Bakterieller Abbau von refraktärem organischem Material im Weserästuar und in der Nordsee

Bacterial decomposition of recalcitrant organic matter in the Weser estuary and in the North Sea

Dissertation

Von der Fakultät für Mathematik und Naturwissenschaften der

der Carl von Ossietzky Universität Oldenburg

zur Erlangung des Grades und Titels einer

Doktorin der Naturwissenschaften

-Dr. rer. nat.-

angenommene Dissertation

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Die vorliegende Doktorarbeit wurde am Institut für Chemie und Biologie des Meeres (ICBM) an der Carl von Ossietzky Universität Oldenburg in der Arbeitsgruppe Biologie geologischer Prozesse angefertigt.

Diese Arbeit wurde von der Deutschen Forschungsgemeinschaft (DFG) im Rahmen des Forschungsprojektes (Si 360/22-1) „*Bacterial decomposition of recalcitrant dissolved organic matter in estuarine systems*“ und im TRR51 gefördert.

Erster Gutachter: Prof. Dr. Meinhard Simon

Zweiter Gutachter: Prof. Dr. Jürgen Rullkötter

Tag der Disputation: 13.04.2011

Für meine Eltern

und

für Michael

Es gibt zwei Arten, sein Leben zu leben:
entweder so, als wäre nichts ein Wunder,
oder so, als wäre alles eines.
Ich glaube an Letzteres...

Albert Einstein

Zusammenfassung

In dieser Arbeit wurde erstmalig der bakterielle Abbau von Huminsäuren im Bereich des ostfriesischen Wattenmeeres und des Weserästuars untersucht. Ein zentraler Aspekt hierbei war die Abhängigkeit des Abbaus von der Salinität, da der Salzgehalt beim Transport des DOM (dissolved organic matter) von der Weser ins Wattenmeer permanent ansteigt und zusätzlich im Ästuar tidenabhängige Schwankungen auftreten. Es wurden Inkubationsversuche in Durchflusskulturen mit Inokulaten aus dem Wattenmeer und dem Weserästuar über einen Zeitraum von 71 bis 74 Tagen durchgeführt. Als einzige Kohlenstoffquelle in den Experimenten standen beiden Bakteriengemeinschaften Huminsäureisolate zur Verfügung. Um einen Einfluss der Salinität auf den Huminsäureabbau zu überprüfen, wurden die jeweiligen Bakteriengemeinschaften nicht nur ihrer gewohnten Salinität, sondern auch einer veränderten Salinität ausgesetzt (marines Inokulum aus 28‰ mit ästuarinem Medium von 14‰ bzw. umgekehrt).

Unter beiden Bedingungen wurden die Huminsäuren durch die jeweiligen Bakteriengemeinschaften abgebaut. Es zeigte sich, dass die Bakteriengemeinschaften auf eine veränderte Salinität jeweils mit einem verstärkten Huminsäureabbau reagierten. Der größte Abbau fand innerhalb der ersten 21-28 Tage des Experiments statt. Anschließend ist nur noch frisch zugeführtes Substrat abgebaut worden. Das bereits zum Teil abgebaute Substrat im Kulturgefäß wurde nicht weiter bakteriell abgebaut. In der ersten Phase des Abbaus wurde eine hohe Dynamik der Bakterienzellzahlen und der Proteinproduktion beobachtet. Die Analysen der DGGE-Profile (denaturierende Gradienten Gelelektrophorese) zeigten ebenfalls große Variationen in der Zusammensetzung der Bakteriengemeinschaften während der ersten drei bis vier Wochen.

Zur phylogenetischen Einordnung der huminstoffabbauenden Bakterien ist einerseits eine Vielzahl von Bakterien aus den verschiedenen Ansätzen isoliert und über ihre 16S rRNA Gene klassifiziert worden. Außerdem wurden am letzten Inkubationstag in den Ansätzen vorhandene Bakterien über die Sequenzierung von DGGE-Banden identifiziert. Die Isolierung von huminstoffabbauenden Bakterien aus dem ostfriesischen Wattenmeer sowie die Analyse der DGGE-Banden des letzten Inkubationstages ergab überwiegend Stämme, die den *Alphaproteobacteria* zugeordnet werden konnten. Ein deutlich geringerer Anteil der Isolate und DGGE-Banden fiel in die Gruppen der *Gammaproteobacteria*, *Actinobacteria* und *Firmicutes*. Dem gegenüber wurden die meisten Phylotypen aus dem Weserästuar den *Gammaproteobacteria* und *Actinobacteria* zugeordnet. In deutlich geringerer Zahl wurden *Alphaproteobacteria* und ein Vertreter der *Betaproteobacteria* isoliert bzw. in DGGE-Banden gefunden.

Um strukturelle Veränderungen der Huminstoffmoleküle zu verfolgen, wurden Proben des Mediums vor und nach der Inkubation mittels Pyrolyse-GC/MS analysiert und verglichen. Der Vergleich von eingesetzten Huminsäuren in beiden Kulturmedien zu den jeweiligen Huminsäurefraktionen nach den Inkubationen erbrachte eine deutliche Reduktion an identifizierbaren Substanzen. Diese Reduktion, zusammen mit der geringen Ausbeute an Huminsäuren nach den

Inkubationen im Vergleich zur eingesetzten Huminsäuremenge bei Experimentbeginn, ist Folge eines primär bakteriellen Abbaus. Die Isolierung von Fulvinsäuren nach den Inkubationen, obwohl lediglich Huminsäuren als Kohlenstoffquelle eingesetzt wurden, ist sowohl auf bakteriellen Abbau als auch auf physiko-chemischen Zerfall zurückzuführen. Dieses zeigte sich anhand der geringen Unterschiede beim Verhältnis aromatischer zu aliphatischen Verbindungen in den Huminsäuren zu Beginn des Experiments verglichen mit den jeweils am Ende isolierten Fulvinsäuren. Insgesamt wurde deutlich, dass die Bakteriengemeinschaften sowohl aus Weserästuar als auch dem Wattenmeer unabhängig von der Salinität bevorzugt aliphatische Verbindungen und wenige aromatische Verbindungen wie z.B. 1,2,3-Trimethoxybenzol abbauen konnten. Bestimmte aromatische Verbindungen wie z.B. Ligninderivate scheinen also bakteriell kaum oder gar nicht abbaubar zu sein, was zu einer Akkumulation im Kulturmedium führte.

Zusammenfassend erbrachten die durchgeführten Experimente deutliche Anhaltspunkte dafür, dass in den Bereichen des ostfriesischen Wattenmeeres und des Weserästuars Bakteriengemeinschaften vorhanden sind, die refraktäres Material terrestrischen Ursprungs abbauen können.

Summary

In this work the bacterial degradation of humic acids in the East Frisian Wadden Sea and the Weser estuary was studied for the first time. A major aspect was the influence of different salinities on the bacterial decomposition process, because during DOM (dissolved organic matter) transport from the river Weser to the Wadden Sea the salinity continuously increases and is tidally affected. Incubations of flow-through cultures with inocula from the Weser estuary and the Wadden Sea were conducted over a period of 71-74 days. In the experiments with these two bacterial communities humic acid isolates were used as sole carbon source. To investigate the effect of salinity on the decomposition of humic acids, the bacterial communities were exposed to different salinities, i.e. marine and estuarine inoculum with 28‰ and 14‰ each.

In both experiments humic acids were decomposed by the two bacterial communities. When exposed to a different salinity, other than the salinity of the inoculum, the bacterial communities responded with an increased decomposition of humic acids. The largest reduction took place within the first 21-28 days of incubation. Afterwards only the incoming fresh substrate was decomposed, whereas the partially decomposed substrate that was already in the culture vessel was not further utilized. In this first decomposition phase, high dynamics of the bacterial cell numbers and the protein production was observed. Analysis of the DGGE profiles (denaturing gradient gel electrophoresis) also showed large variations in the composition of bacterial communities during the first 3-4 weeks.

To get insight in the phylogenetic affiliation of humic acid decomposing bacteria, a large number of bacteria was isolated and classified by their 16S rRNA genes. In addition bacteria were identified by sequencing DGGE bands from the last day of incubation. Sequences of isolates and DGGE bands affiliated to the *Alphaproteobacteria* dominated the humic acid decomposing bacterial community from the East Frisian Wadden Sea. A much smaller proportion of the isolates and DGGE bands fell into the *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes*. In contrast, most phylotypes from the Weser estuary belonged to the *Gammaproteobacteria* and *Actinobacteria*. A much smaller quantity of *Alphaproteobacteria* and one representative of the *Betaproteobacteria* were isolated or identified from the DGGE bands.

To follow the structural changes of humic acid molecules, samples of the medium before and after incubation were analyzed and compared using pyrolysis-GC/MS. Comparison of the humic acid fractions from both culture vessels showed a large reduction of identifiable compounds. This reduction, in addition to the low amount of humic acids after incubation is primarily the result of bacterial decomposition. The isolation of fulvic acids after the incubation, although only humic acids were used as carbon source, is a result of physico-chemical decay but also of bacterial decomposition processes. This became obvious in the small differences in the ratio of the aliphatic to the aromatic compounds of the humic acid fraction from the start of the experiment compared to that of the fulvic acid fraction after the experiment. Overall it was shown that both the marine and the estuarine bacterial community decomposed primarily aliphatic compounds and less aromatic compound, e.g.

1,2,3-trimethoxybenzene. Specific aromatic compounds like lignin derivates were not or only marginally decomposed by the bacteria, resulting in an accumulation in the culture vessel.

In summary, the experiments carried out in this study demonstrated that the bacterial communities in the areas of the East Frisian Wadden Sea and the Weser estuary can considerably reduce terrestrial refractory material.

Liste der Publikationen

Rocker, D., Brinkhoff, T., Dogs, M., Simon, M. 2011. Composition of humic acid degrading estuarine and marine bacterial communities. Submittet to FEMS Microbiology Ecology

Konzept: D.R., M.S., Aufbau und Durchführung der Durchflusskulturen: D.R., Isolierung der Huminstofffraktionen: D.R., molekularbiologische Analyse: D.R., M.D. phylogenetische Analyse: R.D., M.D., T.B., Isolierung und physiologische Tests: D.R., M.D., erster Entwurf des Manuskripts: D.R., M.S.

Rocker, D., Kisand, V., Scholz-Böttcher, B., Kneib, T., Lemke, A., Rullkötter, J., Simon, M. 2011. Differential decomposition of humic acids by marine and estuarine bacterial communities at varying salinities. Submittet to Biogeochemistry.

Konzept: D.R., V.K., M.S., Aufbau und Durchführung der Durchflusskulturen: D.R., DOC-Analytik: D.R., Analyse der bakteriellen Produktion: D.R., A.L., Durchflusszytometer Messungen: D.R., A.L., Isolierung der Huminstofffraktionen: D.R., statistische Analyse: T.K., V.K., massenspektrometrische Analyse: D.R., B.S.-B., erster Entwurf des Manuskripts: D.R., V.K.

Kisand, V., **Rocker, D.**, Simon, M. 2008. Significant decomposition of riverine humic-rich DOC by marine but not estuarine bacteria assessed in sequential chemostat experiments. Aquatic Microbial Ecology 53: 151-160.

Konzept: V.K., M.S., Aufbau und Durchführung der Chemostaten: V.K., DOC-Analytik: V.K., DOC Kontrollexperiment: D.R. Analyse der Bakterienzellzahlen: V.K., Analyse der bakteriellen Produktion: V.K., Manuskript: V.K., M.S.

Beiträge auf internationalen Symposien

Dagmar Rocker, Veljo Kisand and Meinhard Simon. 2007. Bacterial decomposition of recalcitrant organic matter in the Weser estuary. 10th Symposium on Aquatic Microbial Ecology (SAME), Faro, Portugal, September 2- 4, 2007

Dagmar Rocker, Marco Dogs, Veljo Kisand, Thorsten Brinkhoff and Meinhard Simon. 2009. Decomposition of humic acids by estuarine and marine bacterial communities. 11th Symposium on Aquatic Microbial Ecology (SAME), Piran, Slovenia, August, 30 – September 4, 2009

Meinhard Simon, **Dagmar Rocker**, Veljo Kisand, Thorsten Brinkhoff, Marco Dogs, Barbara Scholz-Böttcher, Jürgen Rullkötter. 2011. Decomposition of humic acids by estuarine and marine bacterial communities. American Society of Limnology and Oceanography (ASLO) Aquatic Science Meeting, San Juan, Puerto Rico, February 13-17, 2011

Abkürzungsverzeichnis

AMDIS	Automated Mass Spectral Deconvolution and Identification System
ANOVA	Analysis of Variance
ARISA	Automated Intergenic Spacer Analysis
ASW	Artificial Seawater
ASWM	ASW-Medium
bp	Base Pair
BP	Bakterielle Produktion
C	Carbon
CDOM	Chromophoric DOM
C-Pool	Kohlenstoff-Pool
DF	Degree of Freedom
DGGE	Denaturing Gradient Gelelectrophoresis
DNA	Desoxyribonucleic acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
Est	Estuarine
Est-M	Huminsäuremedium für Chemostaten, die mit Bakterien aus dem Weserästuar inokuliert wurden
ESI FT-ICR-MS	Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
FA	Fulvic Acid
FAME	Fatty acid methyl ester
FISH	Fluorescence *in situ* hybridization
FL	Fluorescence
GE	Growth Efficiency
GWS	German Wadden Sea
HA	Humic Acid
HMW-DOM	High-Molecular-Weight DOM
HPA	Hydrophilic Acids
HS	Humic Substances
LMW-DOM	Low-Molecular-Weight DOM
Mar	Marine
Mar-M	Huminsäuremedium für Chemostaten, die mit Bakterien aus dem Strandbereich von Neuharlingersiel inokuliert wurden
MB	Marine Broth
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OD	Optical Density
PAH	Polyaromatic Hydrocarbon
PCR	Polymerase Chain Reaction
POC	Particulate Organic Carbon

