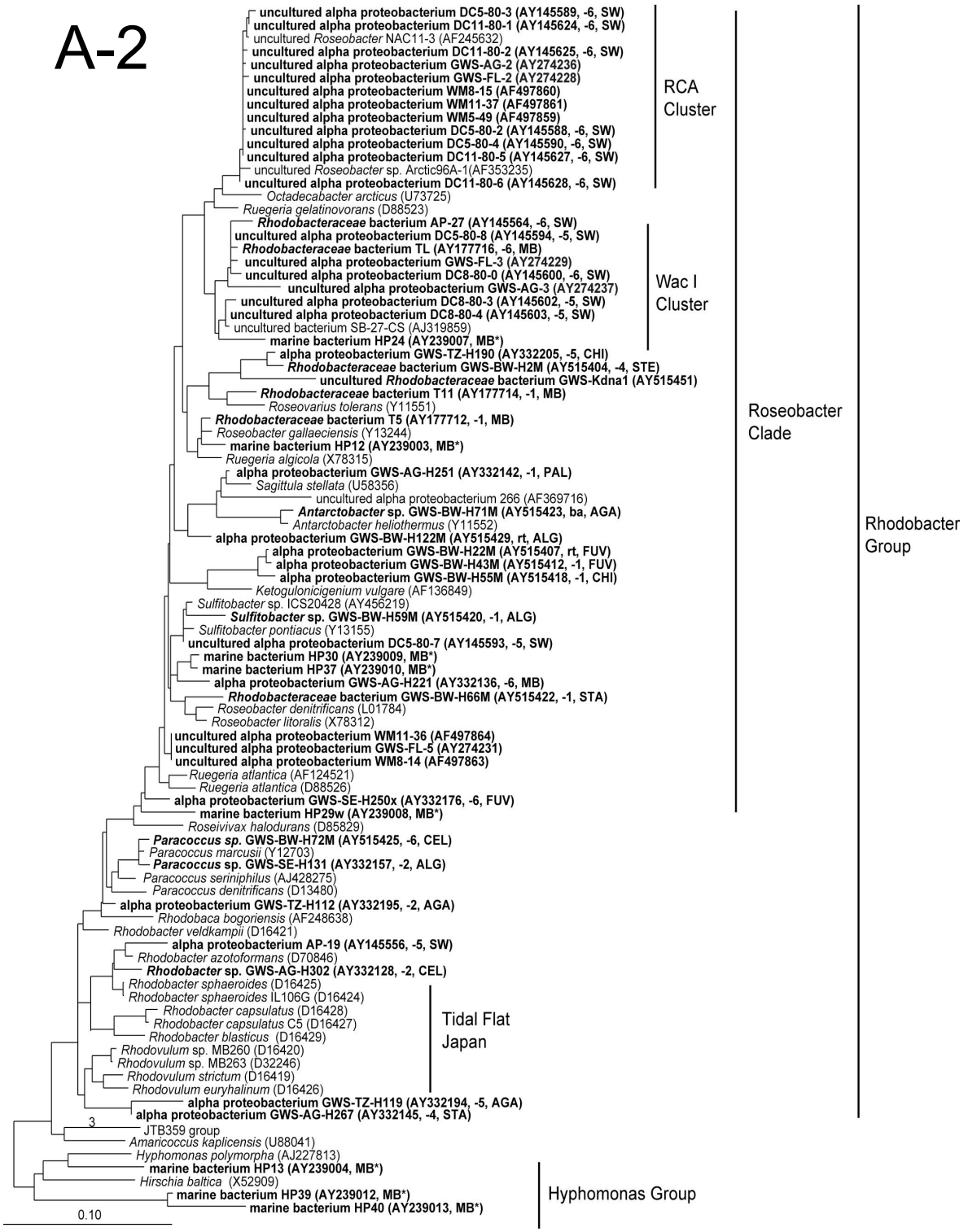
A total of 120 16S rRNA gene sequences from the Wadden Sea affiliate to the α-*Proteobacteria* (Fig. 3A-1 and 3A-2). Ninety sequences derived from isolates, 15 sequences from DGGE bands from enrichment dilution cultures, 13 from DGGE bands of environmental samples, and two from our clone library. Fifty-eight sequences affiliate to the *Rhodobacteraceae*, and 51 of these (28 isolate-derived sequences, 10 DGGE bands of environmental studies, 12 from DGGE bands of dilution cultures and one from a clone library) affiliate to the *Rhodobacter* group of the *Rhodobacteraceae* (Fig. 3A-2). Within the *Rhodobacter* group sequences obtained from the Wadden Sea form two distinct clusters (Fig. 3A-2). One is the recently discovered RCA-cluster (Selje *et al*., 2004) which contains exclusively uncultured organisms, permanently abundant in the Wadden Sea. The other cluster, Wac I (Wadden Sea alpha cluster I), branches also in the upper part of the *Rhodobacter* group and contains DGGE bands from environmental samples of the Wadden Sea detected year-round [GWS-AG-3, GWS-FL-3 (Stevens *et al*., 2004a)], four sequences from high dilution steps of enrichment cultures (10^{-5} and 10^{-6}), and three isolates, of which two also stemmed from high dilution steps (10^{-6}). Sequences of both clusters were obtained during different studies and from different locations (Selje and Simon, 2003; Brinkhoff *et al*., 2004; Grossart *et al*., 2004; Stevens *et al*., 2004a; Selje *et al*., 2004; Selje and Simon, 2004).

Large sequence groups containing more than 10 sequences affiliate to the *Phyllobacteriaceae* (20 isolates), and the *Sphingomonadaceae* (11 isolates and 1 sequence from a dilution culture). The high number of isolates in these groups and the very different substrates they were obtained with indicate that these organisms are metabolically divers and obviously easy to isolate. Six strains were found to be related with the *Rhizobiaceae*. Sequences of these groups were also obtained in different studies (Grossart *et al*., 2004; Stevens *et al*., 2004b; this study). Other sequences from the Wadden Sea are scattered within α-*Proteobacteria* and affiliate with several groups, including two sequences derived from DGGE-bands (WM11-40 and WM6-1; accession-no. AF497862 and AF497866, respectively), which fall into the SAR11 cluster. Sequences, which could not be classified, affiliate loosely to *Hyphomicrobium* (GWS-TZ-218), to *Devosia* (7 isolates), and fall within the *Rickettsiales* (one clone derived sequence GWS-K33). A sequence derived from a dilution culture (DC5-80-1) falls within the SAR116 cluster (Giovannoni *et al*., 1990), and one isolate-derived sequence (AP-25) is loosely affiliated to it. Two isolate-derived sequences from high dilutions, and one clone from a dilution culture (all from dilution step 10^{-5}), as well as one isolate from the lowest dilution step of a dilution series form another new cluster, named the Wac II cluster, and affiliate loosely to the JTB260 and the endosymbiont group.

On the following pages:

Fig. 3. Maximum likelihood trees of isolates and clones obtained from the German Wadden Sea (bold) showing the affiliation within *Proteobacteria* and *Bacteroidetes*. Sequences <1300 bp were added to the tree with maximum parsimony. Isolates from May are marked with an "M" at the end of the name, isolates from rolling tanks with an "rt", and isolates from batch cultures are marked by "ba". If available dilution step and substrate are indicated (CAS = casein, CEL = cellulose; CHI = chitin, FUV = *Fucus vesiculosus*; LAM = laminarin, PAL = palmic acid; STA = starch; MB = Marine Broth 2216, MB* = Marine Broth 2216 prepared with sea water; Grossart *et al*., 2004; SW = autoclaved seawater amended with trace elements and vitamins; Selje and Simon, 2004b. The habitat can be derived from the name of the May and October isolates (BW = bulk water, AG = aggregates, SE = sediment surface, TZ = oxic /anoxic transition zone of the sediment). (A-1) α-*Proteobacteria*; (A-2) *Rhodobacter*-group; (B-1) γ-*Proteobacteria*; (B-2) SAMMIC-group within the γ-*Proteobacteria*; (C) δ-*Proteobacteria*; (E) *Bacteroidetes*. The scale bars indicate the percentage of the sequence divergence.


A-1
alpha proteobacterium GWS-BW-H152 (AY332102, -1, CEL)
manganese-oxidizing bacterium S185-9A1 (U53824)
alpha proteobacterium GWS-SE-H129 (AY332155, -3, ALG)
alpha proteobacterium GWS-TZ-H134 (AY332199, -4, ALG)
Aurantimonas coralicida (AY065627)
Aurantimonas sp. ICS20433 (AY456221)
Fulvimarina pelagi (AY178860)
Rhodobium marinum (D30790)
Rhodobium orientis (D30792)
"Nitratireductor aquibiodomus" (AF534573)
alpha proteobacterium GWS-BW-H225 (AY332114, -2, MB)
alpha proteobacterium GWS-TZ-H181 (AY332209, -1, CHI)
Defluvibacter lusatiensis (AJ132378)
Ahrensia kielensis (D88524)
alpha proteobacterium GWS-SE-H176 (AY332174, -1, CHI)
alpha proteobacterium GWS-BW-H29M (AY515410, -1, CEL)
alpha proteobacterium MBIC3923 (AB016848)
alpha proteobacterium GWS-SE-H244 (AY332186, -2, PAL)
alpha proteobacterium GWS-BW-H52M (AY515416, -1, LAM)
alpha proteobacterium GWS-BW-H49M (AY515414, -1, FUV)
alpha proteobacterium LIg-6 (AJ439365)
alpha proteobacterium GWS-SE-H155 (AY332159, -3, CEL)
Phyllobacteriaceae bacterium GWS-SE-H103 (AY332151, -2, AGA)
Phyllobacteriaceae bacterium GWS-SE-H229 (AY332178, -8, MB)
Phyllobacteriaceae bacterium GWS-BW-H238 (AY332116, -2, PAL)
Phyllobacteriaceae bacterium TK (AY177715, -5, MB)
Phyllobacteriaceae bacterium GWS-AG-H110 (AY332127, -1, AGA)
Phyllobacteriaceae bacterium GWS-BW-H257 (AY332120, -4. STA)
Phyllobacteriaceae bacterium GWS-AG-H210 (AY332135, -1, FUV)
Phyllobacteriaceae bacterium GWS-AG-H249 (AY332139, -2, PAL)
Mesorhizobium loti (X67229)
Phyllobacterium myrsinacearum (D12789)
alpha proteobacterium GWS-BW-H54M (AY515417, -1, CHI)
Bartonellaceae
Brucella melitensis (L26166)
Ochrobactrum sp. GWS-TZ-H138 (AY332201, -1, ALG)
Ochrobacter grignonense (AJ242581)
Agrobacterium tumefaciens (D14500)
Agrobacterium sp. GWS-TZ-H187 (AY332203, -8, CHI)
Agrobacterium sp. GWS-SE-H184 (AY332168, -5, CHI)
marine bacterium HP33 (AY239011, MB*)
Rhizobiaceae bacterium GWS-BW-H42M (AY515411, -1, ALG)
Rhizobium sp. ICS20412 (AY456211)
Rhizobium vitis (X67225)
Rhizobium sp. ICS20406 (AY456208)
alpha proteobacterium GWS-BW-H48M (AY515413, rt, FUV)
alpha proteobacterium GWS-BW-H51M (AY515415, rt, FUV)
Ensifer adhaerens (AF191739)
95
Rhodobacteraceae I
6
5
Endosymbiont Group
JTB260 Group
alpha proteobacterium AP-20 (AY145557, -5, SW)
alpha proteobacterium AP-21 (AY145558, -5, SW)
alpha proteobacterium GWS-BW-H85M (AY515426, -1, FUV)
uncultured alpha proteobacterium DC5-80-6 (AY145592, -5, SW)
6
JTB131 Group
Bradyrhizobium japonicum (X66024)
Methylobacterium organophilum (D32226)
Beijerinckia indica (M59060)
Methylocystis parvus (M29026)
Ancylobacter aquaticus (M27803)
Ancylobacter sp. GWS-SE-H130 (AY332156, -2, ALG)
Hyphomicrobium vulgare (Y14302)
alpha proteobacterium GWS-TZ-H218 (AY332219, -1, FUV)
Roseibium denhamense (D85832)
unknown alpha proteobacterium GWS-SE-H146 (AY332161, -2, CEL)
Stappia aggregata (D88520)
alpha proteobacterium GWS-SE-H253 (AY332189, -1, PAL)
alpha proteobacterium GWS-BW-H6M (AY515405, rt, ALG)
Stappia stellulata (D88525)
Stappia sp. GWS-BW-H226 (AY332115, -1, MB)
alpha proteobacterium GWS-BW-H142M (AY515430, -1, CAS)
alpha proteobacterium GWS-BW-H94M (AY515428, -4, CEL)
alpha proteobacterium GWS-BW-H61M (AY515421, -1, CAS)
alpha proteobacterium GWS-BW-H56M (AY515419, -1, AGA)
alpha proteobacterium GWS-BW-H80M (AY515425, -1, MB)
alpha proteobacterium GWS-BW-H28M (AY515409, -6, CEL)
marine bacterium HP22 (AY239006, MB*)
uncultured alpha proteobacterium BIri24 (AJ318163)
alpha proteobacterium GWS-BW-H162M (AY515431, -1, CHI)
alpha proteobacterium GWS-BW-H239 (AY332117, -2, PAL)
alpha proteobacterium GWS-TZ-H180 (AY332208, -1, CHI)
Devosia neptuniae (AF469072)
Devosia riboflavina (D49423)
uncultured alpha proteobacterium DC5-80-5 (AY145591, -5, SW)
alpha proteobacterium GWS-SE-H151 (AY3321158, -4, CEL)
Sphingobium sp. GWS-SE-H76 (AY332160, -2, CEL)
Sphingobium yanoikuyae (D16145)
Caulobacter leidyi (AB008391)
Sphingomonas paucimobilis (U37337)
Blastomonas nataoria (Y13774)
alpha proteobacterium GWS-SE-H241 (AY332182, -2, PAL)
alpha proteobacterium GWS-BW-H24M (AY515408, -1, CAS)
marine bacterium HP18 (AY239005, MB*)
alpha proteobacterium AP-22 (AY145559, -5, SW)
Sphingopyxis macrogoltabida (D84530)
Sphingomonas sp. GWS-BW-H17M (AY515406, -5, MB)
alpha proteobacterium GWS-BW-H92M (AY515427, -4, CEL)
Erythrobacter sp. GWS-BW-H262 (AY332123, -2, STA)
Erythrobacter citreus (AF118020)
Citromicrobium sp. ICS20409 (AY456209)
Citromicrobium sp. ICS20442 (AY456225)
Sphingomonadaceae bacterium T3 (AY177713, -5, MB)
Erythrobacter longus (M96744)
Erythrobacter sp. GWS-SE-H247 (AY332188, -2, PAL)
Porphyrobacter neustonensis (M96745)
Brevundimonas vesicularis (AY456200)
uncultured alpha proteobacterium DGGE band GWS-FL-4 (AY274230)
Brevundimonas subvibrioides (X94470)
Brevundimonas sp. GWS-BW-H203 (AY332109, -5, FUV)
Brevundimonas diminuta (M59064)
16
SAR 11
Holospora obtusa (X58198)
Caedibacter caryophilus (X71837)
uncultured alpha proteobacterium GWS-K33 (AY515452)
"Anaplasma marginale" (AF414877)
"Wolbachia pipientis" (U23709)
Rickettsia prowazekii (M21789)
uncultured alpha proteobacterium MB12F11 (AY033316)
uncultured proteobacterium OCS28 (AF001636)
uncultured alpha proteobacterium DC5-80-1 (AY145587, -5, SW)
uncultured alpha proteobacterium SAR116 (L35463)
uncultured proteobacterium OCS24 (AF001637)
alpha proteobacterium AP-25 (AY145562, -6, SW)
2
Rhodospirillum rubrum (D30778)
Acetobacteraceae
0.10
Phyllobacteriaceae
Rhizobiaceae
Wac II
Rhodobacteraceae II
Sphingomonadaceae
Rickettsiaceae
SAR 116


A-2
uncultured alpha proteobacterium DC5-80-3 (AY145589, -6, SW)
uncultured alpha proteobacterium DC11-80-1 (AY145624, -6, SW)
uncultured Roseobacter NAC11-3 (AF245632)
uncultured alpha proteobacterium DC11-80-2 (AY145625, -6, SW)
uncultured alpha proteobacterium GWS-AG-2 (AY274236)
uncultured alpha proteobacterium GWS-FL-2 (AY274228)
uncultured alpha proteobacterium WM8-15 (AF497860)
uncultured alpha proteobacterium WM11-37 (AF497861)
uncultured alpha proteobacterium WM5-49 (AF497859)
uncultured alpha proteobacterium DC5-80-2 (AY145588, -6, SW)
uncultured alpha proteobacterium DC5-80-4 (AY145590, -6, SW)
uncultured alpha proteobacterium DC11-80-5 (AY145627, -6, SW)
uncultured Roseobacter sp. Arctic96A-1(AF353235)
uncultured alpha proteobacterium DC11-80-6 (AY145628, -6, SW)
Octadecabacter arcticus (U73725)
Ruegeria gelatinovorans (D88523)
Rhodobacteraceae bacterium AP-27 (AY145564, -6, SW)
uncultured alpha proteobacterium DC5-80-8 (AY145594, -5, SW)
Rhodobacteraceae bacterium TL (AY177716, -6, MB)
uncultured alpha proteobacterium GWS-FL-3 (AY274229)
uncultured alpha proteobacterium DC8-80-0 (AY145600, -6, SW)
uncultured alpha proteobacterium GWS-AG-3 (AY274237)
uncultured alpha proteobacterium DC8-80-3 (AY145602, -5, SW)
uncultured alpha proteobacterium DC8-80-4 (AY145603, -5, SW)
uncultured bacterium SB-27-CS (AJ319859)
marine bacterium HP24 (AY239007, MB*)
alpha proteobacterium GWS-TZ-H190 (AY332205, -5, CHI)
Rhodobacteraceae bacterium GWS-BW-H2M (AY515404, -4, STE)
uncultured Rhodobacteraceae bacterium GWS-Kdna1 (AY515451)
Rhodobacteraceae bacterium T11 (AY177714, -1, MB)
Roseovarius tolerans (Y11551)
Rhodobacteraceae bacterium T5 (AY177712, -1, MB)
Roseobacter gallaeciensis (Y13244)
marine bacterium HP12 (AY239003, MB*)
Ruegeria algicola (X78315)
alpha proteobacterium GWS-AG-H251 (AY332142, -1, PAL)
Sagittula stellata (U58356)
uncultured alpha proteobacterium 266 (AF369716)
Antarctobacter sp. GWS-BW-H71M (AY515423, ba, AGA)
Antarctobacter heliothermus (Y11552)
alpha proteobacterium GWS-BW-H122M (AY515429, rt, ALG)
alpha proteobacterium GWS-BW-H22M (AY515407, rt, FUV)
alpha proteobacterium GWS-BW-H43M (AY515412, -1, FUV)
alpha proteobacterium GWS-BW-H55M (AY515418, -1, CHI)
Ketogulonicigenium vulgare (AF136849)
Sulfitobacter sp. ICS20428 (AY456219)
Sulfitobacter sp. GWS-BW-H59M (AY515420, -1, ALG)
Sulfitobacter pontiacus (Y13155)
uncultured alpha proteobacterium DC5-80-7 (AY145593, -5, SW)
marine bacterium HP30 (AY239009, MB*)
marine bacterium HP37 (AY239010, MB*)
alpha proteobacterium GWS-AG-H221 (AY332136, -6, MB)
Rhodobacteraceae bacterium GWS-BW-H66M (AY515422, -1, STA)
Roseobacter denitrificans (L01784)
Roseobacter litoralis (X78312)
uncultured alpha proteobacterium WM11-36 (AF497864)
uncultured alpha proteobacterium GWS-FL-5 (AY274231)
uncultured alpha proteobacterium WM8-14 (AF497863)
Ruegeria atlantica (AF124521)
Ruegeria atlantica (D88526)
alpha proteobacterium GWS-SE-H250x (AY332176, -6, FUV)
marine bacterium HP29w (AY239008, MB*)
Roseivivax halodurans (D85829)
Paracoccus sp. GWS-BW-H72M (AY515425, -6, CEL)
Paracoccus marcusii (Y12703)
Paracoccus sp. GWS-SE-H131 (AY332157, -2, ALG)
Paracoccus seriniphilus (AJ428275)
Paracoccus denitrificans (D13480)
alpha proteobacterium GWS-TZ-H112 (AY332195, -2, AGA)
Rhodobaca bogoriensis (AF248638)
Rhodobacter veldkampii (D16421)
alpha proteobacterium AP-19 (AY145556, -5, SW)
Rhodobacter azotoformans (D70846)
Rhodobacter sp. GWS-AG-H302 (AY332128, -2, CEL)
Rhodobacter sphaeroides (D16425)
Rhodobacter sphaeroides IL106G (D16424)
Rhodobacter capsulatus (D16428)
Rhodobacter capsulatus C5 (D16427)
Rhodobacter blasticus (D16429)
Rhodovulum sp. MB260 (D16420)
Rhodovulum sp. MB263 (D32246)
Rhodovulum strictum (D16419)
Rhodovulum euryhalinum (D16426)
alpha proteobacterium GWS-TZ-H119 (AY332194, -5, AGA)
alpha proteobacterium GWS-AG-H267 (AY332145, -4, STA)
3
JTB359 group
Amaricoccus kaplicensis (U88041)
Hyphomonas polymorpha (AJ227813)
marine bacterium HP13 (AY239004, MB*)
Hirschia baltica (X52909)
marine bacterium HP39 (AY239012, MB*)
marine bacterium HP40 (AY239013, MB*)
0.10
RCA Cluster
Wac I Cluster
Roseobacter Clade
Rhodobacter Group
Tidal Flat Japan
Hyphomonas Group

γ-*Proteobacteria*

Altogether 84 sequences from oxic habitats of the German Wadden Sea could be assigned to the γ-subdivision of *Proteobacteria* (Fig. 3B-1 and 3B-2). All sequences from isolates (n = 62) could be classified into validly described families, i.e. into the *Vibrionaceae* (n = 13), the *Enterobacteriaceae* (n = 1), the polyphyletic family *Alteromonadaceae* (n = 28), *Oceanospirillaceae* (n = 2), *Halomonadaceae* (n = 9), *Alkanivoraxaceae* (n = 2), *Pseudomonadaceae* (n = 3), and the *Piscirickettsiaceae*, to which 4 isolates affiliate. Within the latter group only sulfur-oxidizing bacteria were obtained with specific substrates (Brinkhoff and Muyzer, 1997). All strains of the *Vibrionaceae* were obtained from samples taken in October 1999 at position B (Fig. 1) during two studies (Stevens *et al*., 2004b; Brinkhoff *et al*., 2004). Organisms of the other two large groups, i.e. the *Alteromonadaceae* and the *Halomondaceae* were isolated from two locations (Fig. 1, position A and B). One clone from the Jadebusen bay (*Beggiatoa* Dangast A9 [Mussmann *et al*., 2003]) affiliates to the *Thiotrichaceae*, and one DGGE band to the *Halomonadaceae*. All other sequences derived from clones affiliate to clusters with sequences of only or mainly uncultured bacteria. One clone from the clone library affiliates to the SAR86 cluster (Rappé *et al.*, 2000), for which currently no cultured isolates are described. Three sequences fall in the previously described NOR5 cluster. This cluster was found to be abundant in the open North Sea and contains only one isolate (Eilers *et al.*, 2001).