PSU	Practical Salinity Unit
Py-GC/MS	Pyrolysis-Gas Chromatography/Mass Spectrometry
RNA	Ribonucleid Acid
rRNA	Ribosomal RNA
SSC	Side Scatter
SPM	Suspended Particulate Matter
TGGE	Temperature Gradient Gel Electrophoresis
TMAH	Tetramethylammonium Hydroxide
TNT	Trinitrotoluol
TOC	Total Organic Carbon
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultraviolet

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1

Einleitung

1 Einleitung

1.1 Vorkommen und Entstehung von Huminstoffen

Huminstoffe sind ubiquitär in der Umwelt vorhanden und kommen somit in Ozeanen, Flüssen, Seen, Sedimenten, aber auch im Grundwasser und in Böden vor (Stevenson, 1985). Diese verschiedenen Gebiete und die dazu gehörigen Transportsysteme sind alle miteinander verbunden (Abb. 1). In den unterschiedlichen Bereichen können Huminstoffe oftmals 50%, in aquatischen Bereichen bis zu 70% des gesamten organischen Materials ausmachen und somit eine bedeutende Kohlenstoffquelle für Mikroorganismen darstellen. Aufgrund ihrer äußerst diversen Struktur, bedingt durch Genese, Abbau und Umweltrelevanz dieser refraktären Substanzen, beeinflussen sie ihre Umgebung auf die unterschiedlichsten Arten. Sie verfügen über hydrophile als auch hydrophobe Eigenschaften, können verschiedene Stoffe wie Metalle und Xenobiotika binden und somit deren Bioverfügbarkeit beeinflussen, werden durch UV-Licht beeinflusst und verändert, was ebenfalls das betreffende Ökosystem beeinflusst (s. Kap. 1.2), und stellen einen großen Kohlenstoff-Pool für verschiedene Organismen dar. So verbreitet Huminstoffe in der Umwelt sind, so unterschiedlich sind die Theorien zur Genese dieser Substanzen. An der Entstehung von Huminstoffen sind mehrere Faktoren beteiligt. Stevenson (1982, 1985) hat vier Haupttheorien zu einem Modell über die Bildung von Huminstoffen zusammengefasst (s. Abb. 2). Die Lignin-Theorie (1) von Waksman (1936) ist lange Zeit die Führende gewesen. Dieser Theorie zufolge wird das nur unvollständig

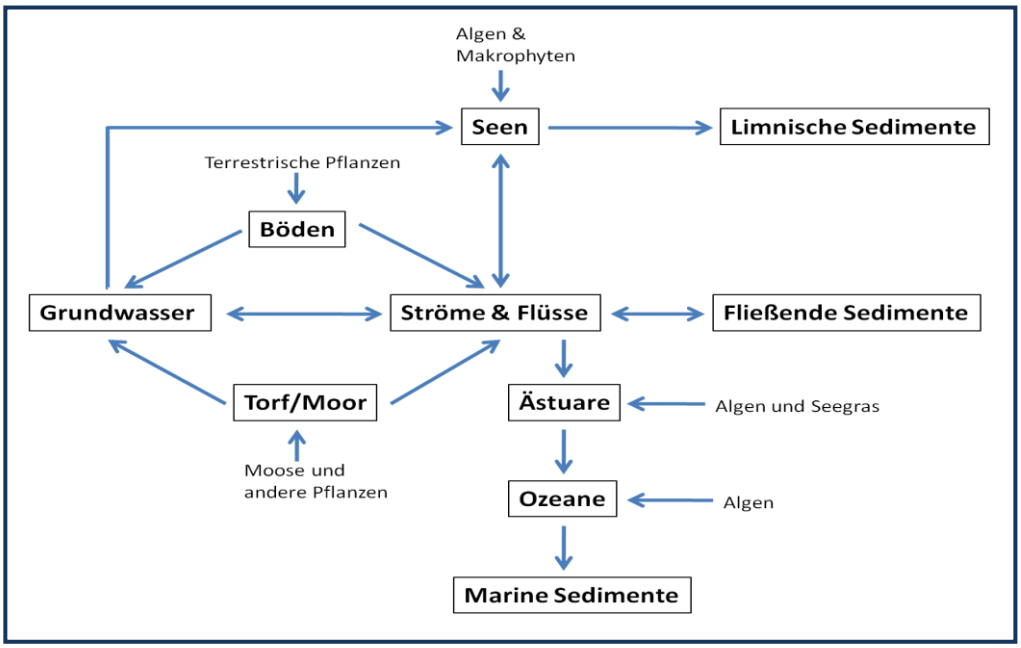

Abbildung 1: Mögliche Flüsse von Huminstoffen in der Umwelt (mod. nach Aiken 1985)

abgebaute Lignin abgestorbener Pflanzen später modifiziert. Denkbarer Grund für diese Modifizierung ist zum Einen eine Demethylierung der einzelnen Lignin-Bestandteile und zum Anderen eine Oxidation der Seitenketten der „Lignin-Monomere“, welche zwar die einzelnen Komponenten des Lignins (z.B. Coniferylalkohol und Sinapylalkohol) enthalten, nicht aber Stickstoffatome, die in den Huminstoffmolekülen enthalten sind. Das Auftreten dieses Elements in Huminstoffen wird über Kondensationsreaktionen von Ligninmolekülen mit Proteinen erklärt. Weiterführende Untersuchungen von Mattson und Koutler-Andersson (1942) konnten Reaktionen von Lignin mit NH_2-Gruppen nachweisen, womit die Präsenz von Stickstoffatomen in den Ringstrukturen der Huminstoffmoleküle erklärt werden kann.

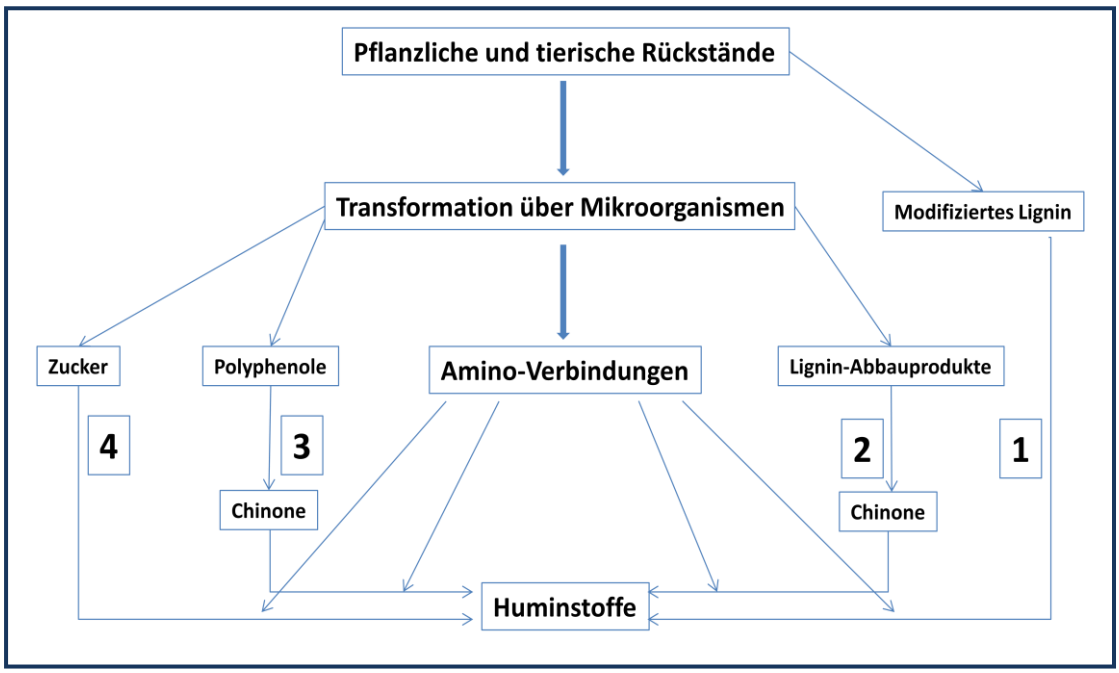

Abbildung 2: Hypothetische Synthesewege von Huminstoffen (Ziffern 1-4) modifiziert nach Stevenson (1985)

In Theorie 2 ist wie in der zuvor beschriebenen Theorie von Waksman ebenfalls Lignin Ausgangssubstanz für die Huminstoffbildung. Die verschiedenen phenolischen Verbindungen (v.a. Aldehyd- und Säureverbindungen) werden über mikrobielle Aktivität zu Chinonen umgebaut. Diese wiederum polymerisieren und integrieren gegebenenfalls stickstoffhaltige Moleküle in ihr Gerüst.

Eine weitere Hypothese (3) der Huminstoffgenese setzt sich aus zwei Schritten zusammen. Grundlage dieser Theorie ist die Bildung von Polyphenolen aus unterschiedlichen Vorstufen. Diese werden anschließend enzymatisch zu Chinonen umgebaut, welche wiederum weiter polymerisieren. Es kommt dann zu weiteren Reaktionen mit Aminosäuren, was zum

Einbau von Stickstoff in das Molekül führt. Der Unterschied zwischen den beiden Ansätzen liegt in den verschiedenen Vorstufen für die Polyphenole.

Die vierte dargestellte Hypothese (4) ist die Zucker-Amin-Kondensation. Diese beinhaltet keinerlei Lignin-Komponenten beim Aufbau der Huminstoffe. Grundlage dieser Theorie ist die Bildung von Schiff'schen Basen aus mikrobiell gebildeten Zuckern und Amino-Verbindungen. Über verschiedene anschließende Reaktionen wird ein braunes Polymer unbekannter Struktur, welches als Melanoidin bezeichnet wird, gebildet. Wie von Stevenson (1982) beschrieben, wird die Theorie über die Bildung von Huminstoffen mittels Polyphenol-Hypothese (zusammengesetzt aus Hypothese 2 und 3) favorisiert. Dieses liegt u.a. daran, dass diese Theorie von einer sehr großen Anzahl von Vorläufermolekülen ausgeht, die ubiquitär verfügbar sind. Eine Schlussfolgerung aus dieser Hypothese ist, dass vermutlich kaum ein Huminstoffmolekül in der Natur dem anderen zu 100% gleicht, was ebenfalls von McCarthy (2001) postuliert wurde. Es ist aber anzunehmen, dass auch die anderen Theorien in der Natur realisiert sind und zur Bildung der Huminstoffe beitragen, betrachtet man die Komplexität und den Aufbau von Huminstoffmolekülen (s. Kap. 1.2).

Für aquatische Huminstoffe hingegen wird die sogen. Melanoidin-Hypothese favorisiert, da sie ohne das Vorhandensein von Lignin, welches Bestandteil von höheren Pflanzen ist, auskommt (autochthone HS). Die Ausgangssubstanzen für die aquatischen (autochthonen) HS sind abgestorbene Algen und Phytoplankton (Ikan et al., 1992). Allerdings können auch die anderen Bildungswege mit Lignin als Vorläufer nicht ausgeschlossen werden, da über Flüsse und Ästuare terrestrisches Material in den marinen Bereich eingetragen werden (allochthone HS). Diese Annahme wird auch gestützt durch die Gegenüberstellung terrestrischer und mariner Huminstoffe von Rashid (1985, Tab.1), wo gezeigt ist, dass beide Fraktionen annähernd gleiche Bestandteile (wie z.B. Polyphenole und Polyaromaten als verwandte Strukturen des Lignins) aufweisen, sich aber nur im Gehalt unterscheiden.

Allerdings wurden auch weitere Theorien formuliert, die im Gegensatz zu einigen der oben genannten, v.a. der Lignin-abhängigen stehen. Als ein Beispiel sei hier Hatcher et al. (1980, 1981) genannt, der anhand von NMR-Untersuchungen eine Beteiligung von Lignin anzweifelt. Es wird aber in vielen Zusammenfassungen zum Thema Huminstoffe und Huminstoffgenese immer wieder betont, dass vermutlich viele Entstehungswege für diese Substanzgruppe existieren. Welcher dieser Wege präferiert wird, ist vermutlich abhängig von dem Habitat, den dort vorliegenden Stoffen und den beteiligten Organismen.

Aquatische Huminstoffe	Terrestrische Huminstoffe
Sind reich an aliphatischen Bestandteilen; Aliphaten doppelt so häufig wie Aromaten vorhanden	Besitzen einen aromatischen Kern, insgesamt sind aromatische Komponenten überwiegend
Polyphenolische und polyaromatische Komponenten sind niedrig konzentriert und kaum substituiert	Aliphatische Ketten sind am aromatischen Kern gebunden
Kohlenstoffgehalt ist bei Fulvinsäuren 45-50%, bei Huminsäuren 50-55%	Der Kohlenstoffgehalt ist variabler, allerdings ist er meist bei 65%
Relativ gesehen ist der Wasserstoffgehalt höher	Niedrige Konzentration an Wasserstoff
Huminstoffe, die aus abgestorbenen marinen Organismen bestehen haben einen höheren Stickstoffgehalt	Der Stickstoffgehalt ist relativ niedrig
Alkoholische Hydroxylgruppen sind reich vorhanden	Wenige alkoholische Hydroxylgruppen
Huminstoffe beinhalten 20-30% an funktionellen Gruppen	Terrestrische Huminsäuren besitzen ca. 25%, Fulvinsäuren ca. 50-60% an funktionellen Gruppen

Tabelle 1: Unterschied zwischen marinen und terrestrischen Huminstoffen (zusammengefasst nach Rashid, 1985)

1.2 Untersuchung und strukturelle Aufklärung von Huminstoffen

Als „Entdecker“ der Huminstoffe gilt Franz Carl Achard, der mit Alkali aus Torfproben ein dunkles Präzipitat erhielt (Frimmel, 1988). Von Saussure (1804) wurde der Begriff Humus (lat. Boden) für dunkel gefärbte Bodenbestandteile geprägt, woraus sich später der Begriff Huminsäure entwickelt hat. Genauere Beschreibungen von Huminstoffen, also für alkalilösliche und säureunlösliche Fraktionen organischer Materie, gehen auf den schwedischen Chemiker Berzelius (1839) zurück, der in seinen Untersuchungen genauere Angaben zu Isolierung, Elementzusammensetzung und Metallbindung gemacht hat (Stevenson, 1982). Die Prägung des Begriffs Fulvinsäure (von fulvus-gelb) geht auf Oden (1919) zurück. Ein weiterer Begriff für diese Substanzgruppe wurde von Kurt Kalle (1937) eingeführt, der gelbe organische Säuren isolierte und sie Gelbstoffe nannte, ein Begriff, der noch lange in der Literatur verwendet wurde. Einen guten Überblick über die Begrifflichkeiten von Huminstoffen, Huminsäuren und Fulvinsäuren ist bei Waksman (1936) zu finden, der Untersuchungen und Begriffsverteilung von Substanzen zusammengefasst und bereinigt hat. Im weiteren Verlauf des 20. Jahrhunderts wurden Huminstoffe immer intensiver

untersucht. Als eine wichtige Arbeit beschreibt Stevenson (1982) die Forschung von Shmuk (1930), welcher in seiner Abhandlung resümiert, dass es sich bei Huminsäuren um eine Zusammensetzung strukturell nah verwandter Komponenten handelt. Des Weiteren konnte er in diesen Molekülen Esterbindungen nachweisen und schloss aus seinen und anderen Arbeiten, dass Huminsäuren aus zwei Hauptbestandteilen zusammengesetzt sind, einem organischen Stickstoff-haltigen Bestandteil (Protein) und einem aromatischen Ring, welcher aus dem Lignin hervor geht.