Five sequences from DGGE bands from environmental samples and one from a dilution culture, as well as 10 clones from the clone library fall into a newly defined cluster. Eight of the nine identical sequences from the clone library and affiliating with γ-*Proteobacteria* were identical with sequences of this new cluster. The remaining sequence was identical with clone K5-4 (SAR86 cluster). Overall, we found 76 sequences belonging to this cluster from different marine habitats and only three are from isolated strains (see below). We propose the name SAMMIC group (Surface Attached Marine MICrobes, Fig. 3B-2), because all sequences within this group were derived from surfaces of marine habitats, either from sediment, a marine sponge (isolate HNSS31), or from suspended and resuspended aggregates occurring in high amounts in the water column of the Wadden Sea. Sequences from DGGE bands falling in this cluster were only obtained from sediment samples and suspended aggregates in the Wadden Sea (Stevens *et al*., 2004a). Sequences in the SAMMIC group occur globally, but mostly in polar habitats, i.e. on the Antarctic continental shelf [e.g., "MERTZ"-clones from Bowman and McCuaig (2003)], on Arctic sea ice (clone ARKICE-74, Brinkmeyer *et al.*, 2003), but also in temperate regions, i.e. on the sediment above gas hydrates off the Oregon coast, and on sediments of the Aegean Sea. Within this group four clusters can be distinguished (Fig. 3B-2): The Wadden Sea Cluster I contains five clone sequences and three DGGE bands from the Wadden Sea, as well as one DGGE band from a 10^{-6} dilution of an enrichment culture. The DGGE bands were derived from aggregates and from the sediment surface, and were detected from spring 1999 to spring 2000 (Stevens *et al*., 2004a).

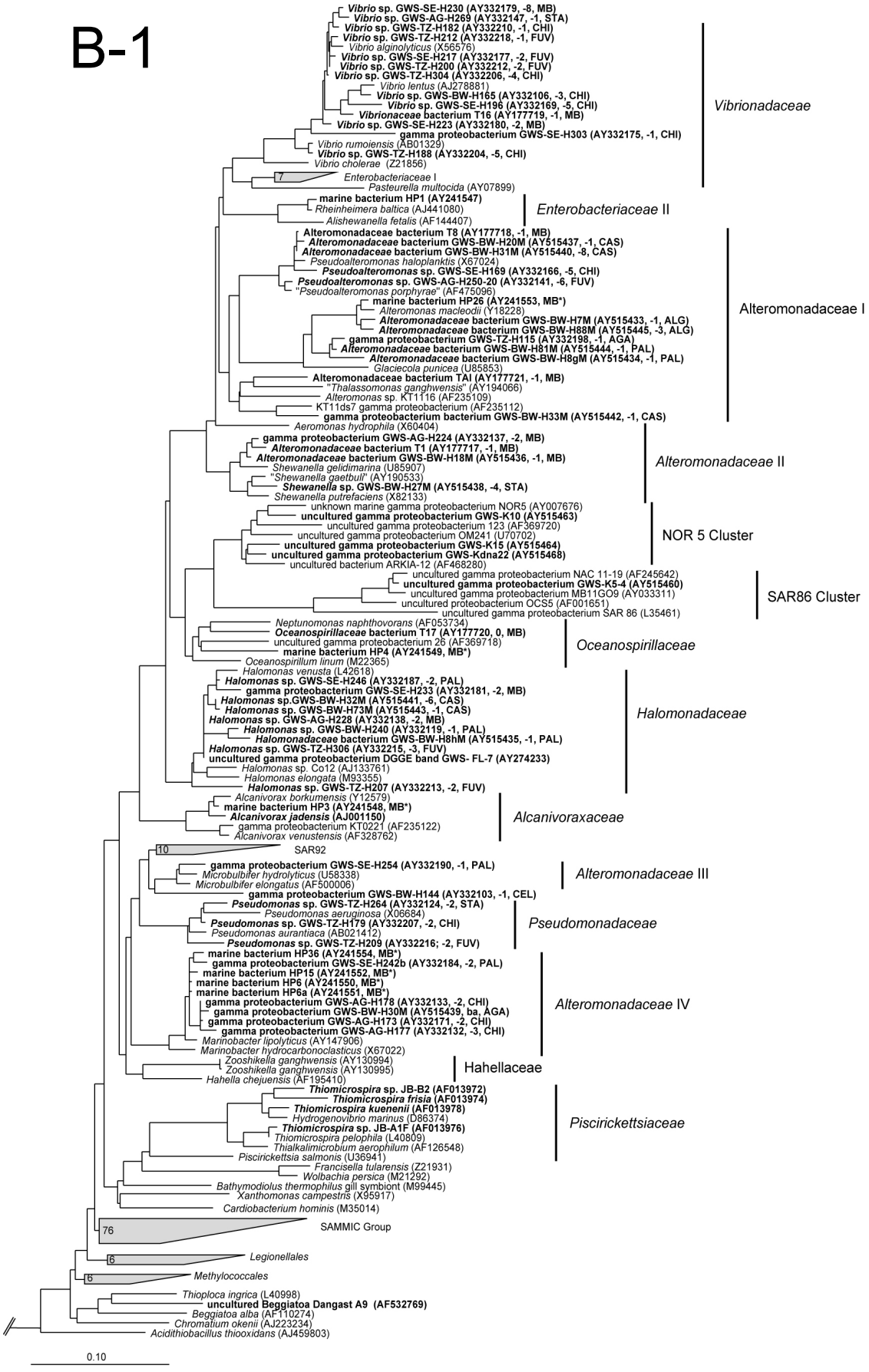
The Wadden Sea Cluster II contains Wadden Sea sequences from three clones and one aggregate-derived DGGE band, which was also detected from April 1999 to June 2000 (Stevens *et al*., 2004a). The two other clusters within the SAMMIC group, BPC036 and JTB255 [named after sequences from O´Neill *et al*., unpublished, and Li *et al*. (1999), respectively], contain no Wadden Sea sequences. The BPC036 cluster contains two sequences from isolates of the SAMMIC group (ODIII6 and NDII1.2), both sulfur-oxidizing bacteria from sediments near hydrothermal vents of the Aegean Sea, and the JTB255 cluster the sequence of the third isolate (HNSS31).

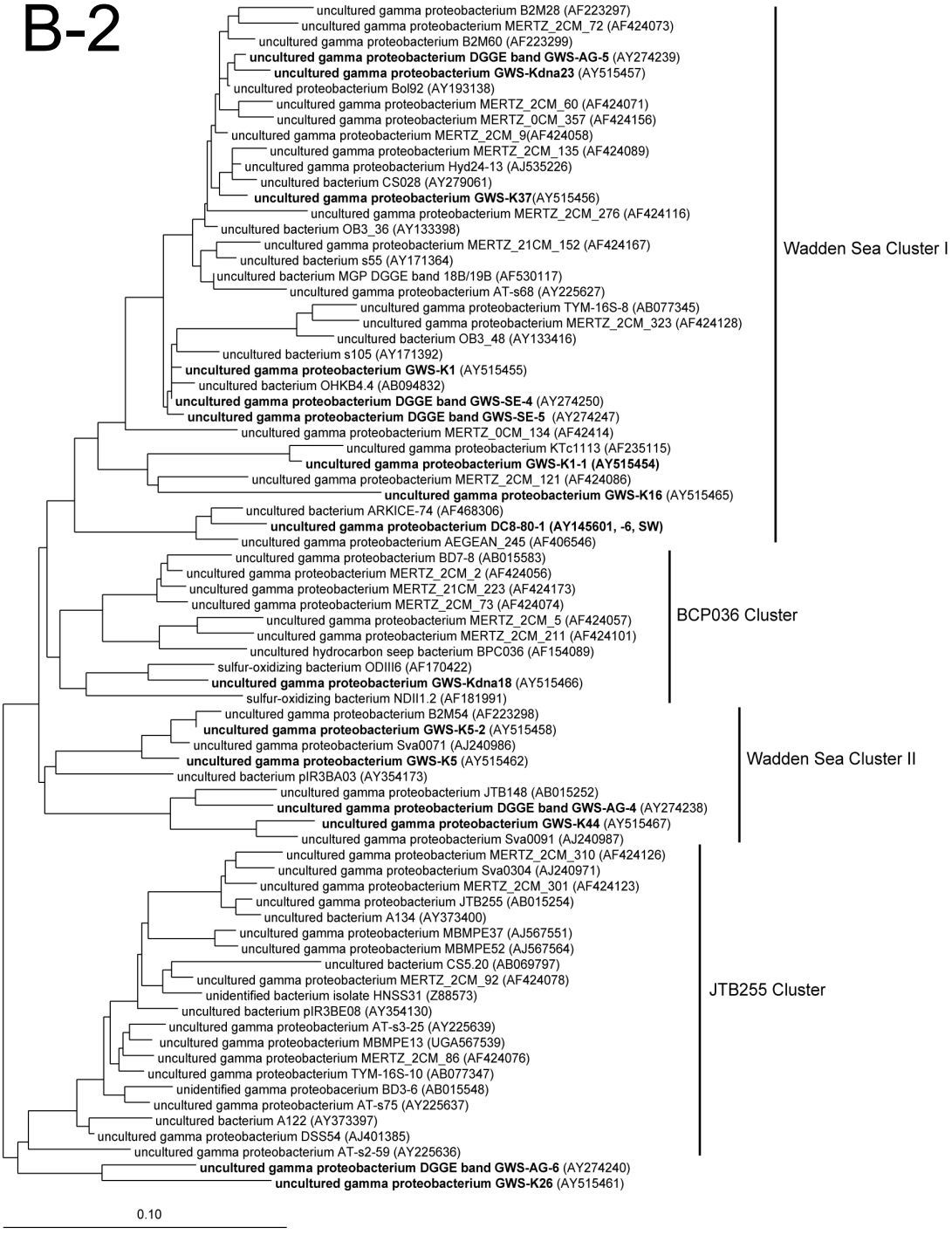
Sequences of δ-*Proteobacteria*

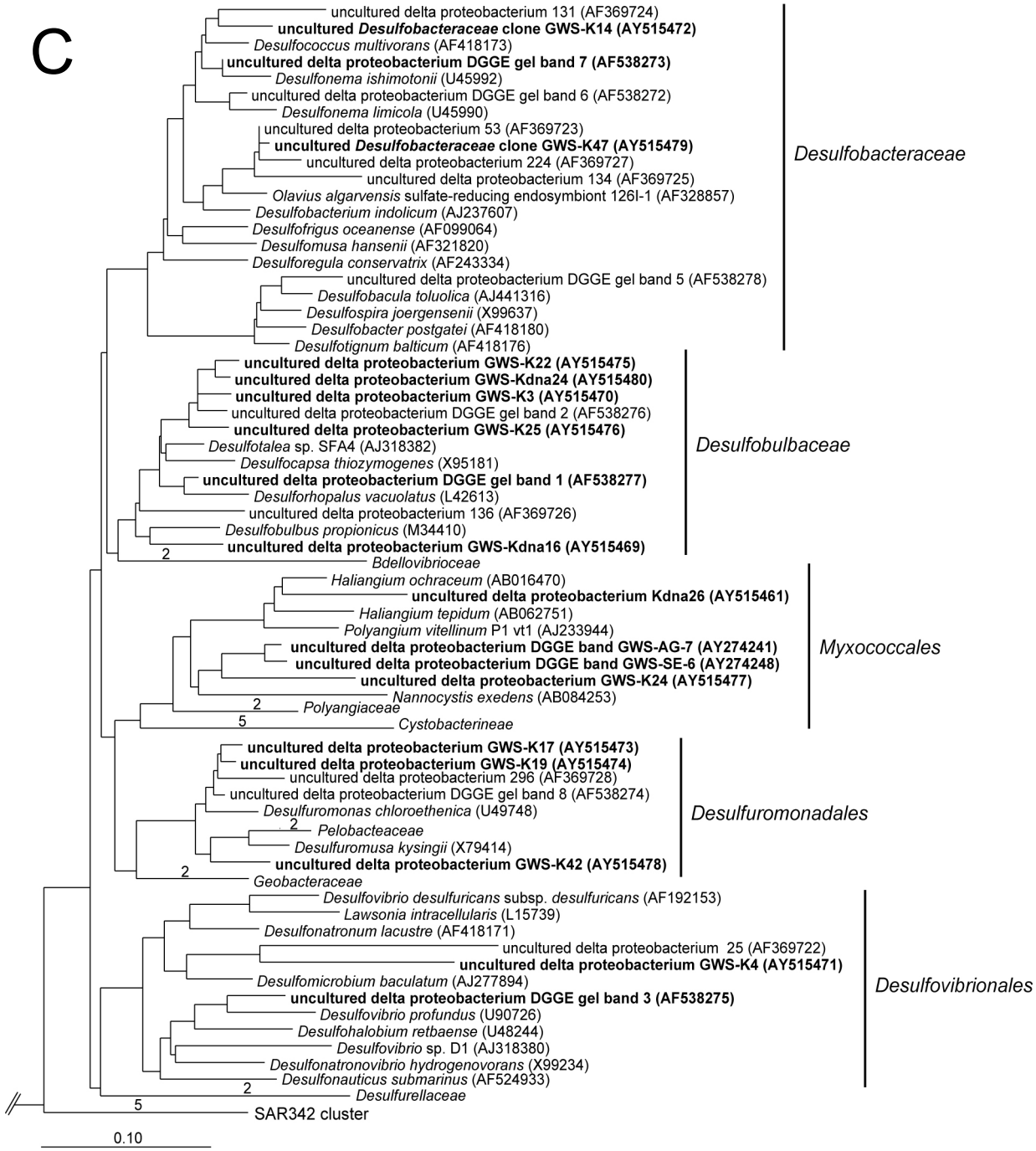
Eighteen sequences from oxic habitats of the German Wadden Sea affiliate to δ-*Proteobacteria* (Fig. 3C). Within the *Desulfobacterales* they affiliate to the *Desulfobacteraceae* [one DGGE band from the sediment surface (Llobet-Brossa *et al*., 2002), two from the clone library from bulk water], and to the *Desulfobulbaceae* [one DGGE band from the sediment surface (Llobet-Brossa *et al*., 2002), 5 from the clone library]. Other sequences affiliate to the *Desulfuromonadales* (3 from the clone library), and to the *Desulfovibrionales* [one DGGE band (Llobet-Brossa *et al*., 2002), and one from the clone library]. Two sequences from annually persisting DGGE bands (Stevens *et al*., 2004a) and two from the clone library affiliate to the strictly aerobic *Myxococcales*. The sequence of clone GWS-K47 within the *Desulfobacteraceae* is related with a symbiont of *Olavius algarvensis* (AF328857), which is capable of reducing sulfate under microaerophilic conditions. The related sequences of all other Wadden Sea sequences affiliate to bacteria which grow strictly anaerobic

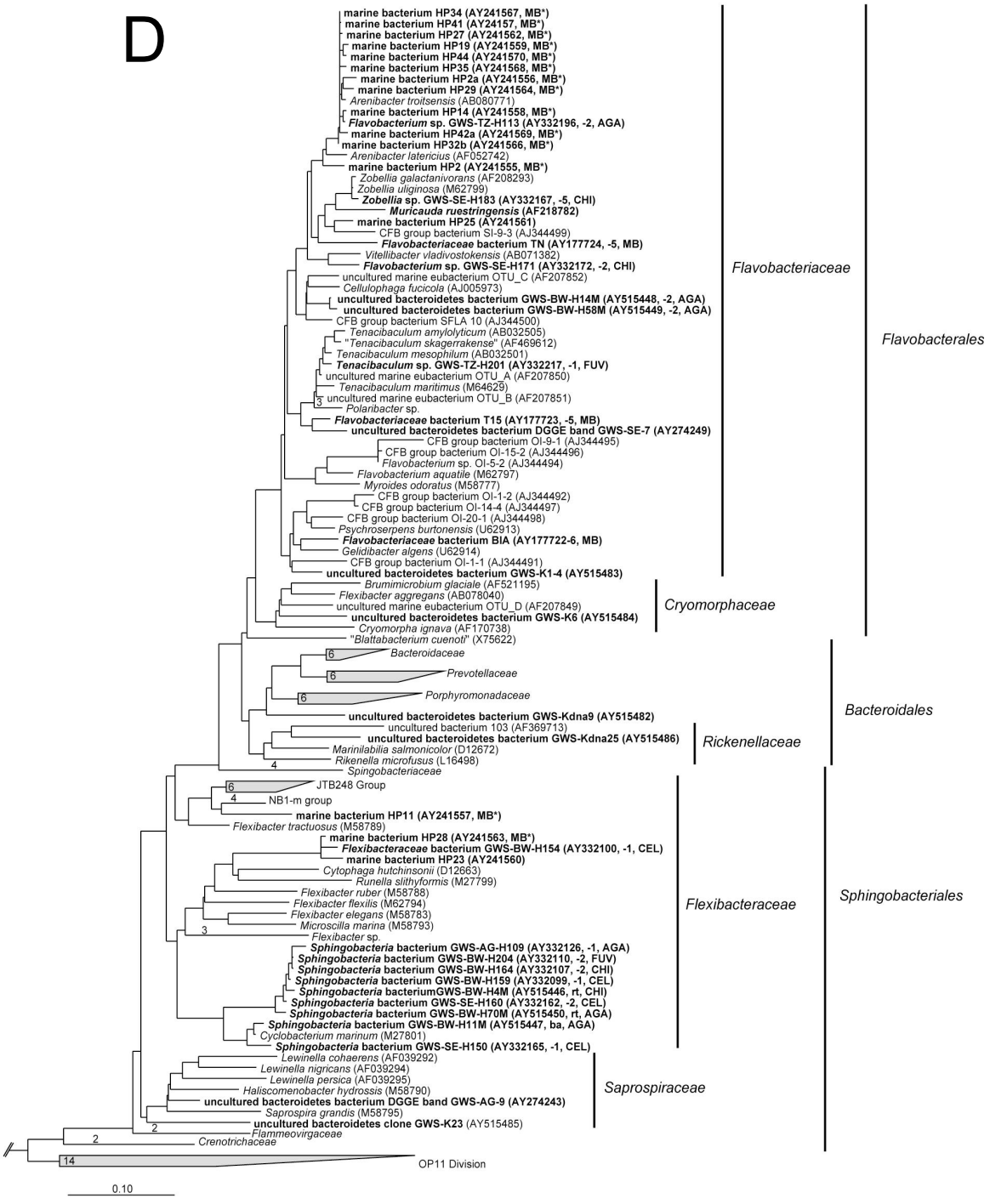
Sequences of *Bacteroidetes*

Forty-three sequences from the German Wadden Sea affiliate to *Bacteroidetes* (Fig. 1D). Most of them could be classified as *Flavobacteriaceae* (23 isolates, one clone, and one DGGE band). Twelve isolates are highly related, affiliate to the genus *Arenibacter*, and, except one strain (GWS-TZ-H113), were isolated with one substrate (Grossart *et al*., 2004). The sequence of the DGGE band (GWS-SE-7), which was obtained from an environmental sample of the sediment surface, is very similar (100 % in overlapping segment) to the sequence of strain T15, isolated from a high dilution (10^{-6}) of a MPN series (Brinkhoff *et al*., 2004). Sequences of 13 isolates fall into the *Flexibacteraceae* and nine of these affiliate with *Cyclobacterium marinum*. Seven of the latter are also highly related, however, they were obtained with several different substrates and from two locations (Fig. 1, positions A and B). One clone-derived sequence affiliates to the *Cryomorphaceae*, one to the *Rikenellaceae*, and one clone and one DGGE band of an environmental sample affiliate to the *Saprospiraceae*. One clone (GWS-Kdna9) could not be assigned to an existing family.