Weiterführend ist das Modell von Haworth (1971, Abb. 3), welches er aus eigenen Ergebnissen und denen anderer Forscher entwickelte. Hierbei geht er von einem Kern polyzyklischer Aromaten mit daran gebundenen Kohlenhydraten, phenolischen Säuren, Peptiden und Metallen aus. Eine der wichtigsten Arbeiten in diesem Gebiet ist sicherlich die Formulierung einer zusammenfassenden, bis heute gültigen Definition der Huminstoffe von Aiken et al. (1985). Hier werden Huminstoffe definiert als *eine Gruppe biogen gebildeter, heterogener organischer Substanzen, die allgemein als gelb bis schwarz gefärbt, hochmolekular und refraktär charakterisiert werden können*. Er unterteilte diese sehr heterogene Substanzgruppe anhand ihres Löslichkeitsverhaltens in Humine, Huminsäuren und Fulvinsäuren. Demnach sind Fulvinsäuren diejenige Fraktion, die in wässriger Flüssigkeit bei jedem pH-Wert in Lösung gehen kann. Die Huminsäuren sind nur über einem pH-Wert von 2 löslich, wohingegen die Humine gänzlich unlöslich in wässrigen Systemen sind. Diese Beobachtung macht sich Aiken auch zunutze in der Isolierung von Huminstoffen aus aquatischen Habitaten (Aiken, 1985). Auch in anderen physikalischen Eigenschaften unterscheiden sich die verschiedenen Huminstofffraktionen. Die klassische Unterscheidung von Fulvinsäuren bis hin zu den Huminsäuren ist von Stevenson (1985) zusammengefasst worden (Tab. 2, 3).

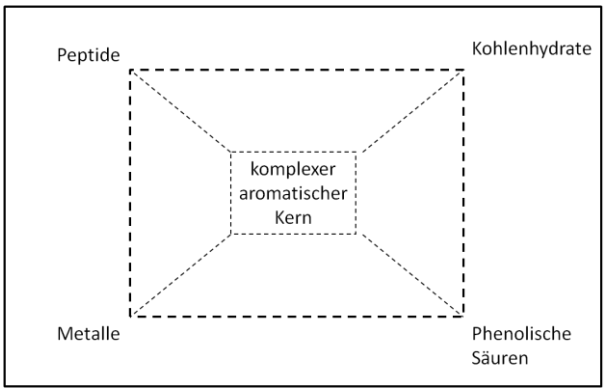

Abbildung 3: Modell des strukturellen Aufbaus von Huminsäuren (Haworth, 1971)

Neben der Unterteilung der einzelnen Huminstofffraktionen ist zum Einen auch die Einteilung in autochthon und allochthon sehr wichtig, zum Anderen wird auch nach aquatischen und terrestrischen HS, wie sie von Rashid (1985) und Steelink (1985) untersucht wurden, unterschieden (s. Kap. 1.1).

Aufgrund ihrer komplexen Struktur sind bisher nur sehr eingeschränkte Modelle über die Struktur von Huminstoffen vorhanden. Die bisherigen Untersuchungen befassen sich in erster Linie mit Strukturformeln von Fulvinsäuren. Eine klassische Strukturformel für eine terrestrische Huminsäure wurde von Fuchs (1931) postuliert und stellt ein polyaromatisches System dar. Dieses Modell wurde in den folgenden Jahren immer weiter modifiziert. Das wohl eindrucksvollste Modell einer Huminsäure wurde von Schulten und Leinweber (1996) veröffentlich (Abb. 4).

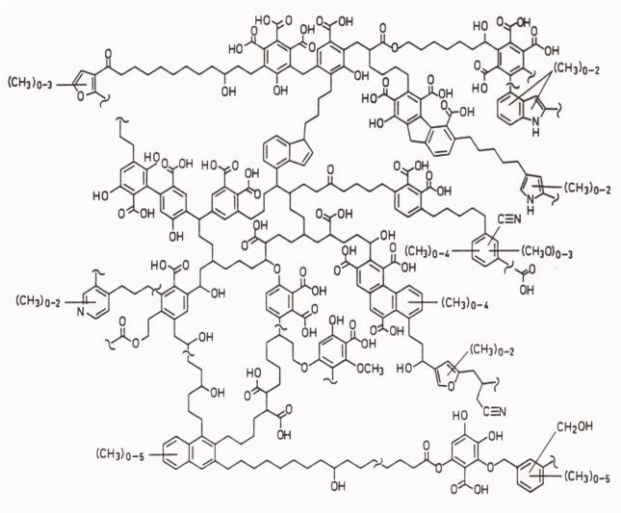

Abbildung 4: Modell einer Huminsäure nach Schulten und Leinweber (1996, rechts)

Dieses komplexe Gebilde beruht auf Ergebnissen aus der Curie-Punkt-Pyrolyse, spektroskopischen und chemischen Analysen sowie anschließender Computerauswertungen. Durch immer weiter fortschreitende Analysemethoden wird auch dieses Modell in Zukunft erweitert und modifiziert werden können. Eine allumfassende Strukturformel für Huminsäuren, bzw. Huminstoffe allgemein wird aber aufgrund der diversen Genesewege (s. Kap. 1.1) und der Komplexität der Moleküle auch in Zukunft nicht möglich sein.

1.3 Eigenschaften von Huminstoffen

Da es sich bei Huminstoffen um eine sehr heterogene Substanzgruppe handelt, sind diese nicht in eine klassische chemische Stoffklasse einzuordnen. Sie werden anhand ihres Löslichkeitsverhaltens in wässrigen Systemen in Humine, Huminsäuren und Fulvinsäuren unterteilt (s.a. Kap. 1.2). Humine sollen hier nicht weiter betrachtet werden, da sie in wässrigen Systemen nicht löslich sind. Huminstoffe sind aus einer Reihe von Verbindungen mit unterschiedlichen funktionellen Gruppen wie z.B. stickstoffhaltigen Amino- und Iminogruppen, Aldehyd-, Hydroxyl- und Carboxylgruppen, Chinonen und Peptiden zusammengesetzt (Stevenson, 1982). Die Elementzusammensetzung der einzelnen Huminstofffraktionen (Tab. 2) variiert ebenfalls, genauso wie ihre physikalischen Eigenschaften (Tab. 3). Im Vergleich zu Fulvinsäuren sind die Huminsäuren komplexer und von höherem Molekulargewicht. In Verbindung mit einem größeren Anteil an aromatischen Verbindungen bedingt dieses auch eine unterschiedliche Bioverfügbarkeit für Mikroorganismen. In den verschiedensten Bereichen hat das Auftreten von Huminstoffen Auswirkungen auf Mensch und Umwelt. In den unterschiedlichen Habitaten bilden Huminstoffe für Mikroorganismen einen bedeutenden Kohlenstoff-Pool. Eine Reihe von Pilzen und Bakterien (s. Kap. 1.4) können Huminstoffe abbauen und in ihren Stoffwechsel integrieren.

Element	Fulvinsäuren	Huminsäuren
C (%)	40-50	50-60
O (%)	44-50	30-35
H (%)	4-6	4-6
N (%)	<1-3	2-6
S (%)	0-2	0-2

Tabelle 2: Anteile einzelner Elemente an Huminsäuren und Fulvinsäuren im Vergleich (Stevenson, 1982)

Fulvinsäuren	→→→→→	**Huminsäuren**
	→ Zunahme des Polymerisierungsgrades →	
~2000	→ Zunahme des Molekulargewichtes →	~300000
45%	→ Zunahme des Kohlenstoffgehaltes →	62%
48%	→ Abnahme des Sauerstoffgehaltes →	30%
	→ Abnahme der Löslichkeit in wässrigen Systemen →	

Tabelle 3: Vergleich der Eigenschaften von Fulvinsäuren und Huminsäuren (verändert nach Stevenson, 1982)

In verschiedenen Studien wurde ein deutlich messbarer Abbau von DOM (dissolved organic matter) allgemein, aber auch von separierten Huminstoffen und Huminstofffraktionen durch Bakterien in den unterschiedlichen Habitaten gezeigt (z.B. Moran und Hodson, 1990; Bussmann, 1999; Kisand et al., 2002; Kisand et al., 2008; Yanagi et al., 2008). Weiterhin sind unter anoxischen Bedingungen Bakterien in der Lage Huminsäuren als Elektronendonatoren und -akzeptoren zu verwenden (Coates et al., 1998; Lovley et al., 1999). Die Huminstoffmoleküle werden durch UV-Strahlung gespalten, was eine Verringerung der UV-Belastung des Gewässers darstellt. Dadurch stellen Huminstoffe in Gewässern einen wirksamen UV-Schutz für die verschiedenen Organismen dar. Ein weiterer Effekt dieses abiotischen Huminstoff-„Abbaus“ ist eine bessere Bioverfügbarkeit dieser nun weniger hochmolekularen Stoffe, was eine Vergrößerung des verfügbaren C-Pools bedeutet (Moran et al., 2000).

Weiterhin interessant an Huminstoffen ist ihre hormonähnliche Wirkung auf verschiedene Tiere. Steinberg et al. (2004, 2008) konnten an Fischen, Invertebraten und Amphibien eine hormonähnliche Wirkung in Richtung Feminisierung der Populationen zeigen. In der therapeutischen und kosmetischen Anwendung wurden Huminstoffe bereits im antiken Babylon und Römischen Reich verwendet (Klöcking und Helbig, 1993). Durch Moorbäder, aber auch durch innere Anwendung sollten rheumatische und gynäkologische Leiden und auch Magen-, Darm- und Leberleiden behandelt werden. Eine antiphlogistische Wirkung wurde bereits vielfach in der Human- und Tiermedizin beobachtet. Mechanismen dieser Wirkung wurden von Klöcking und Helbig (1993) dargestellt und ein Zusammenhang mit bestimmten Synthesewegen von Schmerzmediatoren in Mensch und Tier gezeigt. Aber auch die Metallbindekapazität von HS kann von therapeutischer Bedeutung sein, um z.B. Schwermetallintoxikationen bei Tieren zu verhindern (aus Klöcking und Helbig, 1993). Die Eigenschaft der Huminstoffe, Metalle, aber auch in die Umwelt gelangte Toxine zu binden, kann für verschiedenste Bereiche der Umwelt von negativer Bedeutung sein. Dieses betrifft insbesondere Gewässer und mögliche Belastungen durch Huminstoff-vermittelte Schwermetallablagerungen z.B. in Sedimenten, aber auch die Aufnahme dieser Schwermetalle und anderer Toxine (z.B. TNT, Li et al., 1997). Ein weiterer wichtiger Aspekt ist eine mögliche Grundwasserbelastung, welche sich möglicherweise negativ auf die Trinkwasserversorgung auswirken kann. Dieses sind einige Gesichtspunkte, weshalb eine Akkumulation von Huminstoffen in bestimmten Bereichen der Umwelt nicht wünschenswert ist.

1.4 Abbau durch Mikroorganismen

Huminstoffe sind ubiquitär und stellen für Mikroorganismen eine potentielle Quelle für Kohlenstoff, Stickstoff und andere Elemente dar. Bereits seit den 30er Jahren wurden Pilze untersucht, die Lignin, welches strukturell mit Huminstoffen verwandt ist, abbauen können. Man beobachtete, dass oftmals *Basidiomycota* die Holzbestandteile, v.a. das Lignin, angriffen und zerstörten (Falck, 1926; Rösch, 1961), was z.B. für die Weißfäule an Bäumen verantwortlich ist. Neben *Basidiomycota* können auch *Ascomycota* Lignin abbauen und modifizieren, was durch Jones und Farmer (1967) gezeigt wurde.

In frühen Untersuchungen seit den 60er Jahren sind in erster Linie Pilze untersucht worden. Paul und Mathur (1967) beobachteten bei *Penicilium frequentans,* dass dieser bevorzugt aromatische Bestandteile angreift. Ein weiterer Reaktionsmechanismus beim Abbau von Huminstoffen, über den dieser Organismus zu verfügen scheint, sind Spaltungen von Etherbindungen innerhalb der komplexen Moleküle (Paul und Mathur 1967). In der folgenden Zeit wurde eine große Anzahl an weiteren Pilzen, die sich als effektive Degradierer herausstellten, zumeist aus Böden isoliert. Die bereits aus dem Lignin-Abbau bekannten *Basidiomycota* sind ebenfalls auf Huminstoff-Abbau überprüft worden. Dehorter und Blondeau (1992) zeigten für zwei Lignin-abbauende Organismen, *Phanaerochaete chrysosporium* und *Trametes versicolor*, eine Entfärbung der Medien, was einen Abbau der Huminstoffe bedeutet. Des Weiteren konnten sie zeigen, dass beide Pilze über eine Lignin-Peroxidase und eine Mangan-abhängige Peroxidase verfügen, wobei *T. versicolor* diese erst bildet, wenn Huminstoffe dem Medium zugegeben werden. Gramss et al. (1998) konnten für 36 verschiedene Pilze (*Basidiomycota* und *Ascomycota*) sowie für *Bacillus sp., Alcaligenes sp.* und *Pseudomonas sp*. eine deutliche Abnahme der Absorption in Huminstoffmedien nachweisen, die durch eine Veränderung der Molekularstruktur verursacht wird und eine Reduktion der Molekulargewichte der Huminstoffe bedeutet. Untersuchungen von Blondeau (1989) an *Phanerochaete chrysosporium* zeigten ebenfalls einen deutlichen Abbau von Huminsäuren.