B-1
Vibrio sp. GWS-SE-H230 (AY332179, -8, MB)
Vibrio sp. GWS-AG-H269 (AY332147, -1, STA)
Vibrio sp. GWS-TZ-H182 (AY332210, -1, CHI)
Vibrio sp. GWS-TZ-H212 (AY332218, -1, FUV)
Vibrio alginolyticus (X56576)
Vibrio sp. GWS-SE-H217 (AY332177, -2, FUV)
Vibrio sp. GWS-TZ-H200 (AY332212, -2, FUV)
Vibrio sp. GWS-TZ-H304 (AY332206, -4, CHI)
Vibrio lentus (AJ278881)
Vibrio sp. GWS-BW-H165 (AY332106, -3, CHI)
Vibrio sp. GWS-SE-H196 (AY332169, -5, CHI)
Vibrionaceae bacterium T16 (AY177719, -1, MB)
Vibrio sp. GWS-SE-H223 (AY332180, -2, MB)
gamma proteobacterium GWS-SE-H303 (AY332175, -1, CHI)
Vibrio rumoiensis (AB01329)
Vibrio sp. GWS-TZ-H188 (AY332204, -5, CHI)
Vibrio cholerae (Z21856)
Vibrionadaceae
7
Enterobacteriaceae I
Pasteurella multocida (AY07899)
marine bacterium HP1 (AY241547)
Rheinheimera baltica (AJ441080)
Alishewanella fetalis (AF144407)
Enterobacteriaceae II
Alteromonadaceae bacterium T8 (AY177718, -1, MB)
Alteromonadaceae bacterium GWS-BW-H20M (AY515437, -1, CAS)
Alteromonadaceae bacterium GWS-BW-H31M (AY515440, -8, CAS)
Pseudoalteromonas haloplanktis (X67024)
Pseudoalteromonas sp. GWS-SE-H169 (AY332166, -5, CHI)
Pseudoalteromonas sp. GWS-AG-H250-20 (AY332141, -6, FUV)
"Pseudoalteromonas porphyrae" (AF475096)
marine bacterium HP26 (AY241553, MB*)
Alteromonas macleodii (Y18228)
Alteromonadaceae bacterium GWS-BW-H7M (AY515433, -1, ALG)
Alteromonadaceae bacterium GWS-BW-H88M (AY515445, -3, ALG)
gamma proteobacterium GWS-TZ-H115 (AY332198, -1, AGA)
Alteromonadaceae bacterium GWS-BW-H81M (AY515444, -1, PAL)
Alteromonadaceae bacterium GWS-BW-H8gM (AY515434, -1, PAL)
Glaciecola punicea (U85853)
Alteromonadaceae bacterium TAI (AY177721, -1, MB)
"Thalassomonas ganghwensis" (AY194066)
Alteromonas sp. KT1116 (AF235109)
KT11ds7 gamma proteobacterium (AF235112)
gamma proteobacterium bacterium GWS-BW-H33M (AY515442, -1, CAS)
Alteromonadaceae I
Aeromonas hydrophila (X60404)
gamma proteobacterium GWS-AG-H224 (AY332137, -2, MB)
Alteromonadaceae bacterium T1 (AY177717, -1, MB)
Alteromonadaceae bacterium GWS-BW-H18M (AY515436, -1, MB)
Shewanella gelidimarina (U85907)
"Shewanella gaetbull" (AY190533)
Shewanella sp. GWS-BW-H27M (AY515438, -4, STA)
Shewanella putrefaciens (X82133)
Alteromonadaceae II
unknown marine gamma proteobacterium NOR5 (AY007676)
uncultured gamma proteobacterium GWS-K10 (AY515463)
uncultured gamma proteobacterium 123 (AF369720)
uncultured gamma proteobacterium OM241 (U70702)
uncultured gamma proteobacterium GWS-K15 (AY515464)
uncultured gamma proteobacterium GWS-Kdna22 (AY515468)
uncultured bacterium ARKIA-12 (AF468280)
NOR 5 Cluster
uncultured gamma proteobacterium NAC 11-19 (AF245642)
uncultured gamma proteobacterium GWS-K5-4 (AY515460)
uncultured gamma proteobacterium MB11GO9 (AY033311)
uncultured proteobacterium OCS5 (AF001651)
uncultured gamma proteobacterium SAR 86 (L35461)
SAR86 Cluster
Neptunomonas naphthovorans (AF053734)
Oceanospirillaceae bacterium T17 (AY177720, 0, MB)
uncultured gamma proteobacterium 26 (AF369718)
marine bacterium HP4 (AY241549, MB*)
Oceanospirillum linum (M22365)
Oceanospirillaceae
Halomonas venusta (L42618)
Halomonas sp. GWS-SE-H246 (AY332187, -2, PAL)
gamma proteobacterium GWS-SE-H233 (AY332181, -2, MB)
Halomonas sp.GWS-BW-H32M (AY515441, -6, CAS)
Halomonas sp. GWS-BW-H73M (AY515443, -1, CAS)
Halomonas sp. GWS-AG-H228 (AY332138, -2, MB)
Halomonas sp. GWS-BW-H240 (AY332119, -1, PAL)
Halomonadaceae bacterium GWS-BW-H8hM (AY515435, -1, PAL)
Halomonas sp. GWS-TZ-H306 (AY332215, -3, FUV)
uncultured gamma proteobacterium DGGE band GWS- FL-7 (AY274233)
Halomonas sp. Co12 (AJ133761)
Halomonas elongata (M93355)
Halomonas sp. GWS-TZ-H207 (AY332213, -2, FUV)
Halomonadaceae
Alcanivorax borkumensis (Y12579)
marine bacterium HP3 (AY241548, MB*)
Alcanivorax jadensis (AJ001150)
gamma proteobacterium KT0221 (AF235122)
Alcanivorax venustensis (AF328762)
Alcanivoraxaceae
10
SAR92
gamma proteobacterium GWS-SE-H254 (AY332190, -1, PAL)
Microbulbifer hydrolyticus (U58338)
Microbulbifer elongatus (AF500006)
gamma proteobacterium GWS-BW-H144 (AY332103, -1, CEL)
Alteromonadaceae III
Pseudomonas sp. GWS-TZ-H264 (AY332124, -2, STA)
Pseudomonas aeruginosa (X06684)
Pseudomonas sp. GWS-TZ-H179 (AY332207, -2, CHI)
Pseudomonas aurantiaca (AB021412)
Pseudomonas sp. GWS-TZ-H209 (AY332216; -2, FUV)
Pseudomonadaceae
marine bacterium HP36 (AY241554, MB*)
gamma proteobacterium GWS-SE-H242b (AY332184, -2, PAL)
marine bacterium HP15 (AY241552, MB*)
marine bacterium HP6 (AY241550, MB*)
marine bacterium HP6a (AY241551, MB*)
gamma proteobacterium GWS-AG-H178 (AY332133, -2, CHI)
gamma proteobacterium GWS-BW-H30M (AY515439, ba, AGA)
gamma proteobacterium GWS-AG-H173 (AY332171, -2, CHI)
gamma proteobacterium GWS-AG-H177 (AY332132, -3, CHI)
Alteromonadaceae IV
Marinobacter lipolyticus (AY147906)
Marinobacter hydrocarbonoclasticus (X67022)
Zooshikella ganghwensis (AY130994)
Zooshikella ganghwensis (AY130995)
Hahella chejuensis (AF195410)
Hahellaceae
Thiomicrospira sp. JB-B2 (AF013972)
Thiomicrospira frisia (AF013974)
Thiomicrospira kuenenii (AF013978)
Hydrogenovibrio marinus (D86374)
Thiomicrospira sp. JB-A1F (AF013976)
Thiomicrospira pelophila (L40809)
Thialkalimicrobium aerophilum (AF126548)
Piscirickettsia salmonis (U36941)
Piscirickettsiaceae
Francisella tularensis (Z21931)
Wolbachia persica (M21292)
Bathymodiolus thermophilus gill symbiont (M99445)
Xanthomonas campestris (X95917)
Cardiobacterium hominis (M35014)
76
SAMMIC Group
6
Legionellales
6
Methylococcales
Thioploca ingrica (L40998)
uncultured Beggiatoa Dangast A9 (AF532769)
Beggiatoa alba (AF110274)
Chromatium okenii (AJ223234)
Acidithiobacillus thiooxidans (AJ459803)
0.10


B-2
uncultured gamma proteobacterium B2M28 (AF223297)
uncultured gamma proteobacterium MERTZ_2CM_72 (AF424073)
uncultured gamma proteobacterium B2M60 (AF223299)
uncultured gamma proteobacterium DGGE band GWS-AG-5 (AY274239)
uncultured gamma proteobacterium GWS-Kdna23 (AY515457)
uncultured proteobacterium Bol92 (AY193138)
uncultured gamma proteobacterium MERTZ_2CM_60 (AF424071)
uncultured gamma proteobacterium MERTZ_0CM_357 (AF424156)
uncultured gamma proteobacterium MERTZ_2CM_9(AF424058)
uncultured gamma proteobacterium MERTZ_2CM_135 (AF424089)
uncultured gamma proteobacterium Hyd24-13 (AJ535226)
uncultured bacterium CS028 (AY279061)
uncultured gamma proteobacterium GWS-K37(AY515456)
uncultured gamma proteobacterium MERTZ_2CM_276 (AF424116)
uncultured bacterium OB3_36 (AY133398)
uncultured gamma proteobacterium MERTZ_21CM_152 (AF424167)
uncultured bacterium s55 (AY171364)
uncultured bacterium MGP DGGE band 18B/19B (AF530117)
uncultured gamma proteobacterium AT-s68 (AY225627)
uncultured gamma proteobacterium TYM-16S-8 (AB077345)
uncultured gamma proteobacterium MERTZ_2CM_323 (AF424128)
uncultured bacterium OB3_48 (AY133416)
uncultured bacterium s105 (AY171392)
uncultured gamma proteobacterium GWS-K1 (AY515455)
uncultured bacterium OHKB4.4 (AB094832)
uncultured gamma proteobacterium DGGE band GWS-SE-4 (AY274250)
uncultured gamma proteobacterium DGGE band GWS-SE-5 (AY274247)
uncultured gamma proteobacterium MERTZ_0CM_134 (AF42414)
uncultured gamma proteobacterium KTc1113 (AF235115)
uncultured gamma proteobacterium GWS-K1-1 (AY515454)
uncultured gamma proteobacterium MERTZ_2CM_121 (AF424086)
uncultured gamma proteobacterium GWS-K16 (AY515465)
uncultured bacterium ARKICE-74 (AF468306)
uncultured gamma proteobacterium DC8-80-1 (AY145601, -6, SW)
uncultured gamma proteobacterium AEGEAN_245 (AF406546)
uncultured gamma proteobacterium BD7-8 (AB015583)
uncultured gamma proteobacterium MERTZ_2CM_2 (AF424056)
uncultured gamma proteobacterium MERTZ_21CM_223 (AF424173)
uncultured gamma proteobacterium MERTZ_2CM_73 (AF424074)
uncultured gamma proteobacterium MERTZ_2CM_5 (AF424057)
uncultured gamma proteobacterium MERTZ_2CM_211 (AF424101)
uncultured hydrocarbon seep bacterium BPC036 (AF154089)
sulfur-oxidizing bacterium ODIII6 (AF170422)
uncultured gamma proteobacterium GWS-Kdna18 (AY515466)
sulfur-oxidizing bacterium NDII1.2 (AF181991)
uncultured gamma proteobacterium B2M54 (AF223298)
uncultured gamma proteobacterium GWS-K5-2 (AY515458)
uncultured gamma proteobacterium Sva0071 (AJ240986)
uncultured gamma proteobacterium GWS-K5 (AY515462)
uncultured bacterium pIR3BA03 (AY354173)
uncultured gamma proteobacterium JTB148 (AB015252)
uncultured gamma proteobacterium DGGE band GWS-AG-4 (AY274238)
uncultured gamma proteobacterium GWS-K44 (AY515467)
uncultured gamma proteobacterium Sva0091 (AJ240987)
uncultured gamma proteobacterium MERTZ_2CM_310 (AF424126)
uncultured gamma proteobacterium Sva0304 (AJ240971)
uncultured gamma proteobacterium MERTZ_2CM_301 (AF424123)
uncultured gamma proteobacterium JTB255 (AB015254)
uncultured bacterium A134 (AY373400)
uncultured gamma proteobacterium MBMPE37 (AJ567551)
uncultured gamma proteobacterium MBMPE52 (AJ567564)
uncultured bacterium CS5.20 (AB069797)
uncultured gamma proteobacterium MERTZ_2CM_92 (AF424078)
unidentified bacterium isolate HNSS31 (Z88573)
uncultured bacterium pIR3BE08 (AY354130)
uncultured gamma proteobacterium AT-s3-25 (AY225639)
uncultured gamma proteobacterium MBMPE13 (UGA567539)
uncultured gamma proteobacterium MERTZ_2CM_86 (AF424076)
uncultured gamma proteobacterium TYM-16S-10 (AB077347)
unidentified gamma proteobacerium BD3-6 (AB015548)
uncultured gamma proteobacterium AT-s75 (AY225637)
uncultured bacterium A122 (AY373397)
uncultured gamma proteobacterium DSS54 (AJ401385)
uncultured gamma proteobacterium AT-s2-59 (AY225636)
uncultured gamma proteobacterium DGGE band GWS-AG-6 (AY274240)
uncultured gamma proteobacterium GWS-K26 (AY515461)
Wadden Sea Cluster I
BCP036 Cluster
Wadden Sea Cluster II
JTB255 Cluster
0.10


C
uncultured delta proteobacterium 131 (AF369724)
uncultured Desulfobacteraceae clone GWS-K14 (AY515472)
Desulfococcus multivorans (AF418173)
uncultured delta proteobacterium DGGE gel band 7 (AF538273)
Desulfonema ishimotonii (U45992)
uncultured delta proteobacterium DGGE gel band 6 (AF538272)
Desulfonema limicola (U45990)
uncultured delta proteobacterium 53 (AF369723)
uncultured Desulfobacteraceae clone GWS-K47 (AY515479)
uncultured delta proteobacterium 224 (AF369727)
uncultured delta proteobacterium 134 (AF369725)
Olavius algarvensis sulfate-reducing endosymbiont 126l-1 (AF328857)
Desulfobacterium indolicum (AJ237607)
Desulfofrigus oceanense (AF099064)
Desulfomusa hansenii (AF321820)
Desulforegula conservatrix (AF243334)
uncultured delta proteobacterium DGGE gel band 5 (AF538278)
Desulfobacula toluolica (AJ441316)
Desulfospira joergensenii (X99637)
Desulfobacter postgatei (AF418180)
Desulfotignum balticum (AF418176)
Desulfobacteraceae
uncultured delta proteobacterium GWS-K22 (AY515475)
uncultured delta proteobacterium GWS-Kdna24 (AY515480)
uncultured delta proteobacterium GWS-K3 (AY515470)
uncultured delta proteobacterium DGGE gel band 2 (AF538276)
uncultured delta proteobacterium GWS-K25 (AY515476)
Desulfotalea sp. SFA4 (AJ318382)
Desulfocapsa thiozymogenes (X95181)
uncultured delta proteobacterium DGGE gel band 1 (AF538277)
Desulforhopalus vacuolatus (L42613)
uncultured delta proteobacterium 136 (AF369726)
Desulfobulbus propionicus (M34410)
uncultured delta proteobacterium GWS-Kdna16 (AY515469)
Desulfobulbaceae
2
Bdellovibrioceae
Haliangium ochraceum (AB016470)
uncultured delta proteobacterium Kdna26 (AY515461)
Haliangium tepidum (AB062751)
Polyangium vitellinum P1 vt1 (AJ233944)
uncultured delta proteobacterium DGGE band GWS-AG-7 (AY274241)
uncultured delta proteobacterium DGGE band GWS-SE-6 (AY274248)
uncultured delta proteobacterium GWS-K24 (AY515477)
Nannocystis exedens (AB084253)
Myxococcales
2
Polyangiaceae
5
Cystobacterineae
uncultured delta proteobacterium GWS-K17 (AY515473)
uncultured delta proteobacterium GWS-K19 (AY515474)
uncultured delta proteobacterium 296 (AF369728)
uncultured delta proteobacterium DGGE gel band 8 (AF538274)
Desulfuromonas chloroethenica (U49748)
Desulfuromonadales
2
Pelobacteaceae
Desulfuromusa kysingii (X79414)
uncultured delta proteobacterium GWS-K42 (AY515478)
2
Geobacteraceae
Desulfovibrio desulfuricans subsp. desulfuricans (AF192153)
Lawsonia intracellularis (L15739)
Desulfonatronum lacustre (AF418171)
uncultured delta proteobacterium 25 (AF369722)
uncultured delta proteobacterium GWS-K4 (AY515471)
Desulfomicrobium baculatum (AJ277894)
uncultured delta proteobacterium DGGE gel band 3 (AF538275)
Desulfovibrio profundus (U90726)
Desulfohalobium retbaense (U48244)
Desulfovibrio sp. D1 (AJ318380)
Desulfonatronovibrio hydrogenovorans (X99234)
Desulfonauticus submarinus (AF524933)
Desulfovibrionales
2
Desulfurellaceae
5
SAR342 cluster
0.10