Im weiteren Verlauf der Huminstoff-Untersuchungen gerieten auch Bakterien immer stärker in den Fokus. In unterschiedlichen Habitaten sind verschiedene Gruppen von Bakterien am Abbau beteiligt. In erster Linie wurden Bodenbakterien, später aber auch aquatische Mikroorganismen auf ihr Abbaupotential untersucht. Als Huminstoff-Abbauer sind in Böden oftmals typische Vertreter dieses Ökosystems identifiziert worden. Mathur und Paul (1967) stellten bei *Bacillus sp., Pseudomonas sp., Agrobacterium sp.* und *Arthrobacter sp.* dieses Abbaupotential fest. Tuyev und Emtsev (1984) konnten einen Abbau bei *Clostridium*

sporogenum zeigen. Swift et al. (1987) waren in der Lage, anhand des Abbaus durch *Pseudomonas maltophilia* zu zeigen, dass während der Inkubation hochmolekulare Huminstoff-Bestandteile abnahmen und niedermolekulare zunahmen. Weitere Bodenbakterien wurden von ihnen ebenfalls positiv auf die Verwertung dieses Substrats als Kohlenstoff- und Stickstoffquelle getestet. Filip et al. (1998) untersuchten Phenoloxidase-bildende *Actinobacteria* und Pilze und deren Verwertung von Huminstoffen aus Grund- und Flusswasser und stellten fest, dass Huminsäuren aus Grundwasser geringfügig abgebaut wurden. Die aus Flusswasser stammenden hingegen wurden durch zwei *Steptomyces viridosporus*-Stämme zu ca. 45%, durch *S. michiganensis* zu 16% abgebaut. Bei *S. eurythermus* hingegen war eine Neubildung von Huminsäuren zu beobachten. Dieses, so vermuten die Verfasser, ist auf die Tatsache zurückzuführen, dass einige Enzyme, welche im Abbauprozess von Huminstoffen eine Rolle spielen, Reaktionen katalysieren können, die auch im Aufbauprozess wichtig sind.

Für die Betrachtung aquatischer Organismen sind in erster Linie die Untersuchungen von Bakteriengemeinschaften in DOM-reichen Gebieten mit undefiniertem DOM als Kohlenstoffquelle durchgeführt worden, weniger an separaten Huminstoffen. In huminstoffreichen Seen wurden von Hutalle-Schmelzer et al. (2009) v.a. *Betaproteobacteria* als Huminstoff-Abbauer identifiziert, aber auch spezifische Vertreter der *Alphaproteobacteria* und *Bacteroidetes*. Burkert et al. (2003) waren in der Lage, v.a. *Betaproteobacteria* und *Actinobacteria* aus dem See „Große Fuchskuhle“ in huminstoffreichem Wasser anzureichern. Haukka et al. (2005) konnten eine hohe Abundanz von *Actinobacteria* und *Verrucomicrobia* in Seen mit unterschiedlichen Huminstoffgehalten nachweisen. In anschließenden Anreicherungskulturen aus den entsprechenden Seen dominierten zunächst *Proteobacteria*, die im Weiteren von *Actinobacteria* in den Hintergrund gedrängt wurden. Der Abbau von isolierten Huminstoffen durch spezialisierte Bakteriengemeinschaften in weiteren Habitaten ist wenig untersucht. Covert und Moran (2001) zeigten durch Anreicherungskulturen aus Ästuaren, dass hochmolekulare Fraktionen des DOM, welche hauptsächlich aus Huminstoffen bestehen, durch Vertreter der *Alpha-*, *Beta-* und *Gammaproteobacteria* sowie verschiedene *Bacteroides* abgebaut wurden. Ein ähnliches Bild fanden Hilyard et al. (2008) bei der Untersuchung des Abbaus von polyzyklischen Aromaten durch Bakterien im Flusssediment. Im Öre-Ästuar gehören verschiedene Vertreter der *Bacteroidetes-Gruppe* (insbesondere der *Cytophaga-Flexibacter*-Gruppe) und einer der *Gammaproteobacteria* zu den dominanten Abbauern von DOM aus Flüssen (Kisand et al., 2002).

Für den Bereich der Nordsee und des Weserästuars hingegen liegen kaum Informationen über potentielle Huminstoff-Abbauer vor. Kisand et al. (2008) konnten bereits zeigen, dass v.a. marine Bakterien Huminstoffe abbauen können, ästuarine allerdings nicht. Welche Bakteriengemeinschaften daran beteiligt sind, ist hingegen noch unklar. Auch im Bereich des Wattenmeeres sind zwar Daten über allgemeinen Abbau von DOM, aber nicht über spezialisierte Bakteriengemeinschaften vorhanden, welche Huminstoffe bzw. Huminsäuren abbauen können.

Neben der taxonomischen Einordnung der Bakterien und der analytischen Untersuchung sind in einigen Untersuchungen am Huminstoff-Abbau beteiligte Enzyme nachgewiesen worden. Die beiden hierbei wichtigen beteiligten Enzymklassen sind Oxidoreduktasen und Hydrolasen (Münster und de Haan, 1998). Wichtig für die Spaltung von aliphatischen und aromatischen Komponenten sind verschiedene Oxidoreduktasen. Zu ihnen zählen die bereits oben genannten, welche in Pilzen nachgewiesen wurden, sowie Phenol-Oxidasen und -Peroxidasen (Münster und de Haan, 1998; Schinner und Sonnleitner, 1996). In einem aquatischen *Arthrobacter* wurden durch zugegebene Fulvinsäuren Anhaltspunkte gefunden für eine Induzierbarkeit von Catechol-1,2-oxygenase, welche einen wichtigen Schritt im β-Ketoadipat-Weg katalysiert, , was auf eine Beteiligung dieses Enzyms am Abbau schließen lässt (de Haan, 1976). Dieser Weg ist ein weit verbreiteter Abbauweg von polyzyklischen und homocyclischen Aromaten (Harwood und Parales, 1996) und spielt eine zentrale Rolle in Pro- und Eukaryonten. Von Buchan et al. (2001, 2004) wurde dieser Weg detailliert im Zusammenhang mit dem mikrobiellen Abbau von Lignin analysiert. Bei Untersuchungen der *pca*-Gene zum Abbau aromatischer Substanzen (aus dem β-Ketoadipat-Weg) in marinen Bakterien konnte gezeigt werden, dass diese bei den *Alphaproteobacteria*, insbesondere aus der *Roseobacter*-Gruppe, sehr verbreitet sind (Buchan et al. 2001). Bei den hydrolytischen Enzymen in diesem Zusammenhang sind alkalische und saure Phosphatasen, Aminopeptidasen, Lipasen, Esterasen und Glycosidasen wichtig für Spaltungsreaktionen innerhalb der Huminstoffmoleküle (Münster und de Haan, 1992). Für den Abbau von HS wurden bei Pilzen ähnlich dem Ligninabbau Laccasen (Claus und Filip, 1998; Scheel et al., 1999), Mangan-Peroxidasen (Ziegenhagen und Hofrichter, 1998) und Lignin-Peroxidasen (Steffen et al., 2002) nachgewiesen.

1.5 Huminstoffeintrag in das Weserästuar und Wattenmeer

Die beiden Gebiete, aus welchen wir Inokulate entnommen haben, um den Huminsäureabbau durch verschiedene Bakteriengemeinschaften zu untersuchen, sind das Weserästuar und die Nordsee (Abb. 5). Ästuare sind die tidebeeinflussten Mündungsbereiche von Flüssen und somit Übergangsbereiche in die Küstengewässer. An gezeitenabhängigen Ästuaren wie dem der Weser findet man eine typische Trichterform. Es findet hier eine Durchmischung der verschiedenen Wasserkörper, des salzarmen aus dem Fluss und des marinen aus der Nordsee, statt und beeinflusst nachhaltig abiotische und biotische Faktoren. Nach der Elbe ist die Weser mit einem Gesamteinzugsgebiet von 49.000 km^2 der zweitgrößte Fluss, der Wasser und darin enthaltene Frachten (versch. Kohlenstoff-, Stickstoff- und Phosphat- Spezies und andere Substanzen) in die Deutsche Bucht entlässt. In der Weser gemessene DOC-Konzentrationen z.B. am Standort Brake, ca. 10km flussabwärts des Strömungsmaximum des Weserästuars, bewegen sich zwischen 4,6 und 13 mg/l für das Jahr 2007 und für 2008 zwischen 2,5 und 13 mg/l (Gewässergüteberichte der FGG Weser). Abschätzungen des Gesamtausstroms von DOC, wie sie z.B. für die Elbe (456 µmol/kg, Pätsch und Lenhart, 2004) oder die Ems (1007 µmol/kg, gleiche Quelle) vorliegen, sind für die Weser nicht verfügbar. Ein Vergleich des durchschnittlichen jährliches Ausstroms der Elbe und der Ems (721 m^3s^{-1} bzw. 85 m^3s^{-1} , beides Pätsch und Lenhart 2004) in Relation zu dem Gesamt DOC-Ausstrom macht deutlich, dass viele Faktoren in solche Berechnungen einfließen, so dass eine Abschätzung für die Weser schwierig zu erstellen ist. Allerdings liegt der durchschnittliche jährliche Ausstrom an Wasser aus der Weser in die Nordsee bei ca. 317 m^3s^{-1} (Engel, 1995) und somit im Mittel zwischen der Elbe und Ems, was vermuten lässt, dass sich der DOC-Ausstrom in die Nordsee in einer ähnlichen Größenordnung bewegt. Im Vergleich dazu werden global 430×10^{12} g

Abbildung 5: Probenahmeorte im Weserästuar und im Wattenmeer

terrestrischer Kohlenstoff in die Ozeane eingetragen (Schlünz und Schneider, 2000), wobei der Eintrag der Weser, gemessen an der Größe zu den bedeutenden Strömen der Erde (z.B. Amazonas), sehr gering ist.

Eine wichtige Rolle im Abbau von organischem Material in aquatischen Habitaten wie dem Weserästuar und der Nordsee spielen die unterschiedlichen Bakteriengemeinschaften. Diese wurden in der Vergangenheit in einzelnen Ästuaren untersucht. Allerdings sind solche Studien nicht sehr zahlreich, gemessen an der Vielzahl der global vorhandenen Ästuare (z.B. Kan et al.; 2008, Esham et al.; 2000, Bouvier und del Giorgio, 2002). Ursache hierfür ist zum Teil die Entwicklung der mittlerweile wesentlichen Untersuchungsmethoden für Bakteriengemeinschaften. Die Erschließung der phylogenetischen Zusammensetzung mittels PCR (polymerase chain reaction) und DGGE (denaturing gradient gelelectrophoresis) (auch TGGE, ARISA etc.) gekoppelt mit anschließender Sequenzierung ist erst in den 90er Jahren des letzten Jahrhunderts entwickelt worden. Ebenso ist auch die FISH-Analyse (fluorescence *in situ* hybridisation) ist erst seit ca. Mitte der 90er Jahre üblich. Allgemein konnte v.a. über FISH gezeigt werden, dass in den unterschiedlichen Bereichen des Ästuars verschiedene phylogenetische Gruppen vorherrschend sind. So sind im limnischen Bereich in erster Linie *Betaproteobacteria* anzutreffen. Weiter im marinen Bereich hingegen sind die *Alphaproteobacteria* und da insbesondere Vertreter der *Roseobacter*-Gruppe und auch die *Gammaproteobacteria* abundant.

Für die Weser liegen bereits Untersuchungen von Selje et al. (2003, 2005) bezüglich der Bakteriengemeinschaften vor. Eine Veränderung der Bakteriengemeinschaften konnte entlang des Salinitätsgradienten vom Frischwasserbereich bei Bremen bis in den marinen Bereichs des Ästuars bei Bremerhaven (Selje und Simon, 2003) gezeigt werden. Generell wird anhand von FISH-Daten eine Dominanz der *Cytophaga* (*Cytophaga-Flavobacteria*-Gruppe) deutlich, die aber vom Frischwasser zum marinen Bereich abnahm. Einen gleichmäßigen Anteil von ca. 10% der Gesamtbakterien nahmen die *Proteobacteria* (*Alpha-*, *Beta-* und *Gamma-*) ein, allerdings mit einer großen Variabilität innerhalb der Gruppe in Bezug auf die sich ändernde Salinität. In nachfolgenden Sequenzierungen von prominenten DGGE-Banden wurden z.B. keine *Gammaproteobacteria* und *Cytophaga*-Sequenzen in der marinen Sektion des Ästuars gefunden. In nachfolgenden Arbeiten mit Verdünnungskulturen zeigte sich eine ähnliche Verteilung (Selje et al., 2005). Dominant waren hier die *Alpha-* und *Betaproteobacteria*, gefolgt von der *Flavobacteria/Sphingobacteria* Gruppe und von den *Actinobacteria*. Auch hier waren die *Gammaproteobacteria* kaum nachweisbar. In beiden Arbeiten konnte eine breite Verteilung innerhalb der auftretenden Bakteriengemeinschaft im

Ästuar dargestellt werden. Welche Mikroorganismen in der Weser hingegen für den Abbau der eingetragenen Huminstoffe verantwortlich sind, wurde bisher noch nicht geklärt.