D
marine bacterium HP34 (AY241567, MB*)
marine bacterium HP41 (AY24157, MB*)
marine bacterium HP27 (AY241562, MB*)
marine bacterium HP19 (AY241559, MB*)
marine bacterium HP44 (AY241570, MB*)
marine bacterium HP35 (AY241568, MB*)
marine bacterium HP2a (AY241556, MB*)
marine bacterium HP29 (AY241564, MB*)
Arenibacter troitsensis (AB080771)
marine bacterium HP14 (AY241558, MB*)
Flavobacterium sp. GWS-TZ-H113 (AY332196, -2, AGA)
marine bacterium HP42a (AY241569, MB*)
marine bacterium HP32b (AY241566, MB*)
Arenibacter latericius (AF052742)
marine bacterium HP2 (AY241555, MB*)
Zobellia galactanivorans (AF208293)
Zobellia uliginosa (M62799)
Zobellia sp. GWS-SE-H183 (AY332167, -5, CHI)
Muricauda ruestringensis (AF218782)
marine bacterium HP25 (AY241561)
CFB group bacterium SI-9-3 (AJ344499)
Flavobacteriaceae bacterium TN (AY177724, -5, MB)
Vitellibacter vladivostokensis (AB071382)
Flavobacterium sp. GWS-SE-H171 (AY332172, -2, CHI)
uncultured marine eubacterium OTU_C (AF207852)
Cellulophaga fucicola (AJ005973)
uncultured bacteroidetes bacterium GWS-BW-H14M (AY515448, -2, AGA)
uncultured bacteroidetes bacterium GWS-BW-H58M (AY515449, -2, AGA)
CFB group bacterium SFLA 10 (AJ344500)
Tenacibaculum amylolyticum (AB032505)
"Tenacibaculum skagerrakense" (AF469612)
Tenacibaculum mesophilum (AB032501)
Tenacibaculum sp. GWS-TZ-H201 (AY332217, -1, FUV)
uncultured marine eubacterium OTU_A (AF207850)
Tenacibaculum maritimus (M64629)
3
uncultured marine eubacterium OTU_B (AF207851)
Polaribacter sp.
Flavobacteriaceae bacterium T15 (AY177723, -5, MB)
uncultured bacteroidetes bacterium DGGE band GWS-SE-7 (AY274249)
CFB group bacterium OI-9-1 (AJ344495)
CFB group bacterium OI-15-2 (AJ344496)
Flavobacterium sp. OI-5-2 (AJ344494)
Flavobacterium aquatile (M62797)
Myroides odoratus (M58777)
CFB group bacterium OI-1-2 (AJ344492)
CFB group bacterium OI-14-4 (AJ344497)
CFB group bacterium OI-20-1 (AJ344498)
Psychroserpens burtonensis (U62913)
Flavobacteriaceae bacterium BIA (AY177722-6, MB)
Gelidibacter algens (U62914)
CFB group bacterium OI-1-1 (AJ344491)
uncultured bacteroidetes bacterium GWS-K1-4 (AY515483)
Brumimicrobium glaciale (AF521195)
Flexibacter aggregans (AB078040)
uncultured marine eubacterium OTU_D (AF207849)
uncultured bacteroidetes bacterium GWS-K6 (AY515484)
Cryomorpha ignava (AF170738)
"Blattabacterium cuenoti" (X75622)
6
Bacteroidaceae
6
Prevotellaceae
6
Porphyromonadaceae
uncultured bacteroidetes bacterium GWS-Kdna9 (AY515482)
uncultured bacterium 103 (AF369713)
uncultured bacteroidetes bacterium GWS-Kdna25 (AY515486)
Marinilabilia salmonicolor (D12672)
Rikenella microfusus (L16498)
4
Spingobacteriaceae
6
JTB248 Group
4
NB1-m group
marine bacterium HP11 (AY241557, MB*)
Flexibacter tractuosus (M58789)
marine bacterium HP28 (AY241563, MB*)
Flexibacteraceae bacterium GWS-BW-H154 (AY332100, -1, CEL)
marine bacterium HP23 (AY241560)
Cytophaga hutchinsonii (D12663)
Runella slithyformis (M27799)
Flexibacter ruber (M58788)
Flexibacter flexilis (M62794)
Flexibacter elegans (M58783)
Microscilla marina (M58793)
3
Flexibacter sp.
Sphingobacteria bacterium GWS-AG-H109 (AY332126, -1, AGA)
Sphingobacteria bacterium GWS-BW-H204 (AY332110, -2, FUV)
Sphingobacteria bacterium GWS-BW-H164 (AY332107, -2, CHI)
Sphingobacteria bacterium GWS-BW-H159 (AY332099, -1, CEL)
Sphingobacteria bacteriumGWS-BW-H4M (AY515446, rt, CHI)
Sphingobacteria bacterium GWS-SE-H160 (AY332162, -2, CEL)
Sphingobacteria bacterium GWS-BW-H70M (AY515450, rt, AGA)
Sphingobacteria bacterium GWS-BW-H11M (AY515447, ba, AGA)
Cyclobacterium marinum (M27801)
Sphingobacteria bacterium GWS-SE-H150 (AY332165, -1, CEL)
Lewinella cohaerens (AF039292)
Lewinella nigricans (AF039294)
Lewinella persica (AF039295)
Haliscomenobacter hydrossis (M58790)
uncultured bacteroidetes bacterium DGGE band GWS-AG-9 (AY274243)
Saprospira grandis (M58795)
2
uncultured bacteroidetes clone GWS-K23 (AY515485)
2
Flammeovirgaceae
Crenotrichaceae
14
OP11 Division
0.10
Flavobacteriaceae
Cryomorphaceae
Rickenellaceae
Flexibacteraceae
Saprospiraceae
Flavobacterales
Bacteroidales
Sphingobacteriales

V.4 DISCUSSION

Phylogenetic analysis of the 16S rRNA gene fragments of the clone library indicated a high bacterial diversity in the Wadden Sea, because the 48 different clones affiliated with seven phyla. In contrast, a clone library of a water sample of the adjacent open North Sea included only 16S rRNA gene-sequences of the phylum *Proteobacteria* (Eilers *et al*., 2000). The high diversity might by due to a higher variety of habitats and substrate sources in the Wadden Sea, resulting from the terrestrial influence and that of the open North Sea, as well as from high sedimentation and resuspension processes.

With culture independent approaches or DGGE analyses of high dilution steps of enrichment cultures several sequences were obtained which fell into previously described clusters of uncultured organisms, i.e. the SAR116 cluster (Mullins *et al.*, 1995), the RCA cluster (Selje *et al*., 2004) within the α-*Proteobacteria*, and the SAR86 cluster (Mullins *et al*., 1995) within the γ-*Proteobacteria*, or for which only one isolate is currently described like the SAR11 cluster (Giovannoni *et al*., 1990) and NOR5 cluster (Eilers *et al*., 2001). Bacteria affiliating with these groups seem to be typical members of the marine bacterioplankton, because they have been detected worldwide in marine systems. Therefore it appears not surprising to find them also in the Wadden Sea.

Overall, sequences affiliating with α-*Proteobacteria* were predominant, even though only two were obtained from the clone library. This might be due to a primer bias, because many sequences affiliating with this *Proteobacteria* subclass were obtained with DGGE analyses of environmental samples and enrichment cultures when other primers for the 16S rRNA genes of *Bacteria* were used (Stevens *et al*., 2004a; Selje and Simon, 2004). A dominance of α-*Proteobacteria* was also found in several other studies using either culture-independent (e.g., Gonzalez and Moran, 1997; Cottrell and Kirchman, 2000a), or a combination of culture-dependent and -independent methods (Gonzalez *et al.*, 1996; Eilers *et al*., 2000; Gonzalez *et al.*, 2000).

Bacteria affiliating to the *Roseobacter* clade, a subgroup of the α-*Proteobacteria*, were routinely found in marine waters (Fuhrman *et al.*, 1993; Mullins *et al*., 1995; Rappé *et al.*, 1997; Suzuki *et al*., 1997; Giuliano *et al.*, 1999), and constituted high numbers in coastal seawater (Gonzalez and Moran, 1997). Organisms of two clusters within the *Roseobacter* clade appear to be abundant in the Wadden Sea. Selje *et al*. (2004) detected the RCA cluster to be permanently present in the Wadden Sea and showed that it comprises up to 10% of total bacteria. No bacterial isolate of the RCA cluster so far is available, but three strains of the Wac I cluster were isolated (AP-27, HP24, TL). For the latter cluster we found evidence of a high abundance, because the isolates and phylotypes were frequently obtained from high dilutions of MPN series and the sequences are closely related with those of DGGE bands from environmental samples (GWS-FL-3, GWS-AG-3) (Fig. 3A-2). The DGGE bands at least seasonally persisted, i.e. GWS-FL-3 from February to June 2000, and GWS-AG-3 from July to September 1999 (Stevens *et al*., 2004a).

Many sequences of other subgroups within α-*Proteobacteria* were also obtained from the Wadden Sea and are widely distributed in the phylogenetic tree (Fig.3A-1).

Sporadically isolates or phylotypes were obtained also from high dilutions, but without clear evidence of a high abundance as for the clusters described above.

The second largest group in our study were γ-*Proteobacteria*, which were found to be prominent in marine systems applying culture-independent (Crump *et al.*, 1999) and -dependent methods (Mullins *et al*., 1995). Also in open waters of the North Sea γ-*Proteobacteria* were found in greater proportions than any other phylogenetic group, by FISH (Uphoff *et al.*, 2001), and by clone library analysis and isolation procedures (Eilers *et al*., 2000; Eilers *et al*., 2001). FISH results in the latter study showed, however, that α-*Proteobacteria* and *Cytophaga-Flavobacteria* (*Bacteroidetes*) were also prominent groups. In our study the majority of the isolates affiliating with γ-*Proteobacteria* seemed to be typical "laboratory weed". Most of the isolates were obtained from low dilutions, and only in one case a sequence of an uncultured phylotype (DGGE band GWS-FL-7) affiliates closely to isolates (Fig. 3B-1). γ-*Proteobacteria* were often selectively isolated (e.g., Eilers *et al*., 2000; Fuchs *et al.*, 2000), possibly due to their ability to survive long periods of starvation and to thrive on high substrate concentrations (Amy and Morita, 1983; Nyström, 2001). The discrepancy between the phylogenetic affiliations of our isolates and most sequences of uncultured phylotypes suggest that most of them are of low significance in the environment. Some of our clones from the clone-library affiliate to the clusters NOR5 (Eilers *et al*., 2000) and SAR86 (Giovannoni *et al*., 1990; Fuhrman *et al*., 1993). The former was shown to comprise 8% of total bacterial counts in North Sea surface waters and both seem to have a global distribution. Most of our sequences from DGGE analyses and the clone library, however, affiliate to the newly defined SAMMIC group within γ-*Proteobacteria* (Fig. 3B-1). This group comprises mostly sequences of bacteria from surfaces in marine systems. Within this group the Wadden Sea sequences fall in two distinct clusters, the Wadden Sea Clusters I and II, and two sequences are loosely affiliated with the JTB255 cluster (Fig. 3B-2). Only three sequences of the SAMMIC group are from bacterial isolates. Two of these isolates are sulfur-oxidizing bacteria (ODIII6, NDII1.2), were obtained from a hydrothermal vent system and affiliate with the BPC036 cluster (Sievert *et al*., unpublished), the third isolate (HNSS31) affiliates with the JTB255 cluster. Whether bacteria of the SAMMIC group are truly abundant in marine habitats, and whether they prefer surfaces has to be proven, e.g., by FISH analyses with specific probes.

Surprisingly, δ-*Proteobacteria* constituted the second largest group in our clone library. Most of these sequences have next relatives which are known to be obligately anaerobic. Usually these organisms are not important in the water column and typically occur in anoxic sediments. However, sulfate-reducing bacteria, which are expected to be restricted to the anoxic zones, were shown to be present in the upper, oxic layer in rather high numbers (Sass *et al.*, 1997; Sahm *et al.*, 1999). The importance of resuspension in shallow tidally affected coastal areas has been shown previously (van Leussen, 1996; Jago *et al.*, 2002; Mc Candliss *et al.*, 2002; Stevens *et al*., 2004a). Therefore, we assume that the high resuspension activity in the Wadden Sea causes the transport of these organisms into the water column.

Sequences related closely to our δ-*Proteobacteria* clones were found in a South Korean mud flat in Sunchon Bay (Fig. 3C, clones 25, 53, 131, 134, 136, 224, 296; Lee *et al*., unpublished). However, no information is available whether the respective samples were taken from oxic or anoxic habitats. Sequences affiliating with the obligate aerobic *Myxococcales* were detected by DGGE analyses of environmental samples from the Wadden Sea. These DGGE bands were detected repeatedly in samples taken from the sediment surface and from suspended aggregates in various

seasons (Stevens *et al*., 2004a), indicating that these bacteria are permanent residents of these habitats.

Bacteroidetes occur in nearly every aquatic system, and in marine environments they were often detected as the most abundant phylogenetic group (e.g., Llobet-Brossa *et al*., 1998; Simon *et al*., 1999; Kirchman, 2002). Our analysis provides very scarce evidence for high abundance of isolates or clones of the *Bacteroidetes*. Only in one case we found a 100% sequence similarity between a DGGE band from an environmental sample (GWS-SE-7) and an isolate obtained from a high dilution enrichment culture (T15). Strains affiliating closely with the genus *Arenibacter* and with *Cyclobacterium marinum* were isolated frequently (Fig. 3D), but none of them was isolated from a higher dilution. This indicates that the latter organisms, as some groups within γ-*Proteobacteria*, are obviously easy to isolate, but probably play no major role in the environment.

Bacteria within the *Bacteroidetes* are known to be chemoorganotrophic and especially proficient in degrading various polymers such as cellulose, chitin, and pectin (Reichenbach, 1992; Kirchman, 2002). Our results show that bacteria of other groups are obviously also able to use polymers very efficiently, as many strains from the different groups were isolated with polymers (Fig. 3). Interestingly, organisms we assume to be abundant (see above), were always obtained or enriched with marine broth 2216 or with autoclaved seawater (Selje and Simon, 2004). Recently we reported that highest bacterial numbers with MPN series with samples from the Wadden Sea were obtained with marine broth 2216 or *Fucus vesiculosus* as substrates (Stevens *et al*., 2004b). Single biopolymers resulted in lower numbers, indicating that the more specialized organisms were less abundant in the environmental sample. Thus, more complex media seem to be better suitable for the isolation of abundant bacteria. Low substrate concentrations as present in the autoclaved sea water medium used by Selje and Simon (2004) also appear to favour the enrichment and/or isolation of abundant bacteria. This is in line with findings by Connon and Giovannoni (2002).

Data about bacterial diversity from tidal flats other than the German Wadden Sea are sparse, and it remains to be tested whether other tidal flats contain similar communities. We found evidence for high abundances of organisms of several groups. How abundant these bacteria really are in the Wadden Sea remains to be tested, e.g., by using specific oligonucleotide probes for FISH analyses, as suggest for the SAMMIC group. We do not assume that we detected all abundant groups of bacteria present in the Wadden Sea, however, this study provides a basis for future studies analysing the bacterial communities of the Wadden Sea and other tidal flat systems in more detail, and for a comparison with the communities of other coastal and open ocean habitats.

V.5 EXPERIMENTAL PROCEDURES

Sampling site and procedure

For isolation of bacteria a water sample was collected on 27 May 1999 in the East Frisian Wadden Sea, Germany (53° 37′05′′ N, 07° 08′38′′ E, Fig. 1, sampling site A) at high tide with a black 10 liter plastic jug, rinsed with seawater. The sample was brought to the lab on ice in a cooling box and processed further within 2 h. Water temperature (10°C) and salinity (24.6 psu) were measured in situ by the probe LF 196 (WTW, Weilheim, Germany), respectively. For clone library construction a water sample was collected on 25 October 1999 from the Wadden Sea (53° 42' 20" N, 07° 43' 11" E, Fig. 1, sampling site B) as described above and brought to the lab for further processing. A volume of 250 ml was filtered onto a 0.2 µm Nuclepore filter (47 mm diameter). The filter was immediately frozen at -80°C until DNA-extraction. Water temperature was 9.6°C, salinity 26 psu, the oxygen content of the water was 93%, and the pH value 7.9.

Isolation of bacteria

Ten-fold-dilution series were prepared in 20-ml test tubes. For the dilution series and isolation of bacteria artificial seawater (ASW) described by Atlas (1996), modified as described by Stevens *et al*. (2004b), and amended with 0.1% (w/v) of a carbon source was used. Agar-agar, alginate, casein, cellulose, chitin, dried and pestled *Fucus vesiculosus*, laminarin, marine broth 2216 (Difco, Germany), palmitate, starch and stearic acid were added as carbon source. Preparation of the substrates was performed as described by Stevens *et al*. (2004b). One ml of well-mixed unfiltered seawater was used for inoculation of 9 ml medium. Between every dilution step the samples were vigorously shaken on a vortex mixer. Incubation of the cultures was at 15°C in the dark for eight weeks, and bacterial growth was determined by changes in the OD (660 nm) and by epifluorescence microscopy after DAPI staining (Porter and Feig, 1980). Bacteria were obtained from several dilutions in which growth was detected and isolated on agar plates prepared with the same media as used for the dilution series, amended with 2% (w/v) agar-agar (Difco, Germany). Bacteria were also isolated from 1 liter rolling tanks filled with natural seawater from the sampling site, amended with 0.1% (w/v) agar-agar, alginate, chitin or *Fucus vesiculosus* and incubated for 100 days at 15°C in the dark. Ten ml batch cultures were prepared in test tubes with modified ASW amended with agar-agar or *F. vesiculosus* (see above) as substrates and inoculated with 1 ml Wadden Sea water and incubated at 15°C in the dark on a shaker at 150 rpm. Isolates were compared and checked for purity by DGGE of PCR-amplified 16S rRNA gene fragments as described by Teske *et al.* (2000). Isolates showing a single band on the denaturing gradient gel were sequenced thereafter.

DNA extraction and PCR

Bacterial genomic DNA of pure cultures was isolated by phenol-chloroform extraction with SDS, and isopropanol precipitation after bead-beating, as described previously (Stahl *et al.*, 1988; MacGregor *et al.*, 1997), but modified: Lysozyme treatment was not applied, precipitation done at -20°C, and molecular grade water (Eppendorf, Germany) was used for resuspension at 4°C over night. Primers GM3 (8F) combined with GM4 (1498R) (Muyzer and Ramsing, 1995) or 907RM (Muyzer *et al*., 1998) were used to amplify 1490, or 899 bp fragments of the 16S rRNA genes of the isolates. PCR amplifications were performed as described before (Brinkhoff and Muyzer, 1997). Aliquots (4 µl) of the amplicons were analyzed in 2% (w/v) agarose gels and stained with ethidium bromide (1 µg ml^{-1}) (Sambrook *et al.*, 1989). For subsequent sequence analysis PCR products were purified with the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, USA).

Clone library construction

Bacterial genomic DNA of the water sample was isolated as described before (Stevens *et al*., 2004a). For PCR amplification of almost complete 16S rRNA gene fragments primers GM3F (8F) and GM4R (1492R) (Muyzer *et al.*, 1995) were used. PCR amplifications were performed in triplicates. PCR conditions were as described above. Before cloning PCR products of the three parallels were combined and purified with the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, USA). For cloning the pGEM®-T Vector System II (Promega, Madison, USA) was used according to the technical manual of the manufacturer. Clones were picked randomly and sequenced until 50 different clones were obtained (altogether 65 clones). Sequencing of the clones was performed according to Stevens *et al*. (2004a). Clone sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak *et al.*, 2001).

Phylogenetic analysis

Phylogenetic affiliation on class level of our isolates and clones was determined by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). For comparison with currently available sequences of *Proteobacteria* (α-, γ-, and δ-subgroup) and *Bacteroidetes* from oxic Wadden Sea habitats (water column and oxic sediment layer) we retrieved all available sequences from the NCBI server (http://www.ncbi.nlm.nih.gov) and performed a comprehensive phylogenetic analysis of these and the sequences obtained in our study. The sequences include those of isolates from this and some previous studies (Bruns and Berthe-Corti, 1999; Bruns *et al*., 2001; Brinkhoff and Muyzer, 1997; Brinkhoff *et al*., 2004; Grossart *et al*., 2004; Stevens *et al*., 2004b; Selje and Simon, 2004), and clones from our clone library, and from DGGE bands (Llobet-Brossa *et al*., 2002; Mussman *et al*., 2003; Selje and Simon, 2003; Stevens *et al*., 2004a). The sequences were mainly derived from samples of three locations in the Wadden Sea (Fig. 1). Samples from site A were exclusively taken in this study. Sampling site B (53° 43’ 20’’ N, 07° 43’ 20’’ E) was chosen by Brinkhoff *et al*. (2004), Grossart *et al*. (2004), and Stevens *et al*.

(2004a and b), and site C (53° 36' 04" N, 08° 28' 21" E) by Selje and Simon (2003, 2004). Sampling locations of the other studies were at different sites of the Jadebusen bay (Fig. 1).

Phylogenetic trees were constructed with the ARB software package [http://www.arb-home.de (Ludwig *et al.*, 2002)]. Only sequences with at least 1300 bp were considered for backbone-trees constructed by the maximum likelihood method. Only type strains were used with the following exceptions because no 16S rRNA gene sequence of the type strain was available: *Phyllobacterium myrsinacearum*, D12789; *Hyphomicrobium vulgare*, Y14302; *Brevundimonas vesicularis*, AY456200; *Thioploca ingrica*, L40998; *Polyangium vittelinum*, AJ233944; *Cyclobacterium marinum*, M26788, and *Marinilabilia salmonicolor*, D12672. Not validly described species are marked by quotation. A filter was constructed to exclude alignment positions from the calculations at which less than 50% of sequences of the entire set of data had the same residues. Sequences < 1300 bp were added afterwards by maximum parsimony using the same filter. In the phylogenetic trees, the habitat, substrate and the dilution step were added to the isolate-ID for better estimating the significance of emerging clusters.
The sequences from isolates and clones obtained and described in this study are available from GenBank under accession no. AY515404 to AY515495.