Das andere Entnahme-Gebiet ist das Wattenmeer vor Neuharlingersiel an der Deutschen Nordseeküste. Das Wattenmeer ist in den letzten Jahren Gegenstand intensiver Untersuchungen gewesen, v.a. im Rahmen der Forschergruppe „BioGeoChemie des Watts“ und ist im Jahr 2009 in die Liste der Weltnaturerbe-Stätten der UNESCO aufgenommen (http://www.unesco.org), was die Besonderheit dieses außergewöhnlichen Habitats bekräftigt. Wichtig im Zusammenhang mit der hier vorliegenden Arbeit ist in erster Linie die bakterielle Besiedlung des Freiwassers in der Nordsee, da diese Bakterien auch Gegenstand dieser Arbeit waren. Postma (1981) hat bereits beschrieben, dass das Wattenmeer netto heterotroph ist und als Senke für organische Materie fungiert. Im Wesentlichen werden organische Frachten durch die großen Flüsse Elbe, Weser und Ems eingetragen. Zusätzlich wird auch durch Siele, welche an den verschiedenen Küstenorten verteilt sind, organisches Material aus dem Hinterland in das Wattenmeer eingetragen. Wenig ist über diesen Hinterlandeintrag bekannt, allerdings ergaben Untersuchungen der Esenser Sielacht für das Jahr 2002 und 2003 eine DOC-Exportmenge von 715×10^6 g C a^{-1} und 570×10^6 g C a^{-1} (Gebhardt, 2005).

Verschiedene Faktoren sind für den Abbau des über die Flüsse ins Meer transportierten Materials wichtig. Bedeutend für den Kohlenstoffkreislauf sind die Bakteriengemeinschaften im Freiwasser und Sediment. Mittlerweile liegt eine Reihe von Untersuchungen vor bezüglich der Zusammensetzung der Bakteriengemeinschaften, deren Stoffwechselaktivität, der Abhängigkeit von z.B. den Gezeiten und gelöster und partikulärer Materie und auch von Zusammenhängen mit speziellen Ereignissen im Wattenmeer wie der jährlichen Frühjahrsblüte der Algen. Vor Allem in den letzten Jahren trugen Stevens et al. (2005a, b, 2007, 2009) und Rink et al. (2007, 2008) stark zur Aufklärung der Zusammensetzung der Bakteriengemeinschaften in der Wassersäule bei. So konnte gezeigt werden, dass Freiwasserbakterien in erster Linie von *Alphaproteobacteria*, v.a. der *Roseobacter*-Gruppe, dominiert wird. Weitere abundante Vertreter sind unter den *Gammaproteobacteria*, den *Actinobacteria* und den *Bacteroidetes* zu finden. Über FISH-Analysen konnten Eilers et al. (2000) ebenfalls zeigen, dass die *Roseobacter*-Gruppe bis zu 10% der Gesamtbakterien ausmacht und weiterhin, dass das SAR86-Cluster der *Gammaproteobacteria* und die *Bacteroidetes* abundant sind. Die bakterielle Dynamik dieses Systems im Zusammenhang mit verschiedenen Faktoren wurde von Lemke et al. (2009) untersucht. Es konnte dort gezeigt werden, dass schon der Probenstandort deutliche Auswirkungen auf die bakterielle Dynamik hat, insbesondere auf die hydrographischen und

weiteren Faktoren. Obwohl die beprobten Standorte zum Einen im Einstromgebiet der Tide und zum Anderen im Becken nur wenige km voneinander entfernt sind, konnten deutliche Unterschiede v.a. in der bakteriellen Aktivität und der Zellzahlen der partikelgebundenen Bakterien gezeigt werden, aber auch der POC-Gehalt (particulate organic carbon) und der Chlorophyll a-Gehalt zeigten Unterschiede. Allerdings sind die dort gezeigten Unterschiede zwar im April und Juli deutlich erkennbar, im Winter aber kaum auszumachen. Dieses ist auch in anderen Arbeiten nachgewiesen worden.

Interessant sind die Ergebnisse von Rink et al. (2008), welche drei Tidenzyklen jeweils in verschiedenen Jahreszeiten untersuchten und verschiedene Parameter dabei berücksichtigen konnten. Anhand dieser Ergebnisse konnte gezeigt werden, dass die bakterielle Zusammensetzung unabhängig von der Tide relativ stabil ist, obgleich die meisten anderen Parameter wie SPM (suspended particulate matter), Chlorophyll *a*, POC und Phaeopigmente variierten. Betrachtet man den saisonalen Aspekt dieser Untersuchung, so wird deutlich, dass Schwankungen in sämtlichen gemessenen Parametern im November, also in der Wintersituation, deutlich geringer sind als in der Sommersituation. Gleichzeitig konnten positive Korrelationen von partikelassoziierten Bakterien zu SPM, zu Phaeopigmenten und zur Aktivität der Bakterien gezeigt werden. Auch Grossart et al. (2004) und Lunau et al. (2006) zeigten bereits, dass auch die Bakterienzellzahlen von der Tide abhängig sind, genauso wie SPM, DOC, POC und andere Parameter. Trotz des umfassenden Datenmaterials bezüglich des Wattenmeeres, seiner physikalischen Parameter, seiner Bakteriengemeinschaft, der Bakteriendynamik und der chemischen Zusammensetzung ist auch hier kaum Datenmaterial zum bakteriellen Abbau von Huminstoffen vorhanden.

Für andere Ozeangebiete, Flüsse und Ästuare liegen bereits Untersuchungen zum Abbau refraktärer Substanzen und teilweise der beteiligten Bakterien vor. Ein deutliches Wachstum von Bakterien auf Huminstoffen wurde von Moran und Hodson (1994) gezeigt. Sie isolierten HS aus verschiedenen marinen Bereichen und zeigten einen mikrobiellen Abbau von bis zu 11%. In limnischen Bereichen wie Flüssen und Grundwasser wurden sogar bis zu 26,9% der Huminstoffe von Bakterien abgebaut (Hertkorn et al., 2002). Für die Weser liegt bereits eine Arbeit von Kisand et al. (2008) vor, die eine Abhängigkeit des HS-Abbaus von der Salinität zeigen konnte. Hier wurde deutlich, dass HS insbesondere im marinen Bereich des Weserästuars abgebaut werden, im ästuarinen und limnischen Bereich hingegen fast gar nicht. Allerdings konnte gezeigt werden, dass es zu einer Transformation ohne nachweisbaren Abbau der HS gekommen ist, die in einem Verlust von hochmolekularen HA zugunsten der niedermolekularen FA resultierte. Phylogenetische Untersuchungen dieser Studie wurden

noch nicht veröffentlicht. Arbeiten aus anderen aquatischen Habitaten liegen aber bereits vor. So konnten Esham et al. (2000) aus dem Satilla-Ästuar (Georgia, USA) eine Reihe huminstoffabbauender Bakterien isolieren, die zu den *Alphaproteobacteria*, *Gammaproteobacteria* und Gram-positiven Bakterien gehören. Weiterhin untersuchten Buchan et al. (2000, 2001) eine Reihe von *Alphaproteobacteria*, die der Gruppe der *Roseobacter* zugeordnet wurden, sowie *Gammaproteobacteria* und *Firmicutes*, die Lignin und auch andere aromatische Substanzen über den β-Ketoadipat-Weg abbauen.

In den oben erwähnten Studien aus Weser und Nordsee sind allgemeine DOM-Daten erhoben worden und teilweise Rückschlüsse auf die Bedeutung für die Bakterien gezogen worden. Allerdings konnte bisher noch nicht gezeigt werden, ob und welche spezialisierten Mikroorganismen in diesen Habitaten am Huminstoffabbau beteiligt sind bzw. welche die Schlüsselorganismen in diesem Stoffwechselgeschehen sind.

1.6 Zielsetzung der Arbeit

Ziel dieser Arbeit war es, den Abbau von Huminsäuren durch Bakteriengemeinschaften, welche zwei unterschiedlichen Gebieten, dem Weserästuar und dem Wattenmeer, entnommen wurden, zu untersuchen und zu vergleichen. Hierbei wurden verschiedene Aspekte im Besonderen betrachtet.

Es sollte experimentell untersucht werden, in welchem Ausmaß und durch welche Bakterien HS in den jeweiligen Habitaten abgebaut werden können bzw. durch HS selektioniert werden können. Zu diesem Zweck wurden Chemostaten etabliert, mit Bakterien aus den beiden Habitaten inokuliert und für ca. 10 Wochen in einem Huminsäuremedium mit unterschiedlicher Salinität inkubiert. Die Bakteriengemeinschaften wurden einerseits über PCR-DGGE mit anschließender Sequenzierung prominenter Banden, andererseits über Isolierung unterschiedlicher Bakterien aus den einzelnen Chemostaten mit anschließender Sequenzierung untersucht.

Die Abbaukapazität wurde über DOM-Analysen, Zellzahlbestimmung und bakteriellen Aktivitäten untersucht. Außerdem sollte mittels Pyrolyse GC/MS ermittelt werden, ob und welche strukturellen Änderungen in den Huminstoffmolekülen während der Inkubationen eingetreten sind.

Eine weitere Fragestellung war, ob sich ein Wechsel der Salinität auf die Zusammensetzung und das Abbaupotential der Bakterien auswirkt. Dieses wurde durch Parallelansätze mit gleichem Inokulum und unterschiedlichen Salinitäten des Mediums realisiert.

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2

Composition of humic acid degrading estuarine and marine bacterial communities

Composition of humic acid degrading estuarine and marine bacterial communities

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Running head: humic acid degrading bacteria

Key words: bacteria, DGGE, isolates, humic matter, DOM, flow through culture

Abstract

We carried out experiments to examine the bacterial decomposition of humic acids (HA) by marine and estuarine bacterial communities which served as inocula of flow-through culture systems fed with HA media of salinities of 28 and 14. HA were decomposed to >60% of the initial concentration within 70 days and the foreign salinity yielded the highest decomposition. Denaturing gradient gel-electrophoresis (DGGE) analyses showed that during the decomposition process the composition of the bacterial communities became more and more dissimilar from that of the inoculum and less diverse. A phylogenetic analysis of excised DGGE bands and bacteria isolated on HA as the sole carbon (C) source at the end showed that the communities in the marine flow-through cultures were largely dominated by *Alphaproteobacteria* and *Gammaproteobacteria* whereas the estuarine bacterial communities were dominated by *Gammaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria*. Growth tests of 13 selected isolates showed that 11 were able to grow on HA as the sole C source, 7 on phenol and 3, affiliated to the *Roseobacter* clade, on various aromatic acids. The bacteria retrieved from the various flow through cultures were closely affiliated to organisms capable of degrading humic matter, aromatic and aliphatic compounds and also to other bacteria reported previously from the Wadden Sea and Weser estuary.

Introduction

Humic substances are the most abundant natural organic compounds in aquatic environments. They are usually defined as organic compounds of different solubility at low pH or adsorbed to various types of hydrophobic macroporous resins at low pH and eluted by alkaline solutions and hydrophobic eluants (Thurman & Malcolm, 1981; McKnight & Aiken, 1998). The major compounds isolated by these procedures include hydrophilic acids with the lowest molecular weight, i.e. <600 Da, fulvic acids with a molecular weight of 500 to 1200 Da and humic acids (HA), the high molecular weight fraction. Surprisingly, only scarce information is available on the relative partitioning of each of the three fractions to total humic substances in freshwater as well as marine systems. Kisand *et al*. (2008) reported that HA constitute ~30% of total humic substances in a humic-rich stream and Gebhardt (2005) found that HA constitute 16 to 37% of total humic substances in a drainage channel discharging into the Wadden Sea, southern North Sea. Humic substances in total represent 50-80% of dissolved organic matter (DOM) in freshwaters (Thurman, 1985; McKnight & Aiken, 1998) but only a small fraction (0.7–2.4%) of DOM in the ocean (Opsahl & Benner, 1997). The great discrepancy between high concentrations of humic-rich DOM in freshwaters as compared to the ocean implies that large fractions of humic substances are removed in the coastal zone. In fact, present estimates suggest that terrestrial and thus humic DOM, after mixing with seawater, is degraded rapidly in the coastal zone (Hedges *et al*., 1997; Cauwet, 2002; Mannino & Harvey, 2004).

Flocculation at increasing salinity is one important process of DOM removal during the estuarine passage (Sholkovitz, 1976; Sholkovitz, 1978; Kerner *et al*., 2003) but photochemical and bacterial degradation in estuaries and the coastal zone also contribute to this removal (Moran & Zepp, 1997; Wikner *et al*., 1999; Moran *et al*., 2000; Obernosterer *et al*., 2001; Boyd & Osburn, 2004; Kisand *et al*., 2008). Humic acids appear to be more readily degraded than fulvic acids and hydrophilic acids (Kisand *et al*., 2008). There is very scarce information available, however, which bacteria are capable of and involved in the degradation of humic substances in estuarine and marine systems. It has been shown that the composition of oligohaline bacterial communities changes during the decomposition process of riverine DOM and that *Flavobacteria* are capable of degrading this DOM (Kisand *et al*., 2002; Langeneder *et al*., 2003). In one study marine bacteria were enriched on humic substances, showing that isolates affiliated with *Alpha*- and *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes* were able to grow on this refractory material and on selected aromatic compounds (Esham *et al*., 2000). The growth yield of these isolates, however, was much lower than that

of a natural bacterial community. The fact that some members of the *Roseobacter* clade of *Alphaproteobacteria* are able to degrade lignin and carry genes for cleaving aromatic compounds implies that they are able to decompose humic substances (González *et al*., 1996; Buchan *et al*., 2000; Buchan *et al*., 2001). More recent and detailed information is available from fresh water systems on bacteria degrading humic substances. It has been shown that in particular *Actinobacteria*, specific clusters of *Betaproteobacteria* and the *Sphingomonas* clade of *Alphaproteobacteria* are able and often instrumental in degrading humic substances in lakes (Haukka *et al*., 2005; Hutalle-Schmelzer & Grossart, 2009; Hutalle-Schmelzer *et al*., 2010).