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VI

UNTERSUCHUNG HETEROTROPHER BAKTERIEN AUS DEM WATTENMEER AUF NATURSTOFFPRODUKTION

VI.1 EINLEITUNG

Das biotechnologische Potential mariner Mikroorganismen ist hinsichtlich der Produktion pharmakologisch wirksamer Substanzen vermutlich noch lange nicht ausgeschöpft (Jensen und Fenical, 1994; Kelecom, 2002). Heute werden gleichermaßen Mikro- wie Makroorganismen (Schwämme, Algen) untersucht, wobei jedoch viele vermeintlich von Makroorganismen produzierte Substanzen von mit ihnen assoziierten Bakterien produziert werden (Wagner-Döbler *et al.*, 2002). Nachdem 1966 das erste von einem marinen Bakterium produzierte Antibiotikum beschrieben wurde (Burkholder *et al.*, 1966) stieg die Anzahl der Verbindungen, die aus marinen Bakterien isoliert wurden von Jahr zu Jahr. Nur wenige Substanzen sind jedoch tatsächlich pharmazeutisch aktiv und interessant für den Markt, viele dieser Stoffe befinden sich jedoch schon in vorklinischen Untersuchungen (Faulkner, 2000).

Die meisten Naturstoffe aus marinen Bakterienkulturen wurden aus *Streptomyces* und *Alteromonas* Spezies isoliert (Wagner-Döbler *et al*., 2002), doch scheint die Fähigkeit zur Naturstoffproduktion auch in anderen phylogenetischen Gruppen weit verbreitet zu sein (Long und Azam, 2001; Grossart *et al.*, 2004), so z.B. in den α-*Proteobacteria*, vornehmlich in der *Roseobacter* Gruppe (Böttcher *et al.*, 2000; Gram *et al.*, 2002; Brinkhoff *et al.*, 2004). Zudem haben einige Arbeiten gezeigt, dass ein hoher Anteil an Oberflächen angehefteter Bakterien antagonistische Aktivität zeigen (Long und Azam, 2001) und antibiotisch wirkende Verbindungen produzieren (Burgess *et al.*, 1999). Man vermutet, dass hier der Konkurrenzdruck um Resourcen wie Nahrung (Slattery *et al.*, 2001) und Platz zu effektiven Adaptationen der angehefteten Bakterien geführt hat (Grossart *et al*., 2004). Einige Stämme, die in Reinkultur keine Produzenten waren, haben unter Konkurrenzdruck, z.B. bei Hinzugabe lebender anderer Bakterienzellen, von Zellüberständen oder verschiedener chemischer Verbindungen Antibiotika produziert (Burgess *et al*., 1999).

Bis Anfang der 60er Jahre des letzten Jahrhunderts wurden vornehmlich terrestrische Habitate und Organismen auf Naturstoffproduktion untersucht. Die Häufigkeit isolierter „neuer“ Substanzen schwand rapide. Bei der Suche nach neuen chemischen Strukturen und bioaktiven Verbindungen wurden nun auch „ungewöhnliche“, d.h. vornehmlich „nicht terrestrische Habitate“ (z.B. die Tiefsee oder Hydrothermalquellen; Deming, 1998) untersucht, in der Hoffnung, dass Organismengemeinschaften, die sich an extreme Habitate adaptierten, unterschiedliche, „neue“ Naturstoffe produzieren.

Das Wattenmeer stellt mit seinen ständigen hydrodynamischen Variationen ein solch „ungewöhnliches“ Habitat dar. Dort vorkommende Bakterien müssen sich stetig schwankenden Umweltbedingungen anpassen, einschließlich des schwankenden Salzgehaltes, der Wasserverfügbarkeit und des Nährstoffangebotes (z.B. bei Algenblüten). Die Partikelfracht ist durch die auftretenden klimatischen (Wind) und tidalen Resuspensionsereignisse besonders hoch.

Zur Erfassung des biotechnologischen Potentials von Wattenmeerbakterien wurden insgesamt 41 Bakterienstämme aus dieser Arbeit biologisch und chemisch auf die Produktion neuer Naturstoffe untersucht. Die Stämme wurden aus der Wassersäule und der oberen Sedimentschicht mit verschiedenen Substraten und unterschiedlichen Methoden isoliert und phylogenetisch identifiziert. Dabei sollte

u.a. festgestellt werden, ob die Produktion von Naturstoffen in Wattenmeerbakterien gehäuft bei speziellen phylogenetischen Gruppen auftritt. Gefundene Naturstoffe wurden chemisch aufgereinigt und massenspektrometrisch identifiziert. Mir wurde die Möglichkeit gegeben, für 3 Monate die Labore der Arbeitsgruppe Zeeck (organische Chemie) der Georg-August Universität in Göttingen zu nutzen und 6 meiner Stämme auf die Produktion von Naturstoffen zu untersuchen. Die Ergebnisse dieser Arbeit werden im Folgenden dargestellt.

Um eine Übersicht über die aus den Stämmen dieser Gesamtstudie isolierten Produkte (isoliert und aufgeklärt von Liang *et al.*, 2004) zu geben, wurden sie am Ende der Diskussion in einer Tabelle zusammengefasst.

VI.2 MATERIAL UND METHODEN

A) Isolierung der Bakterien und phylogenetische Analyse

Wasser- und Sedimentproben wurden im Mai 1999 und Oktober 1999 im Wattenmeer der südlichen Nordsee an der Küste bei Neuharlingersiel und Norddeich-Mole entnommen (vgl. Kapitel V.2). Bakterienstämme wurden aus MPN-Verdünnungsstufen (vgl. Kapitel III.2) und aus Rolltankexperimenten (Kapitel IV.2) isoliert und, wie ausführlich in Kapitel III.2 dargestellt, phylogenetisch charakterisiert. Eine Einordnung der Stämme in phylogenetische Stammbäume wurde in Kapitel V vorgenommen.

Zugangsnummern der Sequenzen. Die Sequenzabschnitte der 16S rRNA Gene der hier untersuchten Stämme sind in der GenBank Datenbank unter den Zugangsnummern AY370620 (GWS-BWrt-H120M), AY332175 (GWS-SE-H303), AY515412 (GWS-BW-H43M), AY515407 (GWS-BWrt-H22M), AY515408 (GS-BW-H24M), AY515424 (GWS-BW-H72M) erhältlich.

B) Biologisch-Chemische Untersuchung der Isolate auf Naturstoffproduktion

-Biologische Untersuchungen

Vorkultur. Sechs Bakterienstämme wurden nach Farbe und Form der Kolonien (auf Agar-Platten mit MB 2216, Difco) ausgewählt. Diese und eine unbeimpfte Kontrolle (s. Tabelle VI.1) wurden in Oldenburg in 1 l-Ansätzen (Inokulum: 10 ml) bei 20 °C 2 Tage auf einem Schüttler (150 rpm) in 2 l-Erlenmeyerkolben mit Schikane inkubiert (Medium: Marine Broth 2216, Difco). Der Transport der Proben nach Göttingen erfolgte mit Kühlelementen in Kühltaschen. Sofort nach der Ankunft wurden die biologische Untersuchung der Zellkulturen, die Messung der pH-Werte und die Aufbereitung für die chemischen Untersuchungen (s. Abb VI.1) durchgeführt. Parallel wurde zu alle durchgeführten Versuche und Analyse eine Kontrolle, die nur das verwendete Medium enthielt, mit untersucht .

Agardiffusionstests. Papierfilterplättchen (Durchmesser 9 mm, Dicke: 0.5 mm) wurden mit 25 µl der flüssigen Zellkultur getränkt, unter einer Sterilbank getrocknet und auf Agarplatten mit je einem ausgespatelten Testorganismus (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Mucor hiemalis*, *Candida albicans*, *Chlorella sorokiniana*, *Chlorella vulgaris* und *Scenedesmus suspicans*) aufgelegt. Die Platten wurden einen Tag im Dunkeln bei 37 °C (Bakterien), 27 °C (Pilze) oder 4 Tage bei Tageslicht bei 25 °C (Mikroalgen) inkubiert.

Medien für die Testorganismen (in g/l). Für *S. aureus*, *E. coli*: Bacto nutrient broth 8, $NaCl_2$ 5, Agar-Agar 15; für *B. subtilis*: Glucose 5, Na-Citrat x $2H_2O$ 0.5, KH_2PO_4 3, K_2HPO_4 7, $MgSO_4$ x $7H_2O$ 0.1, $(NH_4)_2SO_4$ 1, Agar-Agar 15; für *C. albicans*: Hefeextrakt 4, Malzextrakt 10, Glukose 4, Agar-Agar 15, pH = 5.5; für Mikroalgen: $NaCl_2$ 0.25, $MgSO_4$ x $7H_20$ 0.75, $NaNO_3$ 2.5, $CaCl_2$ x $2H_2O$ 0.25, K_2HPO_4 0.75, KH_2PO_4 1.75, Fe-EDTA Stammlösung: 1 ml; Spurenelementestammlösung: 1 ml

Fe-EDTA Stammlösung (in g/100 ml): $FeSO_4$ x $7H_2O$ 0.07 g, EDTA 0.093 g; Lösungsmittel: destilliertes Wasser.

Spurenelementelösung (in g/100 ml): $MnSO_4$ x H_2O 0.0169, Na_2MoO_4 x $2H_2O$ 0.13, $Co(NO_3)_2$ x $6H_2O$ 0.1, $CuSO_4$ x $5H_2O$ 0.05, H_3BO_3 0.1, $ZnSO_4$ x $7H_2O$ 0.1; Lösungsmittel: destilliertes Wasser

Die Medienbestandteile wurden von den Firmen Merck, Difco (Bacto nutrient Broth), Gibco (Hefeextrakt) und Brunnengräber (Casein) geliefert.

-Chemische Untersuchungen (s. Abb. VI.1)

Herstellung der Rohextrakte.

Die Kulturen wurden zentrifugiert (40 min, 45000 rpm) und das Pellet wurde mit Ultraschall (10 min) und 150 ml Aceton gelöst. Der Kulturüberstand wurde mit 2N HCL auf pH 7 eingestellt und mit Ethylacetat 2 mal ausgeschüttelt (je 200 ml). Dies wurde sukzessive mit der wässrigen Phase nach Adjustieren auf pH 4 (2N HCL) und pH 10 (2N NaOH) wiederholt.

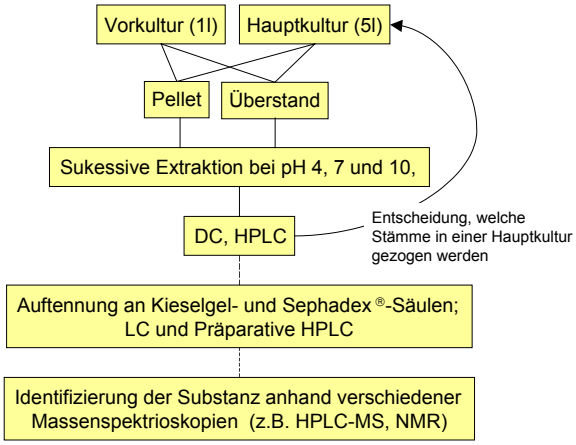


Abb. VI.1. Vorgehensweise bei den chemischen Untersuchungen. Die gestrichelten Linien bezeichnen Vorgänge, die nur mit Extrakten der Hauptkultur durchgeführt wurden. Abkürzungen: s. Text zu den Methoden

Lagerung.

Die jeweiligen Extrakte wurden im Rotationsverdampfer eingeengt (bis zur Trocknung) und bei –20°C gelagert.

Chromatographische Untersuchung der Rohextrakte.

Zur Abschätzung des Vorkommens neuer Produkte in den einzelnen Extrakten wurden diese chromatographisch untersucht. Dazu wurden die Zellextrakte in 500 µl (in Einzelfällen 1 ml) Chloroform:Methanol (CM 1:1) gelöst, die Überstandsextrakte in 500 µl (in Einzelfällen bei pH 10: 3 ml) Methanol:H_2O (MH 9:1) gelöst.

Dünnschichtchromatographie (DC). Acht µl eines jeden Extraktes wurden auf mit Silikagel überzogenen Glasplatten (Nano, MN KG 60; Dicke der Silikachicht: 0.25 mm, Fa. Merck) aufgetüpfelt, und in einer geschlossenen Kammer mit Lösungsmittel (CM 9:1, 20 ml) inkubiert (Laufhöhe: 10 cm). Nach Trocknung wurden die DCs mit der Eigenfarbe und unter UV (UV 254 und UV 366) und nach Anfärbung fotografiert.

Die Anfärbung erfolgte durch Besprühen mit den Färbelösungen Anisaldehyd, Ehrlichs Reagenz und Orcin (Merck, 1980) und anschließender Entwicklung der Farben durch erhitzen der DCs auf ca.
100 °C mit einem Heißluftfön (Merck, 1980).

Analytische High-Performance-Liquid-Chromatography (HPLC). Zellextrakte wurden mit 600 µl, Überstandsextrakte mit 600 µl (in Einzelfällen mit 1500 µl) MH (9:1) gelöst, zentrifugiert und davon 20 µl in die HPLC eingespritzt (Pumpe: 322 System, Autosampler 360, Detektor Diode Array Detektor 440, Hard- und Software: Kroma System 2000, Version 1.6, alles Fa. Kontron; Säule: Knauer RP-18). Das Trägerlösungsmittel war Acetonitril mit 0.1 % Phosphorsäure, die Fließrate betrug 0.5 ml /min, die Dauer eines Laufes 35 min. In den ersten 25 min des Laufes wurde die Acetonitrilkonzentration stetig von 20 % auf 100 % erhöht, die letzten 10 min verblieb sie auf 100 %. Messungen erfolgten bei den Wellenlängen 220, 254, 300 und 400 nm.

Nach Hochskalieren (5-l Ansätze) ausgesuchter Kulturen wurden die DCs und die Messungen mit der analytischen HPLC wiederholt, um die Reproduzierbarkeit gefundener Zonen bzw. Peaks zu überprüfen.

Substanzisolierung.

Säulenchromatografie. Die im DC und in der HPLC beobachteten Produkte wurden an einer Kieselgelsäule (KG80, Lösungsmittel: Methylenchlorid: Dichlormethan 90:10), erhaltene Fraktionen dann an einer Sephadex LH-20 Säule (Pharmacia) in Unterfraktionen aufgetrennt (Lösungsmittel: Methanol).

Präparative HPLC. Die Extrakte aus der Sephadex-Säule wurden in der präparativen HPLC weiter isoliert (Pumpe: PU-1587 System, Detektor: UV-1575 dioden array, Software: Borwin 1.50, Säule: Kromasil 100 C18, 7 µm, 20 x 250 mm, alles Fa. Jasco). Das gleiche Programm und Lösungsmittel wie bei der HPLC-ESI (Electrospray-Ionisation)-MS (s.u.) wurden verwendet.

Substanzidentifizierung.

HPLC-ESI (Electrospray-Ionisation)-MS. Zur Identifizierung der Masse der isolierten Verbindungen wurde die HPLC-ESI-MS (Finnigan LCQ, Pumpe Rheos 4000 flux, UV-Detektor UVIS205 linear, Säule: Grom Supersher 100 RP-18 endcapped 4µm; 2 x 100mm) verwendet. Laufmittel und Lösungsmittel der Extrakte waren Methanol und Wasser, jeweils mit mit 0.05 % Ameisensäure. Der Programmverlauf war wie folgt: von 20 % auf 100 % Methanol in 20 min, 100 % Methanol für 10 min, 100 % Methanol zu 20 % Methanol in 2 min und 20 % Methanol 13 min. Die Lösungen wurden vorher in einem Ultraschallbad (10 min) und mit Helium entgast.

Massenspektroskopie (EI-MS) und 2D-NMR (nuclear magnetic resonance) Spectra. 1H, 1H-Correlated Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Connectivity (HMBC) wurden in den technischen Abteilungen des Fachbereiches als Serviceleistung gemessen. Über die 2D-Spektren kann die Stellung der Atome (C, H) zueinander interpretiert werden, EI-MS dient wie die HPLC-ESI-MS der Massenidentifizierung.

VI.3 ERGEBNISSE

A) Isolierung der Bakterien und phylogenetische Analyse.

Die Ergebnisse der Isolierung und Sequenzierung sind in Tabelle VI. 1 zusammengefasst.

Tabelle VI.1. Auf Produktion von Naturstoffen untersuchte Isolate. Angegeben sind die Isolatbezeichnung (ID) mit dem Subhabitat, die Verdünnungsstufe bzw. Methodik und das Substrat mit der das Isolat gewonnen wurde, die phylogenetische Einordnung, die Sequenzähnlichkeit zu dem nächsten Verwandten nach BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) und dessen Zugangsnummer. GWS = German Wadden Sea; BW = bulk water (ungefiltertes Wasser); SE = Sediment, oxische Schicht; rt = Rolltank; hgc = High GC (*Actinobacteria*); lgc = Low GC. (*Firmicutes*), α = α-*Proteobacteria*; γ= γ-*Proteobacteria*

Isolat-ID	Isolierung	Substrat	Phylogenie	nächster Verwandter (BLAST)
GWS-BWrt-H120	Rolltank	Alginat	hgc	99% *Kokuria* rosea (Y11330)
GWS-SE-H303	-1	Chitin	γ	91% marine bact. DPT1.2 (AJ002567)
GWS-BW-H43M	-1	Fucus	α	98% *Methylarcula* sp. Bio-24 (AJ534207)
GWS-BWrt-H22M	Rolltank	Fucus	α	98% *Methylarcula* sp. Bio-24 (AJ534207)
GWS-BW-H24M	-1	Casein	α	99% Antarctic bact. R-9221 (AJ441010)
GWS-BW-H72M	-6	Cellulose	α	98% *Paracoccus marcusii* (Y12703)
GWS-BW-H79M	-1	Fucus	lgc	99% *Bacillus pumilus* (AY456262)

B) Biologisch-Chemische Untersuchung.

Die Ergebnisse der chemischen Untersuchungen sind graphisch in Abbildung VI.2 zusammengefasst und im Folgenden im Text dargestellt. Die Auswertung der biologischen Tests ergab kein auffälliges Wachstum (Hemmhof / verstärkter Wuchs).

Beschreibung der Rohextrakte.

Nach der Zentrifugation verblieben die Pigmente der farbigen Kulturen meist nur in einer der beiden Fraktionen. Nach der Extraktion mit Aceton erschienen die Farben intensiver und nuancierter als in den Überständen (s. Tabelle 2). Die Kontrolle erschien im Pellet beige und in der Extraktion des Überstandes farblos. Die Farben sind in den Dünnschichtchromatogrammen wiederzufinden (s. Abb. VI.3).

Tabelle VI.2. Farbgebung der untersuchten Isolate und deren Rohextrakte. Angegeben sind die Isolatbezeichnung (ID), die Farben der Flüssigkulturen, der Verbleib der farbigen Pigmente nach der Auftrennung in Überstand und Zellmaterial und die Farbe des in Aceton gelösten Extraktes (in den DCs wiederzufinden). GWS = German Wadden Sea; BW = bulk water (ungefiltertes Wasser); SE = Sediment, oxische Schicht; rt = Rolltank.

Isolat-ID	Farbe der	Pigment nach Trennung		Farbe gelöst in Aceton
	Flüssigkultur	in Überstand	in Pellet	
GWS-BWrt-H120M	rosa		X	rosa-orange
GWS-SE-H303	hellbraun	X		gelb
GWS-BW-H43M	braun	X		gelb
GWS-BWrt-H22M	rot-braun	X	X	leicht orange
GWS-BW-H24M	stark gelb	X	X	stark gelb
GWS-BW-H72M	dunkelbraun	X	(X)	orange
Kontrolle			X	farblos

Auswertung der Dünnschichtchromatographie (DC).

Die Ergebnisse der Dünnschichtchromatographie sind exemplarisch für 3 Stämme in Abb. VI.3 dargestellt. Die Kontrolle zeigt im Eigenlicht nur bei pH 4 eine leicht gelbe Färbung, Mit Anis, Orcin und Ehrlichs Reagenz gefärbt zeigen sich deutlich verschiedenfarbige Bereiche. Das DC der Kultur GWS-BWrt-H120M und GWS-BW-H72M zeigen bei pH 4 und pH 7 gelbe (pH7) bis braune (pH4) Zonen. In der Kontrolle ist eine leicht gelbe Bande in ähnlicher Laufhöhe bei pH 4 zu sehen. Die DC des Stammes GWS-BW-H24M zeigten in der Eigenfarbe auffällige gelbe Zonen im Zellextrakt und v.a. im Extrakt aus dem neutralen pH-Bereich. Diese Zonen sind bei der Anfärbung mit Ehrlichs Reagenz und mit Orcin zu sehen und wirken löschend im UV-Licht mit einer Wellenlänge von 366 nm. Die anderen Stämme zeigten auch Auffälligkeiten, aber die Mengen der evtl. interessanten Substanzen erschienen schon im DC sehr gering, weshalb auf eine weitere Bearbeitung verzichtet wurde. Die Extrakte bei pH 10 ergaben nur sehr schwache (z.B. bei GWS-BW-H24 mit Ehrlichs Reagenz) oder keine Resultate.

Auswertung der analytischen HPLC.

Alle Extrakte wurden in der analytischen HPLC bei 4 verschiedenen Wellenlängen gemessen. Zwei der Extrakte, der Zellextrakt des Stammes GWS-BW-H24M und der Überstandsextrakt (pH4) des Stammes GWS-BWrt-H120M gaben klar abgegrenzte deutliche Peaks. Allgemein ergaben die Zellextrakte und die Überstandsextrakte bei pH 4 die meisten und höchsten vom Medium verschiedenen Peaks.

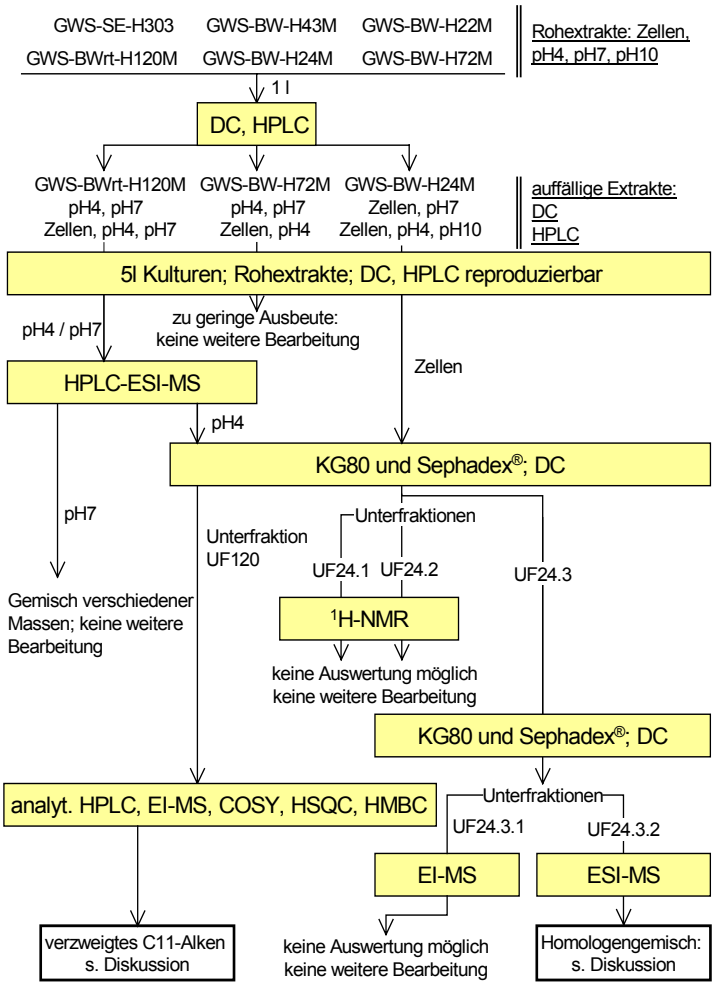


Abb. VI.2. Zusammenfassung der Vorgehensweise und der Ergebnisse der chemischen Untersuchungen. Abkürzungen: s. Text zu den Methoden

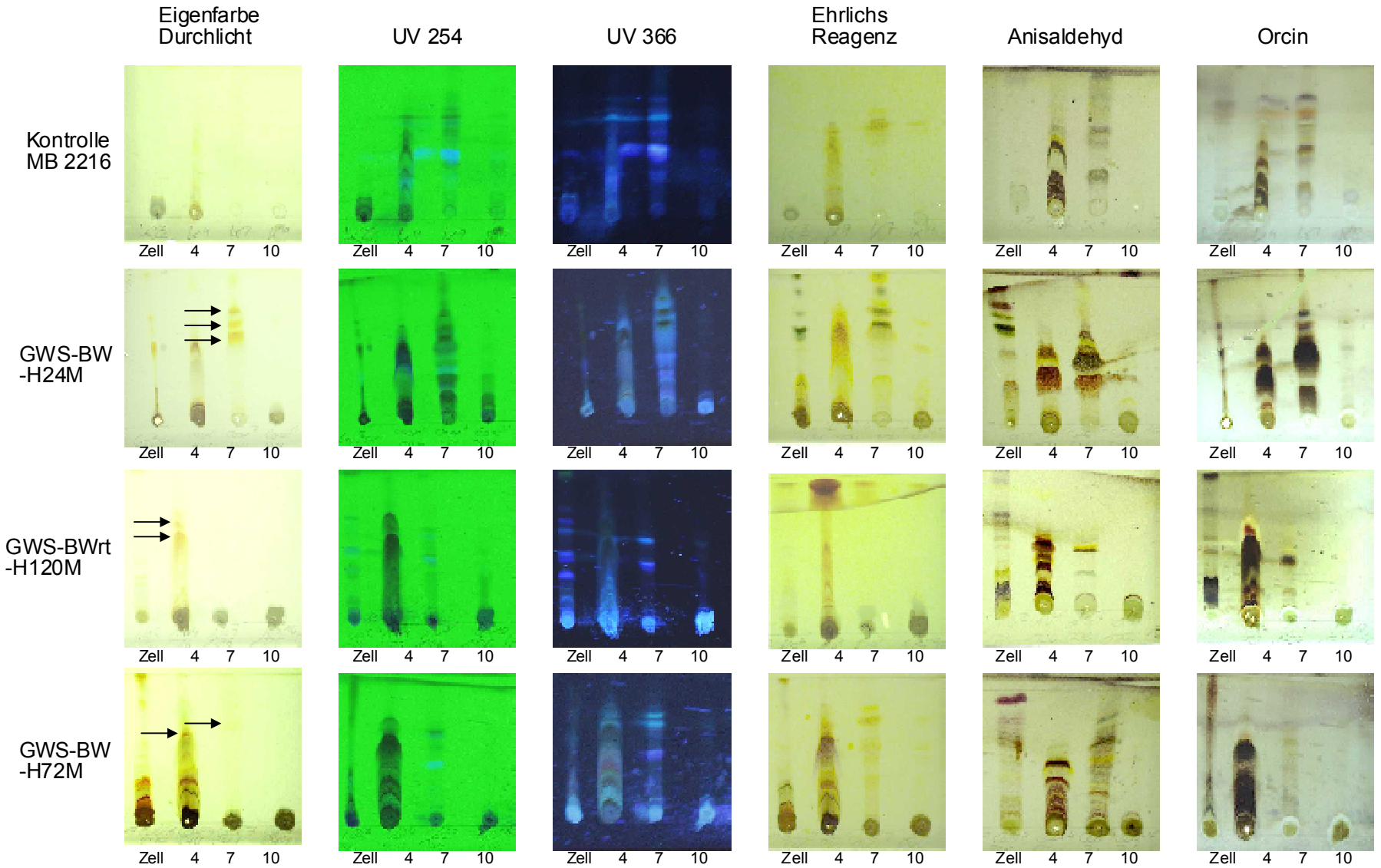


Abb. VI.3. Dünnschichtchromatogramme der Rohextrakte des Mediums MB 2216 und der Stämme GWS-BW-H24M, GWS-BWrt-H120M, GWS-BW-H72M. Auffällige Spots sind mit einem Pfeil Auf der Abbildung mit der Eigenfarbe markiert. Zell = Zellextrakt (Pellet); 4, 7 und 10 = pH- Werte der Extraktion.

Die Zellextrakte von GWS-BW-H24 und GWS-BWrt-H120 zeigten v.a. bei der Detektion bei 220 nm und 254 nm einen bis mehrere Peaks, die nicht im Medium zu finden waren. So hat der Zellextrakt von GWS-BWrt-H120M 4 Peaks mit einer Retentionszeit von 10.8-18.55 min. Der Extrakt des sauren Überstandes hat einen deutlichen Peak bei 10.59 min, der auch (allerdings sehr viel geringer) bei einer Wellenlänge von 254 und 300 nm zu sehen ist. Der bei pH 7 ausgeschüttelte Extrakt zeigt einen Peak mit einer Retentionszeit von 10.64 min (220 nm). Der Zellextrakt von GWS-BW-H24M zeigt einen deutlichen Peak bei 10.31 min in den Wellenlängenbereichen 220, 254, und 300. Der Überstand, extrahiert bei pH 4, zeigt einen Peak mit einer Retentionszeit von 9.51 min
Die Spektren der Extrakte GWS-BW-H72M zeigten im Zellextrakt bei 220 nm (7.43 min) und 300 nm (22.66 min) einen undeutlichen und schwachen Peak und einen bei dem pH 4-Extrakt des Überstandes bei 220 (6.56 min). Der Extrakt bei pH 10 ergab zwar viele hohe Peaks im vorderen und mittleren Bereich (2.77-18 min; bis 2500 mAbs), jedoch ergab schon der Zellextrakt mit einer Produktmenge von 38.2 mg aus einer 5 l-Kultur eine zu geringe Ausbeute, so dass von weiteren Isolierungs- und Aufreinigungsschritten abgesehen wurde (die Ausbeute der Überstandsextrakte war noch geringer, die Daten werden daher nicht gezeigt).
Die Extrakte der anderen Kulturen ergaben kleinere, oft nah zusammenfallende Peaks, die allgemein schlecht zu isolieren sind. Auch hier erfolgte keine weitere Bearbeitung.

Aufbereitung der Stämme GWS-BW-H24M (Zellextrakt) und GWS-BWrt-H120M (Überstandsextrakt, pH4).

Die im DC und in der HPLC entdeckten Substanzen wurden isoliert und aufgereinigt. Mit der präparativen HPLC war eine Auftrennung des gelben Farbstoffes von GWS-BW-H24M nicht möglich. Die Fraktionen konnten jedoch an einer Kieselgelsäule (RP80) und anschließender Sephadex Säule aufgetrennt werden. Im anschließenden Kontroll-DC der erhaltenen Unterfraktionen erschienen 3 gelbe Zonen verschiedener Laufhöhe, deren dazugehörige Fraktionen gepoolt (UF24.1: untere gelbe Zone im DC; UF24.2: mittlere gelbe Zone im DC; UF24.3: obere gelbe Zone im DC) und weiterbearbeitet wurden. UF24.1 und UF24.2 ergaben in einem 1H-NMR kein auswertbares Bild. Von einer weiteren Auftrennung (Sephadex-Säule) von UF24.3 wurden wiederum 2 Unterfraktionen (UF24.3.1, UF24.3.2, je 0.6 mg)

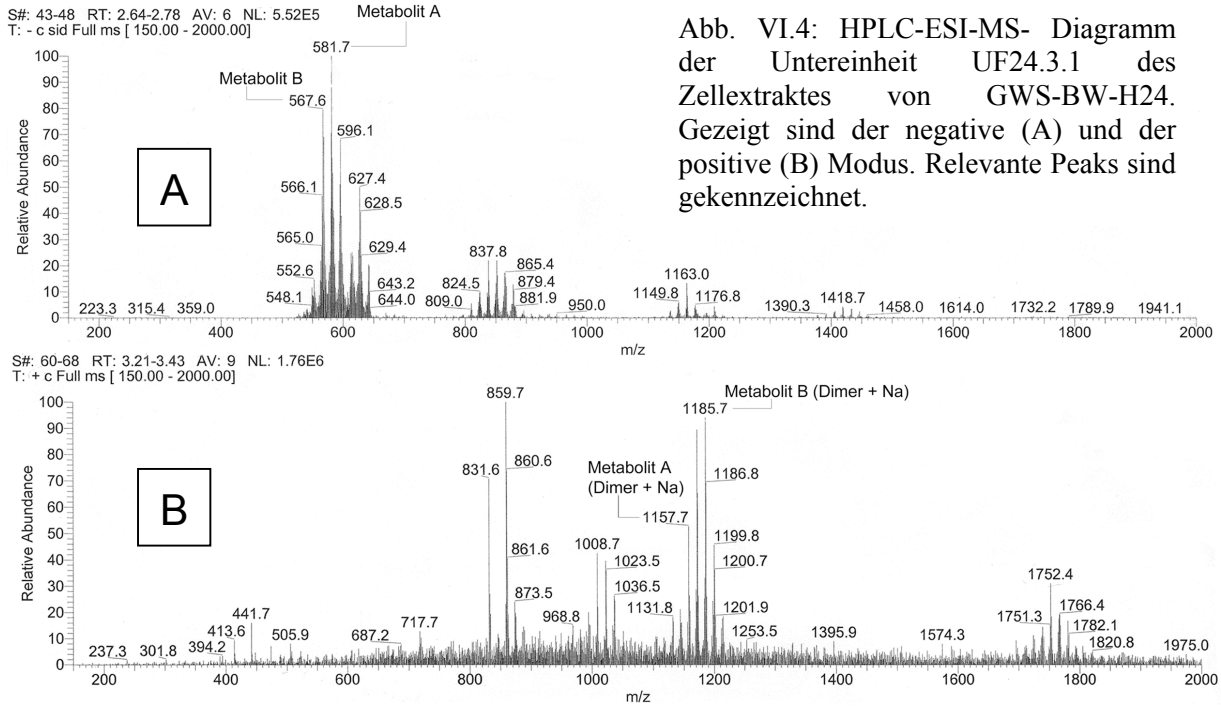


Abb. VI.4: HPLC-ESI-MS- Diagramm der Untereinheit UF24.3.1 des Zellextraktes von GWS-BW-H24. Gezeigt sind der negative (A) und der positive (B) Modus. Relevante Peaks sind gekennzeichnet.

weiterbearbeitet. Die Analysen in der EI-MS und DCI-MS gaben für UF24.3.2 keine auswertbaren Spektren. Die Analyse für UF24.3.1 im ESI –MS zeigte relevante Peaks mit den Massen 1157.7 und 1185.7 im negativen und 581.7 und 567.6 im positiven Modus (s. Abb VI.4).
Der Überstandextrakt pH 7 des Stammes GWS-BWrt-H120M wurde in der HPLC-ESI-MS analysiert. Es zeigten sich folgende Massenpeaks mit ihren Abspaltungen nach ihren Retentionszeiten: Die Masse des Peaks mit der Retentionszeit 4.06 min liegt bei 591.5, die Masse des Peaks bei 7.8 min liegt bei 946.5; Die Masse des Peaks 13.95 min liegt bei 515.5. Diesen Peak kann man auch im positiven wie negativen Modus finden, weswegen er zur Zielsubstanz zu gehören scheint. Für eine weitere Isolierung der Zielsubstanz ist der Ertrag der Mischsubstanz zu gering.
Der in pH 4 erhaltene Extrakt des Stammes GWS-BWrt-H120M konnte in einer Kieselsäule und anschließendem Lauf über eine Sephadex®-Säule aufgetrennt werden. Die Unterfraktion hatte ein Gewicht von 2.2 mg (UF120). Die analytische HPLC zeigte einen Peak bei 10.53 min. Das EI-MS zeigte Peaks mit Molekulargewichtswerten von 42, 55, 69, 83, 93, 112, 126, 137 und 154 und einige sehr kleine Peaks (Abb. VI.5.).

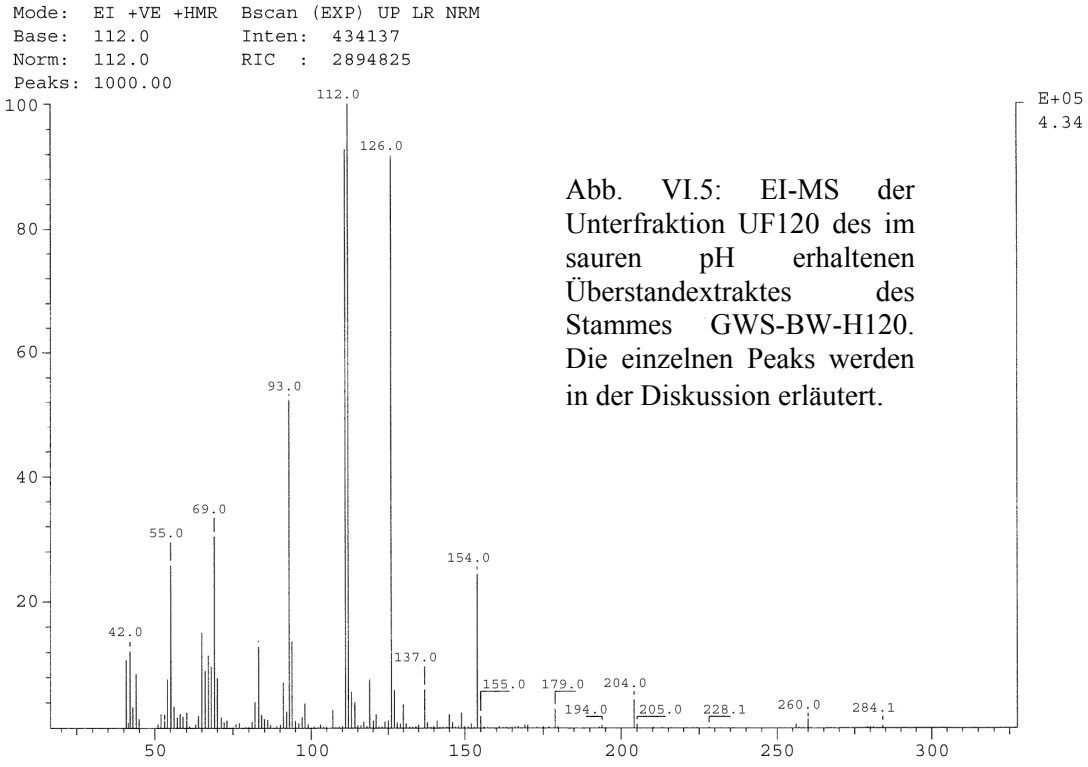


Abb. VI.5: EI-MS der Unterfraktion UF120 des im sauren pH erhaltenen Überstandextraktes des Stammes GWS-BW-H120. Die einzelnen Peaks werden in der Diskussion erläutert.

Die Korrelationspektroskopie (COSY; Correlation SpectroskopY) zeigt zur Substanz gehörende Peaks bei 1.83 ppm[15] bei 5. 61 (d[16]), 6.14 (dd), 6.79 (dd), 6.98 (dd), 7.22 (d) und 7.39 (dd, s. Abb. VI.6 A und B) das sind 9 H-Atome (jeder Peak zeigt ein H-Atom, bei 1.83 liegen jedoch 3). Andere Peaks sind der des Lösungsmittels (bei 3.3

[15] ppm = Einheit der chemischen Verschiebungen δ
[16]Die Signale (Aufspaltungsmuster) von NMR-Spektren sind durch folgende Abkürzungen charakterisiert: s = Singulett, d = Dublett, dd = Dublett von Dublett, t = Triplett, td = Triplett von Dublett, tt = Triplett von Triplett, q = Quartett, qd = Quartett von Dublett, quint = Quintett, m = Multiplett, mc = zentriertes Multiplett. Als „Duplett“ werden direkt benachbarte (über 1C-Atom) H-Atome bezeichnet, „Duplett vom Duplett“ sind 2 direkt benachbarte H-Atome, in deren Nachbarschft sich ein drittes H-Atom befindet.

ppm) und der des Wassers (bei 4.9 ppm). Die Peaks in der Zone 3.4 – 4.0 ppm werden als Verunreinigung der Substanz durch ein zuckerartiges Molekül, das vermutlich aus dem Medium stammt, gewertet und nicht weiter beachtet, da dies ein für Zucker typischer Bereich ist (G. Bach, pers. Mitteilungen). Die Peaks im HMBC zeigen 9 C-Atome der Verbindung, 7 davon werden wie durch Peaks im HSQC gezeigt von einem H-Atom „gesehen“, das heißt, sind an ihm gebunden. 2 C-Atome „sehen“ keine Protonen (HMBC). Tabelle 3 zeigt die H-Atome, die andere H Atome „sehen“ und die dazugehörigen C-Atome.