The Wadden Sea, stretching over 500 km from Den Helder in The Netherlands to Skallingen in Denmark, is the largest tidal flat system globally and therefore of prime importance as a bioreactor for the degradation of terrestrial humic matter in the coastal zone of the North Sea. Humic matter is discharged into the Wadden Sea by large rivers such as rivers Rhine and Elbe but also directly by drainage channels (McQuatters-Gollop *et al*., 2007; Lübben *et al*., 2009). Several studies have been carried out to assess the composition of bacterial communities in this tidal flat system, in particular in its East Frisian section of the German Wadden Sea including the Weser estuary. These studies, applying culture-dependent and culture-independent approaches, have shown that the bacterial communities include phylotypes and isolates of the *Roseobacter* and *Sphingomonas* clades of *Alphaproteobacteria*, of *Flavobacteria* and *Sphingobacteria*, and quite a few members of *Actinobacteria* (Selje & Simon, 2003; Selje *et al*., 2005; Stevens *et al*., 2005; Stevens *et al*., 2007). As the input of chromophoric, i.e. humic-rich, (C)DOM from riverine and terrestrial run off is substantial and a pronounced gradient from the coast to the open North Sea exists (Lübben *et al*. 2009), we hypothesize that the bacterial communities in the Wadden Sea and adjacent estuarine waters are capable of decomposing and important in the removal of humic substances.

To test this hypothesis, flow-through systems were set up to examine the decomposition of HA, the high molecular weight fraction of humic substances, by estuarine and marine bacterial communities and to assess the composition of the bacterial communities during the decomposition process. In addition, bacteria from the late stage of the experiments were isolated on HA as the sole carbon source and examined for their properties to degrade aromatic compounds.

Material and methods

Approach

Flow-through systems were used to assess the decomposition of HA by naturally-derived bacterial communities and to examine the community composition during the decomposition process. In one set of experiments a marine bacterial community was used as inoculum and the decomposition of HA was examined at salinities of 28 and 14. The inoculum was collected on the day of the start of the experiment in June 2007 from the polyhaline (salinity 30) German Wadden Sea at Neuharlingersiel (53° 43' 67" N, 07° 42' 73" E). In a second set of experiments, started in May 2008, we examined the decomposition of the same HA medium at the same salinities but inoculated with an oligohaline (salinity 10) bacterial community. The inoculum was collected near Kleinensiel (Weser estuary, Northwest Germany; 53° 26' 37'' N, 8° 29' 06'' E). The HA were extracted from water collected in a freshwater stream in a peat bog area rich in humic substances near Oldenburg (see below). In each set of experiments three different treatments were tested, one with the salinity close to that of the inoculum, either 14 or 28, one with the same salinity but amended with combusted sediment particles, and one with an enhanced (28) or reduced (14) salinity relative to that of the inoculum. The sediment particles were added to examine whether flocculation and adsorption of HA to these particles enhances abiotic removal of HA or leads to enhanced decomposition of HA by particle-associated bacteria.

Set up of the flow-through systems

Duplicate 10 L glass bottles were filled with 10 L of artificial sea water (ASW) according to Kisand *et al*. (2008) and modified by adding 34.5 µM KNO_3 and $NaNO_3$ and 3.5 µM K_2HPO_4 and KH_2PO_4, respectively. Artificial seawater media of salinities 28 and 14 were prepared from stock solutions of a 10x higher concentration by dilution with ultra-clean water (Sartorius, Göttingen, Germany) and supplemented with HA as described below. Wadden Sea sediment combusted for 2 h at 500 °C was added to one set of duplicates at a concentration of 30 mg L^{-1}. All media were supplemented with HA at a final concentration of 6 mg HA L^{-1}. Media were prefiltered through 0.22 µm capsule filters (Mini capsule filters, HT Tuffryn, Pall Life Sciences, Dreieich, Germany). Peristaltic pumps (8-channel Ismatec MC-MS/CA8) were used to pump the medium into and out of the glass bottles at a dilution rate of 0.01 d^{-1}. The bottles were continuously aerated using room air, filtered through 0.22 µm filters. All glassware were combusted for 2 hours at 500 °C and plastic ware, silicon tubings and glass

syringes were rinsed with 0.1 N HCl. Two hundred fifty mL of the inocula, prefiltered through 5.0 µm polycarbonate filters (47 mm), were added to the flow through systems which were run at room temperature for 71 (marine) and 74 (oligohaline) days, respectively. For the first 3 days they were run in batch mode to avoid washing out of the bacteria. Subsamples for measurements of dissolved organic carbon (DOC) and denaturing gradient gel electrophoresis (DGGE) analyses were withdrawn periodically from the outflow. In order to examine possible abiotic removal of DOC by flocculation two sterile controls of a salinity of 28 were run in parallel to the inoculated experiments in 2007, one for 56 and one for 84 days. These controls were run exactly as the other experimental bottles but without a bacterial inoculum.

Extraction of humic acids

Sixty L of water rich in HS were collected in 20-l PE bottles from Wildenloh (peat bog area near Oldenburg, Northwest Germany) in June 2007 and May 2008 for the experiments inoculated with the marine and estuarine bacterial community, respectively, and pre-filtered through a 0.1 µm cartridge using tangential flow ultrafiltration (Amicon-Millipore, Schwalbach, Germany) and acidified to pH 2.0. The high-molecular-weight (>1000 Da) material of the DOM was isolated by column chromatography of 10-30 L of filtered samples using XAD-8 resins (Sigma-Aldrich, trademark name DAX-8) (Thurman & Malcolm, 1981; Malcolm & MacCarthy, 1992). Humic substances were eluted with 0.1 N NaOH. The HA fraction was obtained by precipitation at pH 1 (overnight at 4°C), centrifuged at 4,000 g and the precipitate redissolved in ultra-clean water. HA were re-concentrated onto the XAD-8 resin and desalted by elution with 0.1 N NaOH. Sodium ions were removed by cation exchange chromatography (Amberlite IR-120(H)), vacuum concentrated, and the concentrate freeze-dried.

DOM analysis

After subsampling an aliquot of 10 mL was immediately filtered through a 0.2 µm polyethersulfon filter (Minisart high flow, Sartorius, Goettingen, Germany) mounted on an acid-rinsed glass syringe. The filtrate was acidified with 40 µL 6N HCl and stored at 4 °C in the dark until analysis. DOC was determined in five replicate analyses of one sample vial by high-temperature combustion with a TOC-V CSH analyzer (Shimadzu) and by using potassium hydrogen phthalate as a standard. The standard deviation of the five replicates was always <5%.

Bacterial abundance

Ten mL subsamples were fixed with glutardialdehyde (Sigma, final concentration 1%) for 45 min. Thereafter, the samples were stored at -80°C until further processing. Enumeration of bacteria was performed with a BD FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Two hundred µL of sample were mixed with 5 µL of SYBR Green I Solution (Molecular Probes, Leiden, The Netherlands, diluted 1:1000 with dimethylsulfoxide, Roth, Karlsruhe, Germany) and 5 µL of Flouresbrite plain microsphere beads (diameter 1 µm, Polysciences, Eppelheim, Germany) solution. The Flouresbrite plain beads were calibrated against BD TruCOUNT™ beads (diameter 1 µm, Becton Dickinson Biosciences, Heidelberg, Germany) every day prior to analysis. Analysis time was usually 1 min and a minimum of 1,000 cells was counted. The side scatter (SSC) gain was adjusted at 390 and the fluorescence 1 (FL1) at 319. The maximum aberration of analysis was 5% (aberration of Flouresbrite plain microsphere beads). Data were processed and bacterial numbers calculated by WinMDI 2.9 (Windows Multiple Document Interface for Flow Cytometry, http://facs.scripps.edu/software.html).

Isolation of humic acid degrading bacteria

On the last day of the incubation subsamples were withdrawn to isolate bacteria. Therefore, dilution series of 10^0 to 10^4 with inocula of each treatment were prepared in combusted 15 mL test tubes. One hundred µL of each dilution step (10^0 to 10^4) of each treatment were spread onto agar plates with HA as the sole C source (6 mg HA L^{-1} in ASW and 15 g agar L^{-1}) and incubated at 20 °C in the dark for 4-6 weeks. Single colonies from the plates inoculated with 10^2 and 10^3 dilution steps were transferred to new HA plates and further incubated. All growing colonies on these plates were singularized at least twice before the transfer to agar plates with complex media (100% Marine Broth 2216, Difco (MB), BD Sciences, Heidelberg, Germany). After incubation of one week cells were harvested in 50 µL of PCR-grade water. All strains were first classified by morphological features and in a second step representative strains were identified by sequencing of excised bands of PCR-amplified 16S rRNA gene fragments separated by DGGE (Teske *et al*., 2000).

Growth tests on substrates:

Strains were tested for growth in i) 10% MB, diluted with autoclaved seawater from Neuharlingersiel, Wadden Sea, and ii) artificial sea water medium (ASWM, containing in 1 L aqua dest 20 g NaCl, 4 g Na_2SO_4, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 3 g $MgCl_2$ x 6 H_2O, 0.5 g

KCl, 0,19g $NaHCO_3$, 0.15 g $CaCl_2$ x 2 H_2O and 1 mL of an autoclaved trace element solution containing in 1 L 5.2 g Na_2–EDTA, 2.1 g $FeSO_4$ x 7 H_2O, 30 mg H_3BO_3, 0.1 g $MnCl_2$ x 4 H_2O, 0.19 g $CoCl_2$ x 6 H_2O, 24 mg $NiCl_2$ x 6 H_2O, 2 mg $CuCl_2$ x 2 H_2O, 0.144 g $ZnSO_4$ x 7 H_2O, 36 mg $NaMoO_4$ x 2 H_2O, was added after autoclaving the ASWM). Both media were autoclaved for 20 min at 121 °C. The ASWM was supplemented with glucose, HA, phenol or aromatic acids (benzoic acid, vanillic acid, salicylic acid, ferulic acid, p-coumaric acid, cinnamic acid) to test for growth on these compounds as the single C source at concentrations of 0.1% (w/v) except for phenol and benzoic acid which were added at 0.05% concentration (Esham *at al*., 2000). For some strains the media had to be supplemented with 2 mL per L ASMW of a 5-fold concentrated vitamin solution containing (per 1 L aqua dest): 10 mg biotin, 25 mg nicotine-acid, 25 mg thiamine-dichloride, 25 mg p-aminobenzoic-acid, 25 mg Ca-D(+)-pantothenate, 50 mg pyridoxamin-dihydrochloride, 5 mg cyanocobalamine (vitamin B12), 10 mg folic acid, 25 mg riboflavine, 25 mg lipoic-acid, sterile filtered (Balch *et al*., 1979). After addition of the substrates and vitamins the media were sterilized by 0.2 µm filtration. Growth experiments were performed in 10 mL test tubes filled with 5 mL of medium and 50 µL of preculture as inoculum. Test tubes were incubated at room temperature in the dark for 22-28 days and growth was monitored visually and photometrically at 600 nm (OD_{600}). Positive growth was defined as $OD_{600} \geq 125\%$ of the initial value.

Isolation of DNA

Eighty mL of the subsamples from the flow through cultures were filtered onto 0.2 µm polycarbonate membranes (47 mm diameter, Whatman, Dassel, Germany). The filters were extracted as described (Zhou *et al*., 1996). The harvested cells of the isolates were treated with “freeze and thaw” or extracted like the cells of the samples of the flow through culture experiments (see above).

PCR und DGGE

PCR and DGGE were performed according to the protocol described by Brinkhoff & Muyzer (1997). DGGE was performed with the INGENY U 2x2 system (INGENY, Leiden, The Netherlands). The gradient of urea and formamide for the DGGE was 40-70%. After a running time of 20 h (100 V) the gels were stained with SybrGold© (Invitrogen) for 1 h and documented using a BioDoc Analyze Transilluminator (Biometra, Göttingen, Germany). Prominent bands of the DGGE gels with samples from the last day of the flow through culture experiment were excised with a sterile scalpel, transferred into 50 µL of PCR grade water and

stored at –20 °C. The DNA was reamplified with primer set 341F and 907RM prior to sequencing.

Sequencing and cloning of DNA fragments

The 16S rRNA gene amplicons were purified with the Gold DNA purification kit (Qiagen©). Few purified PCR-products of the DGGE bands of the 16S rRNA gene fragments could be sequenced directly using sequencing primer GM8R and 907RM (Muyzer *et al.*, 1993). Most bands, however, were overlaid by two or more sequences. The 16S rRNA gene fragments of these bands were cloned using the pGEM T-Vector System II (Promega, Madison, USA) following the instruction manual. Clones were picked by blue-white selection and diluted in 50 µL PCR-grade water. PCR-amplification of the clones was done with primer set pUC/M13f – pUC/M13r (Messing, 1983). Sequencing of 16S rRNA genes of most isolates was done with primer 907RM and of clones with pUC/M13f. Several isolates (HA-est-Is39-14, HA-est-Is113b-14, HA-est-Is122-14, HA-est-Is132b1-14, HA-est-Is185a-28) were sequenced with primer 518F (Muyzer *et al*., 1993) and 907F. The purified 16S rRNA gene amplicons of DGGE bands and most isolates were sequenced by GATC-Biotech AG (Konstanz, Germany). A few amplicons were sequenced with a model 4200 DNA sequencer (LI-COR, Lincoln, USA) as described (Selje & Simon, 2003). Sequences from DGGE bands were checked for chimera formation with Pintail (http://www.bioinformatics-toolkit.org/Pintail/). Sequences were compared with those in GenBank using BLAST of the National Center for Biotechnology Information (NCBI) server (www.ncbi.nlm.nih.gov). For all gene comparisons at least 400 bp for DGGE bands and 700 bp for most isolates were used (HA-mar-Is13-28, HA-mar-Is16-14+28 >500bp).

Statistics and phylogenetic analysis:

Cluster analysis of the DGGE profiles was performed with the program *Gel Compar II* Version 2.5 (Applied maths, Kortrijk, Belgium). Calculations were curve-based, using Pearson correlation and UPGMA.

Phylogenetic trees of DGGE-bands/clones and isolates were constructed using the ARB software package (Ludwig *et al*. 2004, see: www.arb-home.de). The backbone trees were calculated using bootstrap calculation of sequences >1300 bp. Shorter sequences were afterwards added to the backbone tree. No filter was used. The outgroup consists of 16SrRNA sequences of 4 type strains belonging to the *Cyanobacteria* (*Lyngbya* PCC 7419, AJ000714, *Oscillatoria sancta* PCC 7515, AF132933, *Microcystis aeruginosa* PCC 7820, U40339,

Synechococcus sp. PCC 7003, AB015059) Published sequences from the habitats where the inocula for the experiments were collected (Wadden Sea, Weser estuary) and type strains of the respective lineages were used as reference sequences.