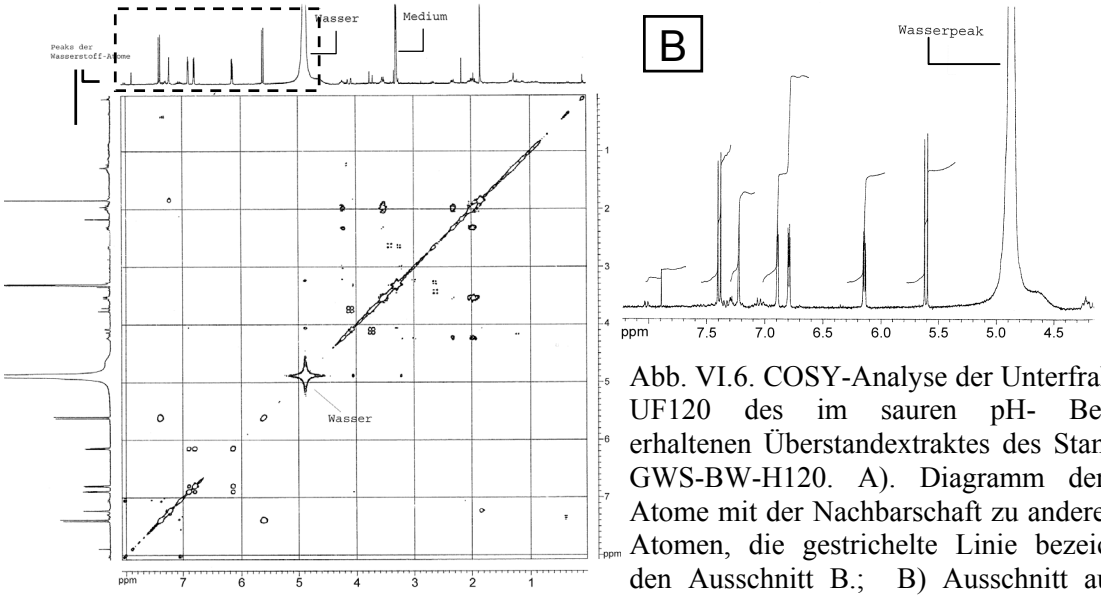


Abb. VI.6. COSY-Analyse der Unterfraktion UF120 des im sauren pH- Bereich erhaltenen Überstandextraktes des Stammes GWS-BW-H120. A). Diagramm der H-Atome mit der Nachbarschaft zu anderen H-Atomen, die gestrichelte Linie bezeichnet den Ausschnitt B.; B) Ausschnitt aus A (Hauptpeaks) zur genaueren Auflösung der Abstände der H-Atome zueinander, dargestellt als Frequenz über den H-Atom-Peaks. Mediums- und Wasser-Peak sind gekennzeichnet.

A

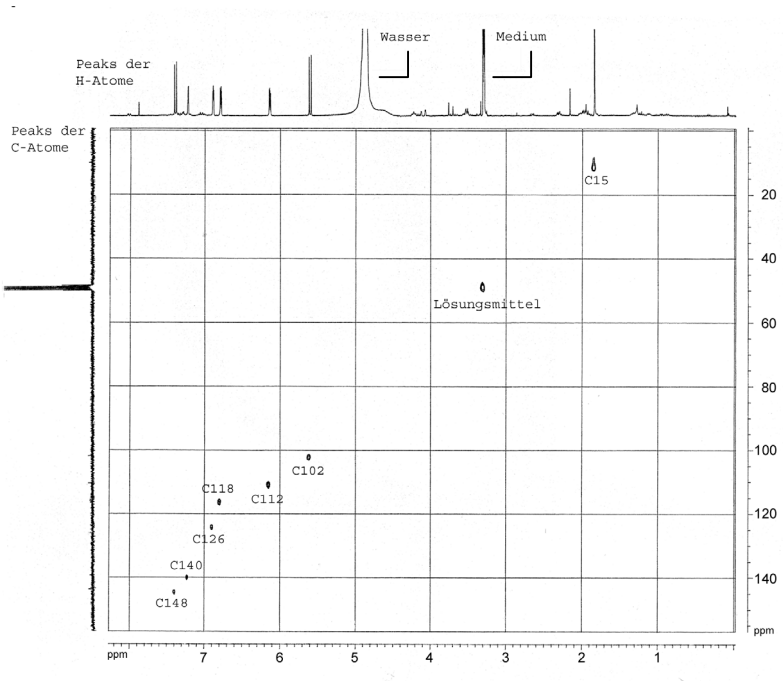


Abb. VI.7. HSQC-Analyse der Unterfraktion UF120 des im sauren pH erhaltenen Überstandextraktes des Stammes GWS-BW-H120. Mediums- und Wasserpeak sowie der Lösungsmittelspot sind gekennzeichnet. Die Spots der Kohlenstoffatome sind mit ihrer Retentionszeit bezeichnet.

VI.4 DISKUSSION

Alle isolierten Substanzen ließen sich nur sehr schwer aufreinigen. Die Unterfraktionen aus den Säulenchromatographien wurden wegen der geringen Ausbeute selbst aus den 5 –l-Kulturen sehr grob geschnitten (d.h. aus vielen Unterfraktionen wurde zu einer Fraktion gepoolt), so dass keine hochreine Substanz erhalten werden konnte. Nur von der Substanz in der Unterfraktion UF120 (Stamm GWS-BWrt-H120M) konnten Strukturvorschläge erarbeitet werden, die im Folgenden diskutiert werden. Die Ergebnisse zu den Substanzen aus der Unterfraktion UF24.3.1 (Stamm GWS-BW-24M) werden im Anschluss daran erläutert.

Unterfraktion UF 120 des Stammes GWS-BWrt-H120M.

Elf C-Atome und 22 H-Atome, ergeben die im EI-MR gesehene Masse von 154, es könnte sich also um ein größeres Molekül handeln als in den NMR-Spektren zu sehen war (die Peaks waren sehr schwach und undeutlich zu erkennen). Bis auf den Peak mit der Masse 93 im EI-MS weist das Spektrum auf ein Alken hin. Peak 42, 55, 69, 122 und 126 könnten dann die C2, C4, C5, C8- bzw. C9 Bruchstücke darstellen. Die Geradzahligkeit der Fragmente 112 und 126 weisen auf eine Doppelbindung hin und kommt vermutlich durch Umlagerung der Protonen zustande. Peak 137 würde das Molekül mit Abspaltung einer C1 (Methylengruppe) bezeichnen.

Tabelle VI.3. Zusammenfassung der Ergebnisse der COSY, HSQC und HMBC – Analysen. Angegeben ist die chemische Verschiebung δ [ppm] der H- und C-Atome, die Größe der Signale ausgedrückt in Hertz [hz], dieden Abstand der H-Atome zum nächsten H-Atom angibt, und die Bezeichnung der Aufspaltungsmustr im NMR-Spektrum.

H-Atom [ppm]	Abstand	Beurteilung	C-Atom [ppm]
1.8	1	d	15
5.6	7.8	dd	102
6.7	3.8 und 15	dd	118
6.9	1.5 und 2.45	dd	126
7.3	7.8	dd	148
7.2	1	d	140
1.8	1	d	15

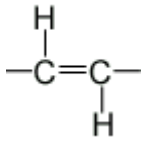
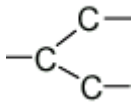
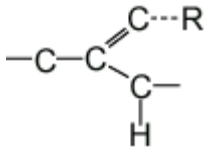
Folgende Schlussfolgerungen lassen sich festhalten:

- Die Substanz ist farblos und polar.
- Sie liegt extrazellulär vor.
- Sie färbt mit Anisaldehyd dunkelbraun.
- Jedes Proton ist an ein Kohlenstoff-Atom gebunden, an dem sich kein zweites Proton befindet, mit Ausnahme einer Methyl- oder Ethylgruppe an 15 ppm-C Atom (es „sieht“ die 3 H-Atome an 1,83 ppm)
- Die Hertz Zahl von 7.8 weist auf trans-Kopplungen in den Doppelbindungen hin (G. Bach, pers. Mitteilungen).

Ungesicherte Eigenschaften sind:

- Das Molekül ist ein verzweigtes Alken.
- Es ist aliphatisch.
- Die Anzahl der Protonen ist mindestens 9.
- Die Massenzahl der Substanz ist 154.
- Die Anzahl der C-Atome ist mindestens 9, vermutet man im EI-MS ein Molekulargewicht von 154, ist die Anzahl der C-Atome 11, die der H-Atome dann 22.
- Das Molekül enthält eine Methylengruppe.

Die Strukturformel lässt sich mit den erhaltenen Hertz-Zahlen nicht festlegen. Folgende Teile sind aber vermutlich enthalten:
A) Es gibt eine trans-Doppelbindung (mit C140 und C102).
B) Es gibt eine Verzweigung.
C) Es gibt 2 C-Atome, die nicht mit einem Proton verknüpft sind.

A)  B)  C) 

Anisaldehyd- Schwefelsäure färbt Zucker, Terpene und Steroide (Merck, 1980). In den NMR-Spektren ist ein Bereich aufgefallen, der Hinweise auf eine Verunreinigung mit einem Zucker gibt. Da mit dem Farbstoff Orcin, Zucker wie Amylosen und Saccharosen auch braun färbt, stammt die Färbung der DCs möglicherweise aus diesem Zucker.

Massenspektrum der Substanz UF24.3.1 des Stammes GWS-BW-24M.

UF24.3.1 ist eine Subtanz mit stark gelber Eigenfarbe, die sowohl intra- als auch extrazellulär vorkommt. Ob die Substanz durch lysieren toter Zellen in den Überstand geraten ist oder tatsächlich aus der Zelle heraustransportiert wurde, kann bisher nicht entschieden werden. Die gelbe Anfärbung mit Ehrlichs Reagenz weist auf ein aromatisches Amin hin (Merck, 1980). Die Massenspektren für diese Substanz waren nicht eindeutig, es handelt sich bei der Substanz wahrscheinlich um ein Homologengemisch, dass sich um eine Methylen (CH_2)-gruppe unterscheidet. Das eine Homologon hat eine Masse von MW = 581,7, das andere eine von MW = 567,6. Die Peaks der Masse 1185,7 und 1157,7 im positiven Modus repräsentieren ihre Dimer mit zusätzlicher Na-Gruppe.

SCHLUSSBETRACHTUNG

In 3 von 6 untersuchten Stämmen konnten eine Reihe interessanter Hinweise auf Produkte verschiedenster Art isoliert werden. Jedoch war keines von ihnen bioaktiv. Im Laufe der Arbeiten stellte sich immer wieder heraus, dass die geringe Ausbeute von Naturstoffen aus marinen Organismen nicht nur auf Makroorganismen beschränkt ist, wo das Ernten des Organismus in ausreichender Menge oft der limitierende Faktor ist (Uemura *et al.*, 1985), sondern sich auch in den Mengen der zu extrahierenden Produkte bei marinen Mikroorganismen zeigt. Zur tatsächlichen Aufklärung einer Struktur ist oft ein Hochskalieren der Kultur auf 50 l nötig, was zeit- und finanzaufwendig ist, so dass der Einsatz von derart großen Fermentern nur bei Verdacht auf eine besondere chemische Struktur gerechtfertigt ist. Aber auch ohne Fermenternutzung und ohne das Problem der geringen Ausbeute ist eine chemische Untersuchung dieser Art nicht nur sehr zeitaufwendig, sondern auch wegen des enormen Materialaufwandes und der Gerätenutzung sehr kostenintensiv.
Die Effizienz der klassischen Methoden kann durch eine Voruntersuchung der Bakterienstämme mit molekularbiologischen Methoden erheblich vergrößert werden. So kann mit spezifischen Primern nach bestimmten für Sekundärsstoffe spezifischen Genen gesucht werden (Metsa-Ketela *et al.*, 1999; Feng *et al.*, 2001; Piel, 2002;2003 Wagner-Döbler *et al*., 2002). Erste Arbeiten auf diesem Feld lieferten Hinweise auf ein weit höheres Potential zur Naturstoffproduktion von Bakterien als bisher mit herkömmlichen Methoden gefunden wurde (Martens *et al.*, 2004). Dies steht in Einklang mit Beobachtungen von Burgess *et al*., 1999, die ein Ein- und Ausschalten der Naturstoffproduktion unter verschiedenen Bedingungen beobachten konnten.
Im Gesamtprojekt Biotechnologie konnten aus dieser Arbeit 250 Reinkulturen aus dem deutschen Wattenmeer zur Verfügung gestellt werden (s. aus dieser Arbeit resultierende Studien auf Seite iv). Einundvierzig dieser Isolate wurden biologisch-chemisch auf Naturstoffproduktion untersucht; die Struktur verschiedener Naturstoffe konnte von 4 Stämmen beschrieben werden (s. Tabelle VI.4), die zu den α- und γ-*Proteobacteria* gehören. Von 2 weiteren Stämmen aus dem Wattenmeer (HP1, AY241547 und T5, AY177712) konnten Produkte isoliert werden (Liang *et al*., 2004). Auch diese Stämme fallen in die α- und γ-*Proteobacteria*.

Obwohl es nach diesen Ergebnissen so aussieht, als würde die mikrobielle Naturstoffproduktion im Wattenmeer vorwiegend in diesen phylogenetischen Gruppen auftreten, könnte sich bei der Untersuchung weiterer Isolate das phylogenetische Spektrum produzierender Bakterien noch erheblich verschieben, da die Anzahl der untersuchten Stämme statistisch gesehen gering ist. Daher kann es sein, dass auch im Wattenmeer Stämme anderer phylogenetischer Gruppen Naturstoffe produzieren, die gewählten Wachstumsbedingungen jedoch v.a. für α- und γ-Proteobakterien zur Naturstoffproduktion ideal waren. Durch Hinweise aus dieser und anderen Arbeiten (Brinkhoff *et al*., 2004; Grossart *et al*., 2004) ist zu vermuten, dass das Potential von Mikroorganismen aus dem Wattenmeer zur Naturstoffproduktion noch lange nicht vollständig erfasst wurde. Wie in der anschließenden Tabelle gezeigt und schon vorher beobachtet wurde (Bode *et al.*, 2002), haben Bakterien, die Sekundärmetabolite produzieren, häufig das Potential, mehr als eine Verbindung zu erzeugen. Dementsprechend könnte zusätzlich zur Erschließung neuer Habitate ein Ausschöpfen der schon vorhandenen Ressourcen, sowohl das Habitat betreffend (wie in Kapitel V gezeigt, sind noch nicht alle abundanten Bakterien isoliert) als auch bezüglich schon vorhandener Bakterienstämme (z.B. Variationen des Produktspektrums bei Medienveränderung), sich als in industrieller wie wissenschaftlicher Hinsicht lohnenswert herausstellen. Eine aussichtsreiche Strategie der Erschließung neuer Naturstoffe ist die Suche nach neuen taxonomischen Gruppen, in denen Naturstoffproduktion gehäuft auftritt mit anschließender gezielter Isolierung der Bakterien dieser Gruppe.

Tabelle VI.4. Struktur und Beschreibung der Produkte der in dieser Arbeit isolierten und untersuchten Stämmen. Gegeben sind die Isolatbezeichnung (ID), Accession-Nummer der Isolate, phylogenetische Einordnung (nach ARB; Ludwig *et al.*, 2002), der Name des isolierten Naturstoffes (falls gegeben), sowie seine kurze Beschreibung.

Isolat-ID (Acc.-No.) phylogen. Einordnung	Naturstoff	Beschreibung des Naturstoffes
GWS-BW-H6M /RK366 (AY515405) α-*Proteobacteria*	1,3-Dihydroxybenzol (Resorcin)	Aromat, farblos, kristallin, süß schmeckend. Rosa im Licht, an der Luft und in Gegenwart von Eisen. Leicht löslich in Wasser, Alkohol, Ether und Glycerin, bakteriozid; Schmelzpunkt: 109-111 °C; Siedepunkt: 280 °C.
GWS-BW-H8hM /RK377 (AY515435) γ-*Proteobacteria*	2,3-Indoldion (Isatin)	gelbrot, kristallin, Schmelzpunkt: 193° C, wenig löslich in Wasser. Ausgangsverb. von Indigo und einigen Pharmaka. Auch aus *Alteromonas* sp., Shrimp-symbionten, *Schizophyllum commun* (Basidomycet).
	2 Hydroxysäuren	farblos, amorpher Feststoff; **neue chemische Struktur.**
	7-Hydroxy-2H-benzo [1,4] thiazin-3-on	farblos, amorpher Feststoff, löslich in Aceton, Methanol und Chloroform, **neu als Naturstoff.**
	Indol-3-Essigsäure	farblos, Feststoff, löslich in Aceton, Methanol und Chloroform, in höheren Pflanzen weit verbreitet, auch isoliert aus marinen Algen, und deren symbiotischen Bakterien. Wirkt in der Wachstumsregulation bei Pflanzen, schon isoliert aus *Rhizobium* sp.
	3-Hydroxy-acetylindol	farblos, Feststoff, schon isoliert von Rotalgen, dem Schwamm *Tedania ignis*, beziehungsweise aus *Micrococcus* sp., der auf *T. ignis* wächst. Als Tryptophan-Metabolit in Myxobakterien bekannt.
	Indol-3-Carboxyl-Indol-3-Carboxy-säure	Auch isoliert von marinen Algen und Pflanzen. Auch isoliert von Rotalgen und Pflanzen.
	Phenylessigsäure	Hauptprodukt des Stammes, kommt in Pilzen als Phytotoxin vor, schon isoliert von marinem Streptomyceten.
	Glusun I + II	Gelber (I) bzw, orangefarbener (II) Feststoff, löslich in Methanol, wenig in unpolaren Lösungsmitteln.
GWS-BW-H52M /RK2207 (AY515416) α-*Proteobacteria*	Bakterio-phäophytin	Dunkelgrüner Feststoff, primärer Elektronen-akzeptor in der Bakterienphotosynthese.
	Spheroidenone	Rot, amorpher Feststoff, Bakteriencarotenoid aus Proteobakterien.
GWS-BW-H260 (AY332122), α-*Proteobacteria*	Bioaktive Compound	Polar, schwach violett, löslich in DMSO, Methanol, gering in unpolaren Lösungsmitteln.

VI.5 LITERATUR

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VII

GESAMTBETRACHTUNG UND AUSBLICK

Im Folgenden werden die Ergebnisse der einzelnen Manuskripte im Zusammenhang diskutiert. Auf eine Wiederholung der in den einzelnen Manuskripten detailliert diskutierten Punke wird hier verzichtet, näher erläutert werden lediglich Aspekte, die in den vorangegangenen Kapiteln nicht oder nur kurz dargestellt werden.

VII.1 EINORDNUNG IN DEN GESAMTZUSAMMENHANG MIT ANDEREN STUDIEN

Ohne eine Charakterisierung der Bakteriengemeinschaft ist keine vollständige ökologische Beschreibung eines Habitates möglich, da Bakterien einen großen Anteil, oft sogar den größten, am Stoffumsatz haben (Löffler, 2001). In der hier vorliegenden Studie wird zum ersten Mal das aerob wachsende heterotrophe Bakterioplankton eines Wattenmeeres mikrobiologisch und phylogenetisch dezidiert in den verschiedenen Kompartimenten Wassersäule, Aggregate und Sedimentoberfläche untersucht. Andere im gleichen Zeitraum erschienene Arbeiten über vorwiegend anaerob wachsende Bakteriengemeinschaft des gleichen Habitats (Llobet-Brossa *et al.*, 1998, 2000; Böttcher *et al.*, 2000; Rütters *et al.*, 2002) und Untersuchungen an Bakterien aus dem marinen Teil des Weserästuars (Selje und Simon, 2003, 2004), ergänzen diese Studie. Andere natürliche Wattenmeere oder tidal beeinflusste Flächen wurden hinsichtlich der Bakteriengemeinschaft nur sehr exemplarisch im Hinblick auf bestimmte physiologische (USA: Steppe und Paerl, 2002, Japan: Fukui *et al.*, 1999) und phylogenetische Gruppen (Südkorea: Hiraishi und Ueda, 1995) untersucht oder dienten als Habitat zur Isolierung neuer Bakterien zur Stammbeschreibung (Südkorea: Yi *et al.*, 2000; Yoon *et al.*, 2003 a und b; Engelhardt, 2001). Eine Publikation diesen Jahres enthält eine weitreichende phylogenetische Studie über Sequenzen einer Mikrobenmatte aus einem Wattenmeer (Harris *et al.*, 2004)

Die hier vorliegende Arbeit grenzt sich von den anderen durch die Vielfalt er angewandten Methoden sowie durch die Untersuchung vornehmlich oxischer Habitate ab.

VII.2 JAHRESZEITLICHE VARIABILITÄT DER BAKTERIENPOPULATION

Die vorliegende Studie zeigt, dass die jahreszeitliche Variabilität der Bakteriengemeinschaften, auch der planktischen, trotz der starken Hydrodynamik relativ konstant ist. Obschon es kleinere saisonale Variationen während der kälteren Jahreszeit gibt, bleiben die Hauptvertreter in allen 3 Kompartimenten über das Jahr detektierbar (und oft auch dominant). Eine Veränderung der Bakteriengemeinschaft ließ sich auch während einer Planktonblüte nicht mit einer DGGE-Analyse detektieren. Andere Arbeiten zeigen ebenfalls eine im Jahresverlauf konstante Bakterienpopulation, wie z.B. im Skagerrak und im Kattegatt anhand von DGGE und PFGE (pulsed field gel electrophoresis) Analysen bei Riemann und Middelboe 2002 (Riemann and Middelboe, 2002) nachgewiesen. Riemann und Middelboe fanden allerdings bei der Untersuchung einer Zone, wo die Wassermassen unterschiedlicher physikalisch-chemischer Parameter (von Skagerrak und Kattegatt) aufeinandertreffen, deutliche Variationen in der Bakterien- und Virenpopulation.

Auch wir konnten im Wattenmeer 2 Wassermassen (eine im Sommer und eine im Winter) bezüglich ihrer Temperatur und ihres Salzgehaltes voneinander unterscheiden (s. Abb. VII.1). Aber auch in den Grenzbereichen der Wasserkörper, d.h. in den Zeiten, wo sie ineinander übergehen, nämlich im Herbst und im Frühjahr, wurden weder mit der DGGE Analyse noch mit der Clusteranalyse Veränderungen detektiert. Allerdings könnten die Übergange in einem so zeitlich begrenztem Rahmen stattfinden, dass unser Probenahmeraster (monatlich) zu grob war, um die jeweilige Veränderung zu erfassen. Zudem scheint in einer Zone, wo verschiedene Wassermassen über einen längeren Zeitraum aufeinandertreffen (wie zwischen Skagerrak und Kattegatt bei Riemann und Middelboe, 2002, das Herausbilden einer spezifischen Bakteriengemeinschaft eher ein räumliches denn ein temporäres Ereignis zu sein.

Man muß jedoch bedenken, dass in der hier vorliegenden Arbeit vorwiegend die Artenvielfalt (*richness*) der Bakteriengemeinschaften anhand von DGGE Profilen ermittelt wurde, die Artenabundanz (*evenness*) jedoch nur eingeschränkt über die MPN-Reihen. Mit FISH ließe sich die *evenness* genauer bestimmen.

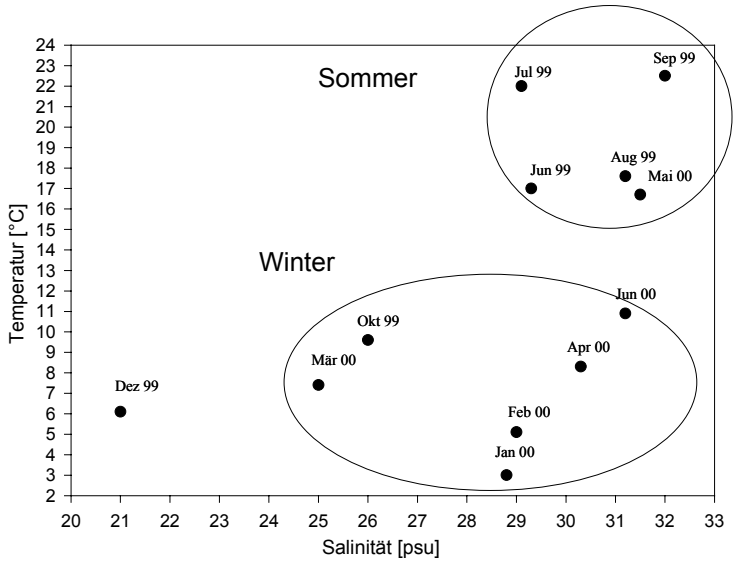

Abb. VII.1. Unterscheidung des Wasserkörpers des Nordseewattenmeeres in einen „Sommer“ und einen „Winter-Wasserkörper“ anhand der Temperatur und des Salzgehaltes im Jahresverlauf von Juni 1999 bis Juni 2000. Die geringe Salinität im Dezember 1999 ist durch Verdünnung des Wasserkörpers durch überdurchschnittlich hohe Niederschläge bedingt (Niederschlagsmessung: Deutscher Wetterdienst, Hamburg).

Der Großteil der abundanten Spezies der Bakterienpopulation des Wattenmeeres scheint also gegenüber weiten Schwankungsbereichen von Temperatur, Wasserverfügbarkeit, Licht und Salzgehalt mehr oder weniger unempfindlich zu sein und viele verschiedene Ressourcen nutzen zu können, sie sind also „Generalisten“. Tatsächlich wurden in dieser Studie auch Generalisten bezüglich der Substratverwertung und der Anpassung an den Lebensraum gefunden: es wurden z.B. Spezies des gleichen Sequenztyps aus allen 3 Kompartimenten und mit diversen Substraten isoliert (Kapitel III), trotzdem sich die frei in der Wassersäule lebende Gemeinschaft gegenüber der der Sedimentoberfläche als signifikant unterschiedlich herausstellte und sich die Gemeinschaften der drei Fraktionen (mit Überschneidungen mit der Aggregatfraktion) deutlich voneinander unterschieden.
Nun konnten jedoch auch in Habitaten, die eine ausgeprägte saisonale Schwankung in der Bakterienpopulation zeigen, Generalisten detektiert werden. So konnten Dominik und Höfle Sequenzen von *Comamonas acidivorans* zu jeder Jahreszeit im Epilimnion eines eutrophen Sees nachweisen (Dominik und Höfle, 2002)

Zudem muss man sich bewusst sein, dass die Detektion saisonaler Gleichförmigkeit der Bakteriengemeinschaft auch methodisch bedingt sein kann, z.B. durch die Wahl der Primer in der DGGE. Auch sind die meisten Isolate gleichen Sequenztyps aus dem Wattenmeer ausschließlich von einem Kompartiment isoliert worden.

VII.3 DIE BAKTERIENPOPULATION IM WATTENMEER

Wie DGGE Analysen zeigten, sind die dominanten Bakteriengruppen in den Kompartimenten unterschiedliche: frei lebend dominieren α- *Proteobakterien* und auf der Sedimentoberfläche assoziiert die γ-*Proteobakterien.* Aggregate werden vorwiegend von Bakterien beider phylogenetischer Gruppen besiedelt, was den vermittelnden Charakter dieses Kompartimentes zwischen den anderen beiden aufzeigt.
Diese Ergebnisse spiegeln sich in denen der Kultivierung wieder. Auch hier lassen sich Bakteriengruppen des Freiwasserkompartimentes und des Sedimentes in der Aggregatfraktion wiederfinden, sieht man von der allgemeinen Dominanz der Actinobakterien im gesamten Kultivierungsansatz ab.
Die Dominanz der Actinobakterien im Kultivierungsansatz (25 %) war ein überraschendes Ergebnis. Nicht nur dass mit kultivierungsunabhängigen Methoden keine so große Abundanz zuvor in marinen Habitaten gefunden wurde, was eine einseitige Ausrichtung der Kultivierungsmethode darstellen kann, auch die hohe Diversität innerhalb dieser Gruppe konnte vormals in marinen Habitaten nicht gezeigt werden (Fuhrman *et al.*, 1993; Jensen und Fenical, 1995; Gray und Herwig, 1996; Suzuki *et al.*, 1997; Rappé *et al.*, 1999; Urakawa *et al.*, 1999; Mincer *et al.*, 2002). Die hohe Diversität scheint also für das Wattenmeer als marines Habitat einzigartig zu sein. Auch in Isolierungsstudien aus Nachbarhabitaten konnten tatsächlich nur wenige Gram-positive Spezies und dementsprechend keine hohe Diversität dieser Gruppe ausgemacht werden (Eilers *et al.*, 2000).
Bezüglich der Abundanz ergab ein Vergleich mit den Verdünnungsstufen, aus denen einige der Actinobakterien isoliert werden konnten, mit der gefundenen Abundanz dieser Gruppe in kultivierungsunabhängigen Ansätzen in einer anderen Studien übereinstimmende Werte von 5-10 % (Llobet-Brossa *et al.*, 1998), was mit Ergebnissen aus einer Klonbibliothek bestätigt werden konnte. Die Diskussion, ob die erhaltenen Isolate tatsächlich inhärent marin sind, wird in Kapitel IV ausführlich behandelt. Es ist jedoch Fakt, dass die Isolate auf „echtem“ marinem Medium gut gediehen und mehrfach übertragbar waren.
Eine weitere Besonderheit ist die geringe Detektion der Klassen *Flavobacteria /Sphingobacteria* in dieser Studie. *Flavobacteria / Sphingobacteria* können in fast jedem Habitat gefunden werden (Kirchman, 2002), in marinen Habitaten werden sie oft als dominierende Bakteriengruppe detektiert, z.B. mit FISH (Simon *et al.*, 1999), anhand der Anfertigung einer Klonbibliothek (Cottrell und Kirchman, 2000b), durch Isolierung aus einem angrenzenden Habitat, der Nordsee (Eilers *et al.*, 2000) oder mit FISH in Wattenmeersedimenten (Llobet-Brossa *et al.*, 1998; Llobet-Brossa *et al.*, 2002).
Es ist bekannt, dass molekulare Methoden durch die Primer im PCR-Schritt gegenüber der Klasse *Flavobacteria* diskriminieren (Eilers *et al.*, 2000; Kirchman, 2002).
Aber auch der kultivierungsabhängige Ansatz in dieser und anderen Studien aus diesem Habitat (Brinkhoff *et al.*, 2004, Grossart *et al*., 2004; Selje und Simon, 2004)

zeigten keine hohe Abundanz der *Flavobacteria / Sphingobacteria*, obwohl in dieser Studie vorwiegend mit Biopolymeren isoliert wurde (Ausnahme MB, das als Vollmedium Biopolymere enthält, aber keines darstellt), für deren Verwertung diese phylogenetische Gruppe bekannt ist (Reichenbach, 1992). Die hier vorliegende Studie hingegen zeigt deutlich, dass die Fähigkeit zur Verwertung von Biopolymeren viele phylogenetische Gruppen (es wurden Spezies aus 7 verschiedenen phylogenetischen Klassen isoliert) umfasst. Besonders die am Probenahmeort in großer Menge vorkommende Braunalge *Fucus vesiculosus* zeigte sich für Anreicherungskulturen gut geeignet. Dies mag daran liegen, dass *F. vesiculosus* gemörsert und mit dem Medium autoklaviert wurde, was nachweislich zur Freisetzung einfach zu verwertender labiler Substrate führt (Rink, 2001).

Das Zusammentragen und die phylogenetische Analyse aller Sequenzen aus dem Habitat zeigt einige neue, vermutlich für das Wattenmeer relevante Cluster, wovon 2 (das Wadden Sea cluster I und II, in die γ-*Proteobacteria* fallen) in eine Gruppe mit Sequenzen ausschließlich von marinen, unkultivierten, oberflächenassoziierten Bakterien (SAMMIC-Group; Surface-Associated Marine MICroorganisms) fallen. Das zeigt, dass besonders γ-*Proteobacteria* die Gemeinschaften auf Oberflächen dominieren.

VII.4 ISOLIERUNG „NEUER BAKTERIEN“

Es konnte eine Vielzahl phylogenetisch sehr diverser Bakterien aus dem Habitat isoliert werden. Sie fallen in 7 verschiedene phylogenetische Ordnungen , sogar ein Stamm der selten isolierten *Planctomycetes* konnte kultiviert werden. Viele dieser Isolate stellen nach Vergleich ihrer 16S rRNA Sequenzen mit den Sequenzen der Datenbanken des NCBI Servers neue Arten (mit einer Sequenzähnlichkeit von ≤96 %) und sogar vermutlich neue Gattungen dar (Sequenzähnlichkeit ≤89 %), von denen einige, gemessen an der Verdünnungsstufe aus der isoliert wurde, abundant im Habitat vorzukommen scheinen. Natürlich lässt eine einmalige Isolierung aus einer hohen Verdünnungsstufe keine gesicherten Rückschlüsse auf die Abundanz zu, bei Bruns et al. konnten in MPN Reihen auch in hohen Verdünnungsstufen keine abundanten Spezies gefunden werden (Bruns *et al.*, 2002), aber in dieser Studie wurden Spezies gleichen Sequenztyps aus unterschiedlichen Kompartimenten aus hohen Verdünnungsstufen isoliert, was den Rückschluss auf eine wahrscheinlich hohe Abundanz erhärtet. Bei Selje et al. konnten so isolierte Spezies mit kultivierungsunabhängigen Methoden als dominant identifiziert werden (Selje und Simon, 2004).

Eine Isolierung von Mikroorganismen ist essentiell für die Gewinnung neuer Naturstoffe in der Biotechnologie und der Pharmaindustrie. Die Isolierung vieler vermutlich neuer Arten gibt außerdem die Möglichkeit, neue wissenschaftliche Erkenntnisse über evtl. neue Stoffwechselwege und damit neue ökologische Zusammenhänge in einem Habitat zu erlangen. Obschon es bereits molekularbiologische Methoden gibt, Stoffwechselaktivitäten einzelner Bakteriengruppe *in situ* zu untersuchen (*STARFISH*, Ouverney und Fuhrman 1999, oder *MIKRO-FISH*, Cottrell und Kirchman, 2000), ist für eine dezidierte physiologische Untersuchung immer noch die Notwendigkeit zur Isolierung und Kultivierung von Bakterien gegeben.

Zudem wird die Aussagekraft der 16S rRNA Gene und ribosomale Proteine für ökologische Fragestellungen bezweifelt: Palys *et al*. führen aus, daß die 16S rRNA zu konserviert sei, da sie meist sog. „Haushaltsgene" trägt, die sich nur gering evolutiv verändern (Palys *et al.*, 1997). Auch würden die meisten Substitutionen neutral sein, d.h., sich nicht auf die Fitness auswirken. Merkmale die die Fitness beeinflussen, lägen zumeist auf Plasmiden kodiert vor und beeinflussen somit die DNA und RNA Sequenzen nicht.

VII.5 RÜCKSCHLÜSSE AUF DAS HABITAT: SCHLUSSFOLGERUNGEN UND HYPOTHESEN

Wie die vorliegende Studie zeigt, ist die Bakteriengemeinschaft des Wattenmeeres nicht erst, wie das Watt als Ganzes (Grimm *et al.*, 1999), bei der Betrachtung mehrerer Jahrzehnte als konstant zu beschreiben, sondern schon bei der Betrachtung eines Jahres.
Trotz des Vorkommens von Störungen, wie den Sturm im Februar 2000, bleibt das Ökosystem bezüglich der in dieser Studie gemessenen Parametern konsistent. Starke Regenfälle im Dezember 1999 sind über den Salzgehalt messbar, und die ungewöhnliche Windrichtung im November 1999 konnte über die Erhöhung der Bakterienzahl als Resuspensionseffekt detektiert werden; die Bakteriengemeinschaft ist aber während all dieser Störungen konstant verblieben. Auch in Mesokosmosexperimenten mit Wasser verschiedener Küsten konnte eine hohe Konsistenz der Bakteriengemeinschaft nach Störung durch künstlich hervorgerufene Resuspension nachgewiesen werden. Nach max. einer Woche waren alle gemessenen Parameter auf dem Status wie vor der Störung (Sloth *et al.*, 1996). Die Bakteriengemeinschaft scheint also ein Konstanzfaktor in einem dynamischen System zu sein und evtl. als stabilisierender Faktor die hohe Elastizität des Systems mit zu bedingen. Auch bei Grimm *et al.*, 1999 wird die Bakteriengemeinschaft des Nordseewattenmeeres als funktionelle Einheit (es wurden v.a. die Stoffumsetzungen und Remineralisierungsprozesse untersucht) als extrem persistent, also fortdauernd und beständig beschrieben. Dadurch, dass Bakterien im Wattenmeer durch Remineralisierung die Hauptnährstoffquelle bilden (bottom-up supply) wirkt sich durch die hohe Hydrodynamik des Systems (die Kompartimente werden ständig vermischt) diese Persistenz auf das gesamte Habitat aus (Grimm *et al.*, 1999). Allerdings gibt es auch Systeme, in denen eine dynamische Bakterienpopulation dem System Stabilität verleiht, wie Reaktorversuche von Fernández *et al.*, 1999 zeigen.
Es wäre interessant, zu klären, ob sich einzelne Bakterienspezies an die unterschiedlichen kurzzeitigen Veränderungen des Habitates anpassen. Mögliche Anpassungsmechanismen Gram-negativer Bakterien werden bei Ramos et al 2001 erläutert (Ramos *et al.*, 2001).
Die Studien über die Abundanz verschiedener phylogenetischer Gruppen zeigten das Wattenmeer als ein typisch marines Küstenhabitat (z.B. Fuhrman *et al.*, 1980; Gonzalez and Moran, 1997; Murray *et al.*, 1998; Crump *et al.*, 1999; Rappé *et al.*, 2000) trotz des recht hohen terrestrischen Eintrags durch Flüsse und dem sog. *runoff* (Abfluss durch über- und unterirdisches Niederschlagswasser). Allein die Abundanz und Diversität von Bakterien der Gruppe *Actinobacteria* gibt Hinweise darauf, dass das Habitat als ein Schmelztiegel aus marinen und terrestrischen Einflüssen bezüglich der Bakteriengemeinschaft gesehen werden kann. Andere Parameter zeigten diesen Einfluss nur bedingt (wie die geringe Salinität im Dezember 1999),

allerdings könnte die große Vielfalt der isolierten phylogenetischen Gruppe ein weiterer Hinweis für die Kennzeichnung des Habitates als terrestrisch-mariner Schmelztiegel sein. Die große Anzahl von isolierten „neuen“ Bakterien und das Auffinden neuer phylogenetischer Cluster aus dem Habitat zeigen, dass das Wattenmeer bezüglich seines Potential der Gewinnung neuer Bakterien noch nicht ausgeschöpft ist. Daher kann es als Reservoir sowohl neuer wissenschaftlicher Erkenntnisse über Bakterien und deren Mitwirkung an den globalen Stoffkreisläufen als auch neuer biotechnologischer Anwendungen angesehen werden.

VII.6 AUSBLICK

Bezüglich der jahreszeitlichen Schwankungen könnte durch die Wahl spezifischer Primer in der DGGE und die Anwendung weiterer Methoden wie z.B. die *in-situ* Detektion verschiedener Bakteriengruppen mit gruppenspezifischen fluoreszierenden Sonden (FISH, Card-FISH; Pernthaler *et al*. 2002) die Auflösung der Detektion erhöht werden, so dass sich eventuell ergebende jahreszeitliche Veränderungen nachweisen ließen.
Auch ließen sich durch die Anwendung weiterer molekularer Methoden (FISH, *real-time* PCR) Abundanzen und auch die Diversität von Actinobakterien im Wattenmeer direkt nachweisen und weiter Erkenntnisse über ihren Ursprung (terrestrisch oder marin) erlangen.
Weiterhin gilt es, die Abundanz und die globale Verbreitung des SAMMIC Gruppe durch das Design spezifischer Nachweissysteme zu untersuchen.
In dieser Studie wurde eine weitreichende Stammsammlung erstellt, die eine wertvolle Grundlage für viele weitere Arbeiten darstellt und noch darstellen wird. Neue Isolate, v.a. Spezies von vermutlich neuen Gattungen, gilt es zu charakterisieren, d.h. die gesamte Länge der 16S rRNA zu entschlüsseln und physiologische Eigenschaften dezidiert zu untersuchen.
Im biotechnologischen Ansatz sind durch die Untersuchung der Isolate dieser Studie bereits Erkenntnisse über die Phylogenie biotechologisch relevanter Bakteriengruppen erlangt worden.
Weiterhin werden Nachweissysteme auf molekularer Ebene entwickelt, die die Ausbeute des klassischen Auswahlsystems erhöhen werden.

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Erklärung

Hiermit bestätige ich, dass ich die vorliegende Dissertation selbstständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

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