Nucleotide sequence accession number:

Sequences are deposited at GenBank under Accession number HM368261 to HM368353

Results

In all flow-through cultures, bacterial abundance increased strongly during the first week and reached highest numbers of $1.2x10^7$ and $2.0x10^7$ mL^{-1} in the flow-through cultures with the marine and estuarine inoculum, respectively. The high numbers persisted for about 8 days, declined thereafter and remained between <1.0 and $2.0x10^6$ mL^{-1}. At least 90% of the bacteria were free-living cells as checked microscopically. Even in the treatments with added sediment particles <10% of the bacteria were particle-associated. There was no systematic difference between the numbers and temporal dynamics of the bacteria in the flow through cultures with the marine (salinity 28) and the estuarine (salinity 14) inoculum and among the various treatments (data not shown). In none of the flow through cultures did visible bacterial growth on the walls of the glass bottles occur. For further details see Rocker *et al.* (2011).

In the experiments with the marine inoculum, 43% (mean of duplicates) of the added HA were decomposed in the treatment with the marine medium and 59.5% in that with the added sediment particles (Table 1). A higher fraction, 65% of the added HA, was decomposed in the treatment with the estuarine medium. In the experiments with the estuarine inoculum, the highest HA decomposition, 70.5% (mean of duplicates) was recorded in the treatment with the marine HA medium, whereas the treatment with the estuarine medium yielded a lower decomposition (56.5%). As in the other experiment, the addition of sediment particles enhanced the HA removal. Most of the HA decrease occurred in the first 3-4 weeks. In the two sterile control experiments with the marine HA medium and without a bacterial inoculum, DOC concentrations decreased by <9% during the incubation periods of 56 and 84 days, respectively. For a more detailed analysis of the HA decomposition in the various treatments see Rocker *et al*. (2011)

Analysis of the bacterial communities by DGGE

DGGE analyses showed a clear succession of the bacterial communities during the course of the experiments. As substantiated by a cluster analyses, the DGGE patterns in the early phase were rather similar to the inoculum and became more and more dissimilar and composed of fewer bands towards the later phases (Fig. 1). The highest number of bands was present from days 6 to 25. Such recurrent patterns were observed in both experiments and all treatments (data not shown). From the experiments with the marine and estuarine inocula 25 and 23 representative bands, respectively, of various treatments, persisting during the last weeks, were excised from the DGGE gel of the last sampling, in most cases cloned, reamplified and sequenced (Table 2).

Eighteen of the 47 16S rRNA gene fragments retrieved from the flow-through cultures are affiliated to *Alphaproteobacteria*, 12 exclusively from treatments with the marine inoculum, 4 exclusively from those with the estuarine inoculum and 2 from both, a treatment with a marine and one with an estuarine inoculum (Tables 2, S1). Several phylotypes were retrieved from more than one treatment. The second largest phylogenetic lineage of the sequenced bands were *Gammaproteobacteria* with 12 different phylotypes. Four phylotypes were retrieved exclusively from the flow-through cultures with the marine inoculum, 7

Table 1. Fractions of humic acids (HA) decomposed in flow through culture experiments inoculated by a marine (salinity 30) and estuarine/oligohaline (salinity 10) bacterial community. The flow through cultures of both experiments were fed by HA media of 3 treatments in duplicates: salinities of 14 and 28 and addition of combusted sediment particles in the salinity close to that of the inoculum.

Marine inoculum				Estuarine/oligohaline inoculum			
Exp ID	Salinity	Sediment	HA decomposed (%) after 62 days	Exp ID	Salinity	Sediment	HA decomposed (%) after 62 days
Mar 1	28	-	48	Est 1	14	-	54
Mar 2	28	-	38	Est 2	14	-	59
Mar 3	28	+	46	Est 3	14	+	63
Mar 4	28	+	73	Est 4	14	+	72
Mar 5	14	-	75	Est 5	28	-	65 *
Mar 6	14	-	55	Est 6	28	-	76

* run for 64 days

exclusively from those with the estuarine inoculum and one phylotype from a treatment with a marine as well as an estuarine inoculum. In addition, 2 phylotypes are affiliated to *Actinobacteria* and to *Sphingobacteria* (*Bacteroidetes*) and one to *Betaproteobacteria* and *Planktomycetales* each. The betaproteobacterial phylotype was retrieved from a flow through culture with the estuarine inoculum and fed with the estuarine HA medium supplemented with suspended sediment particles. Both actinobacterial phylotypes were also retrieved from a treatment with an estuarine inoculum and medium (Tables 2, S1). Each of the two sphingobacterial phylotypes originated from a flow-through culture with a different inoculum but incubated at the same salinity and with suspended sediment particles. The phylotype affiliated with the *Planktomycetales* and rather distantly with its deeply branching novel genus *Phycisphaera* (Fukunaga *et al*., 2009), originated from a flow-through culture with the estuarine inoculum but fed with the marine HA medium.

Fourteen of the alphaproteobacterial 16S rRNA phylotypes are affiliated to the order of *Rhodobacterales*, including 11 of the *Roseobacter* clade, 5 to the order of *Sphingomonadales* and the others to various further lineages within this proteobacterial subclass (Fig. 2a). There was no systematic phylogenetic difference among the phylotypes of the flow-through cultures with a marine or estuarine inoculum and/or the treatments with the marine and estuarine HA media. The gammaproteobacterial phylotypes were equally

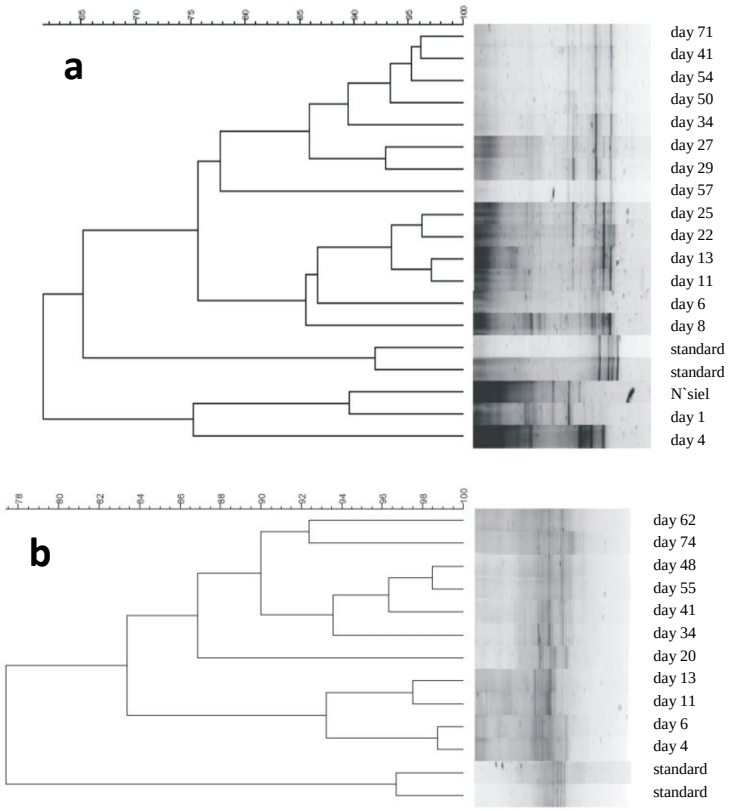


Figure 1: Cluster analysis and DGGE banding patterns of the bacterial communities during the course of a flow through culture inoculated with (a) a marine bacterial community and fed with the estuarine HA medium (flow through culture Mar 5, Table 1) and (b) with an estuarine inoculum and fed with the estuarine HA medium (flow through culture Est 1, Table 1). The standard consists of 16S rRNA gene fragments of the following bacteria: *Bacillus amyloliquefaciens*, *Ruegeria algicola*, *Roseovarius tolerans*, *Phaeobacter gallaeciensis* BS107.

distributed among this subclass (Fig. 2b). However, 5 of the 7 phylotypes retrieved from the flow-through cultures fed with the marine HA medium in both experiments are affiliated to the *Alcanivorax* lineage.

Analysis of isolated strains

In the experiment with the marine inoculum 133 organisms were isolated on HA as the sole carbon source. Strains with a sequence similarity of the 16S rRNA gene fragment (~700 bp) of >97% to the same most closely related validated species were classified into an isolate group. Thus, a total of 19 single isolates or isolate groups were obtained (Tables 2, S2). The great majority of all isolates (74%) and 11 isolate groups (58%) affiliated with *Alphaproteobacteria*. Almost equal percentages were isolated from flow-through cultures fed with the marine and oligohaline HA medium. Seventeen and 15 isolates are affiliated to *Firmicutes* and *Gamma-proteobacteria*, respectively. The isolates of *Firmicutes* fell into 2 isolate groups and those of *Gammaproteobacteria* into 5 groups. Two isolates constituting one isolate group are affiliated to *Actinobacteria*.

The experiment with the estuarine inoculum yielded 139 isolates constituting 26 distinct isolate groups (Tables 2, S2). In contrast to the experiment with the marine inoculum *Alphaproteobacteria* constituted only 27 isolates forming 6 isolate

Table 2: Number and percentages of DGGE phylotypes, isolate and isolate groups of the various phylogenetic lineages retrieved from flow through cultures with a marine or estuarine inoculum or from both treatments (estuarine/marine). Percentages are based on the total number of phylotypes, isolates or isolate groups retrieved from both types of flow through culture.

DGGE Bands Phylogenetic class	Marine		Estuarine		Estuarine/ Marine
	Number	%	Number	%	Number
Alphaproteobacteria	12	71	4	25	2
Betaproteobacteria	0	0	1	6	0
Gammaproteobacteria	4	24	7	44	1
Sphingobacteria	1	6	1	6	0
Actinobacteria	0	0	2	12	0
Planktomycetales	0	0	1	6	0

Isolates Phylogenetic class	Marine		Estuarine		Estuarine/ Marine
	Number	%	Number	%	Number
Alphaproteobacteria	98	76	26	19	2
Betaproteobacteria	0	0	1	1	0
Gammaproteobacteria	11	9	62	46	6
Actinobacteria	2	2	47	35	0
Firmicutes	17	13	0	0	0

Isolate groups Phylogenetic class	Marine		Estuarine		Estuarine/ Marine
	Number	%	Number	%	Number
Alphaproteobacteria	11	58	6	23	0
Betaproteobacteria	0	0	1	4	0
Gammaproteobacteria	5	26	7	27	0
Actinobacteria	1	5	12	46	0
Firmicutes	2	11	0	0	0

groups of which 2 matched with groups of the experiment with a marine inoculum (Table S2). The highest fraction of isolates (46%) is affiliated to *Gammaproteobacteria*, constituting 7 isolate groups (27%) and including those of the flow-through cultures with the marine inoculum. *Actinobacteria* constituted 35% of the isolates but 46% of the isolate groups (12 groups) and the great majority of isolates and isolate groups were obtained from the estuarine treatments. In addition, one betaproteobacterial isolate was obtained on the marine medium.

In the estuarine flow-through cultures with estuarine inoculum fungal isolates of the yeast genus *Sporobolomyces* (*Basidiomycota*) were retrieved. No fungi were obtained from flow-through cultures with a marine inoculum or medium.

Six of the 14 isolate groups of *Alphaproteobacteria* affiliated to the *Roseobacter* clade, five to *Sphingomonadales*, 2 to *Rhodobacteraceae* and one to *Rhodospirillaceae* (Fig. 2a, Table S2). Four of the isolate groups of the *Roseobacter* clade and 2 of *Sphingomonadales* were obtained from the treatments with the marine inoculum and 2 of each lineage from treatments with the estuarine inoculum. In three cases isolate groups affiliated closely to phylotypes retrieved by the DGGE analysis, a *Sulfitobacter* isolate group (AL-3; HA-mar-Is3-14+28, HA-mar-Is16-14+28) with phylotypes HA-mar-B8-28 and HA-mar-B16-28, a *Sphingopyxis* isolate group (AL-8; HA-mar-Is19-14), with phylotype HA-mar-B6-28, and a *Thalassospira* isolate (HA-mar-Is2-14+28) with phylotypes HA-mar-B24-28 and HA-mar-B34-28 (Fig. 2a).

The isolate groups of *Gammaproteobacteria* cover a rather large diversity including the *Pseudomonadales* with 4 groups and the *Alcanivorax* and *Alteromonas* lineages with one isolate group, each (Fig. 2b, Table S2). These groups were closely related to 16S rRNA phylotypes retrieved with the DGGE analysis such as phylotypes HA-est-B74-28 and HA-mar-B60-14 with isolates HA-mar-Is9-28, HA-mar-Is22-14, HA-est-Is39-14, phylotype HA-mar-B10-28 with isolate HA-mar-Is8-14+28 and phylotype HA-est-B17-14 with isolates HA-mar-Is6-28, HA-mar-Is10-28 and HA-est-Is41-14.

The actinobacterial isolate groups covered a broad variety of this phylogenetic class (Fig. 2c, Table S2). Four isolates including that obtained from the flow-through cultures with the marine inoculum are affiliated to *Micrococcaceae* and the others to various lineages such as *Nocardiaceae* (3 isolates) and *Microbacteriaceae* (3 isolates). The isolates did not closely match phylotypes retrieved by the DGGE analysis.

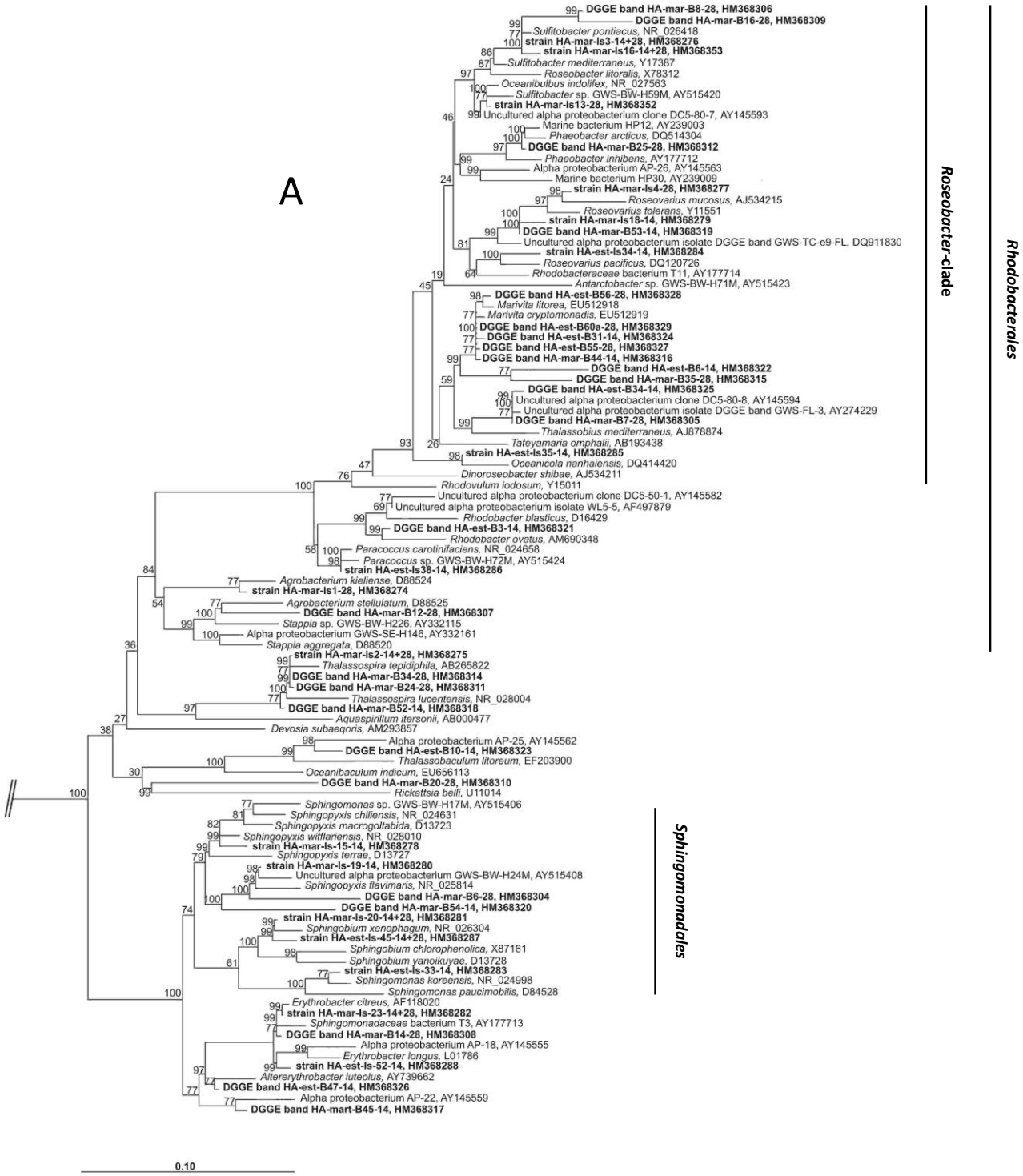
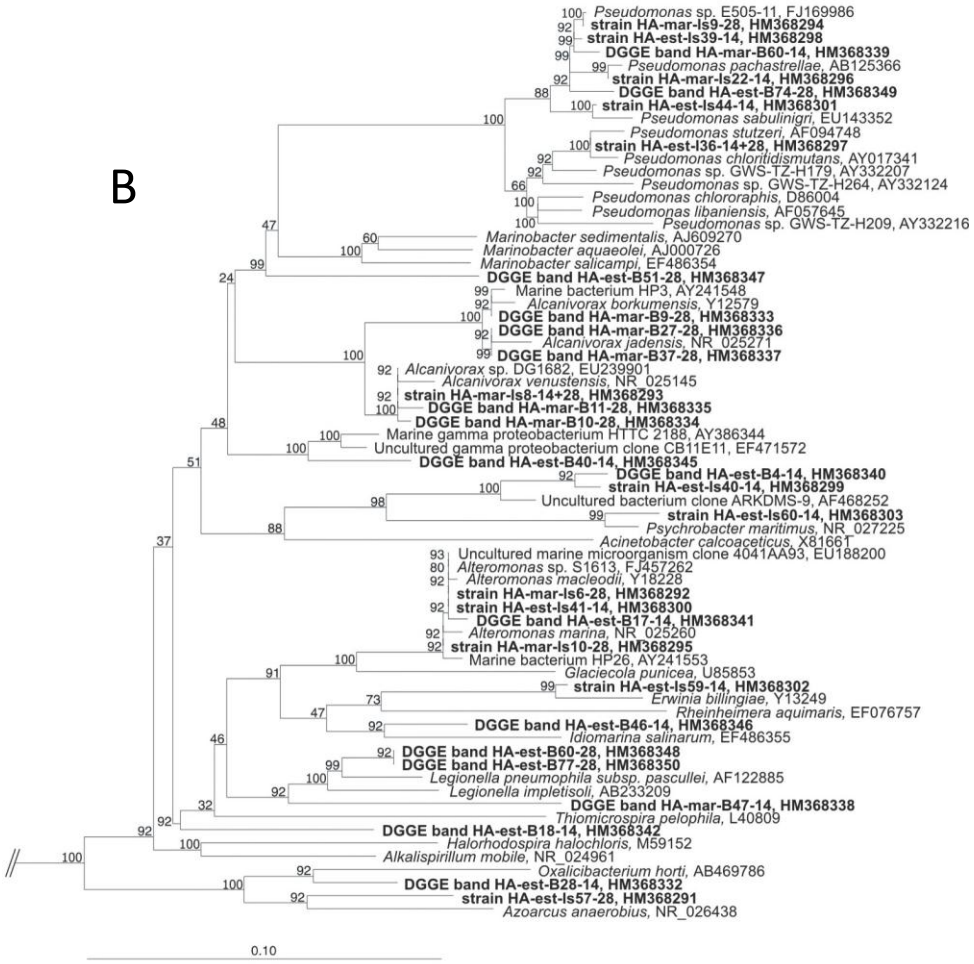
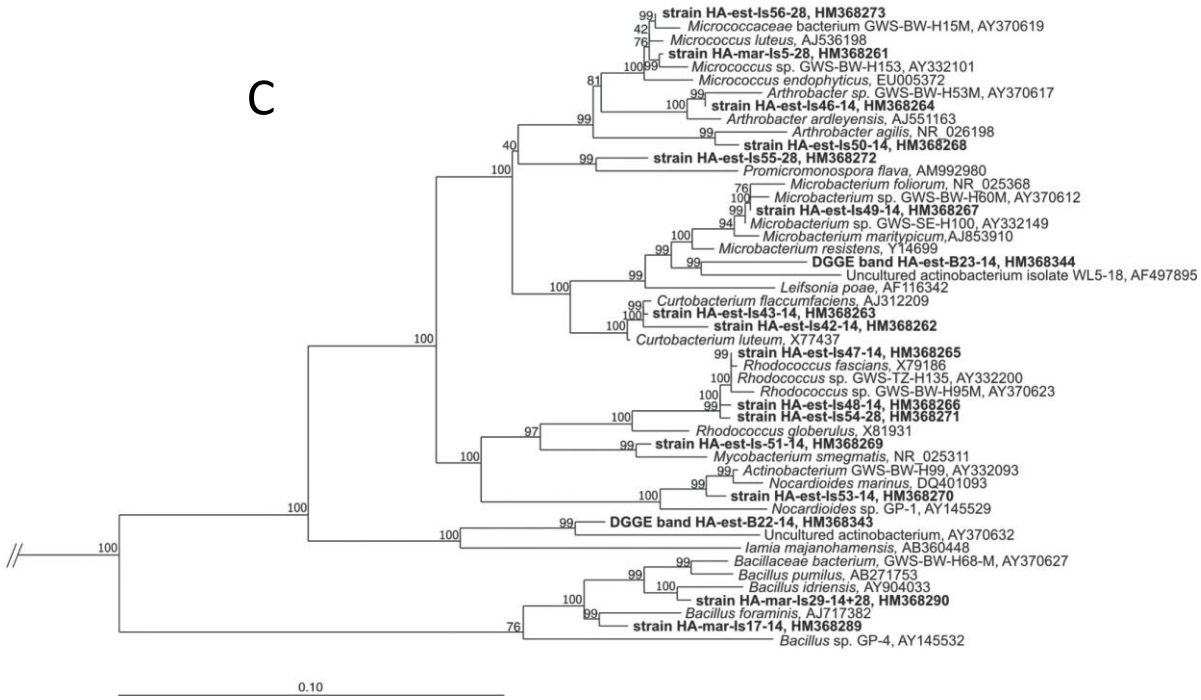


Figure 2: Phylogenetic tree of *Alphaproteobacteria* (A), *Gammaproteobacteria* (B) and *Actinobacteria, Sphingobacteria and* Planktomycetales (C) calculated with Maximum-Likelihood based on 16S rRNA gene fragments of DGGE bands and isolates retrieved from flow through cultures fed with a humic acid (HA) medium of marine or estuarine salinity. Published sequences from the habitats where the inocula for the experiments were collected (Wadden Sea, Weser estuary) and type strains of the respective lineages were used as reference sequences. ID of the sequences and isolates of this study is as follows. DGGE bands (band, B) and isolated (strain) retrieved from a culture with estuarine (est) or marine (mar) inoculum and fed with estuarine (14) or marine (28) medium. When 14+28 is indicated sequences were obtained from cultures fed with estuarine and marine medium. Numbers at nodes are bootstrap values derived from 1000 replicates.


B
Pseudomonas sp. E505-11, FJ169986
strain HA-mar-ls9-28, HM368294
strain HA-est-ls39-14, HM368298
DGGE band HA-mar-B60-14, HM368339
Pseudomonas pachastrellae, AB125366
strain HA-mar-ls22-14, HM368296
DGGE band HA-est-B74-28, HM368349
strain HA-est-ls44-14, HM368301
Pseudomonas sabulinigri, EU143352
Pseudomonas stutzeri, AF094748
strain HA-est-l36-14+28, HM368297
Pseudomonas chlorritidismutans, AY017341
Pseudomonas sp. GWS-TZ-H179, AY332207
Pseudomonas sp. GWS-TZ-H264, AY332124
Pseudomonas chlororaphis, D86004
Pseudomonas libaniensis, AF057645
Pseudomonas sp. GWS-TZ-H209, AY332216
Marinobacter sedimentalis, AJ609270
Marinobacter aquaeolei, AJ000726
Marinobacter salicampi, EF486354
DGGE band HA-est-B51-28, HM368347
Marine bacterium HP3, AY241548
Alcanivorax borkumensis, Y12579
DGGE band HA-mar-B9-28, HM368333
DGGE band HA-mar-B27-28, HM368336
Alcanivorax jadensis, NR_025271
DGGE band HA-mar-B37-28, HM368337
Alcanivorax sp. DG1682, EU239901
Alcanivorax venustensis, NR_025145
strain HA-mar-ls8-14+28, HM368293
DGGE band HA-mar-B11-28, HM368335
DGGE band HA-mar-B10-28, HM368334
Marine gamma proteobacterium HTTC 2188, AY386344
Uncultured gamma proteobacterium clone CB11E11, EF471572
DGGE band HA-est-B40-14, HM368345
DGGE band HA-est-B4-14, HM368340
strain HA-est-ls40-14, HM368299
Uncultured bacterium clone ARKDMS-9, AF468252
strain HA-est-ls60-14, HM368303
Psychrobacter maritimus, NR_027225
Acinetobacter calcoaceticus, X81661
Uncultured marine microorganism clone 4041AA93, EU188200
Alteromonas sp. S1613, FJ457262
Alteromonas macleodii, Y18228
strain HA-mar-ls6-28, HM368292
strain HA-est-ls41-14, HM368300
DGGE band HA-est-B17-14, HM368341
Alteromonas marina, NR_025260
strain HA-mar-ls10-28, HM368295
Marine bacterium HP26, AY241553
Glaciecola punicea, U85853
strain HA-est-ls59-14, HM368302
Erwinia billingiae, Y13249
Rheinheimera aquimaris, EF076757
DGGE band HA-est-B46-14, HM368346
Idiomarina salinarum, EF486355
DGGE band HA-est-B60-28, HM368348
DGGE band HA-est-B77-28, HM368350
Legionella pneumophila subsp. pascullei, AF122885
Legionella impletisoli, AB233209
DGGE band HA-mar-B47-14, HM368338
Thiomicrospira pelophila, L40809
DGGE band HA-est-B18-14, HM368342
Halorhodospira halochloris, M59152
Alkalispirillum mobile, NR_024961
Oxalicibacterium horti, AB469786
DGGE band HA-est-B28-14, HM368332
strain HA-est-ls57-28, HM368291
Azoarcus anaerobius, NR_026438
0.10


C
strain HA-est-ls56-28, HM368273
Micrococcaceae bacterium GWS-BW-H15M, AY370619
Micrococcus luteus, AJ536198
strain HA-mar-ls5-28, HM368261
Micrococcus sp. GWS-BW-H153, AY332101
Micrococcus endophyticus, EU005372
Arthrobacter sp. GWS-BW-H53M, AY370617
strain HA-est-ls46-14, HM368264
Arthrobacter ardleyensis, AJ551163
Arthrobacter agilis, NR_026198
strain HA-est-ls50-14, HM368268
strain HA-est-ls55-28, HM368272
Promicromonospora flava, AM992980
Microbacterium foliorum, NR_025368
Microbacterium sp. GWS-BW-H60M, AY370612
strain HA-est-ls49-14, HM368267
Microbacterium sp. GWS-SE-H100, AY332149
Microbacterium maritypicum,AJ853910
Microbacterium resistens, Y14699
DGGE band HA-est-B23-14, HM368344
Uncultured actinobacterium isolate WL5-18, AF497895
Leifsonia poae, AF116342
Curtobacterium flaccumfaciens, AJ312209
strain HA-est-ls43-14, HM368263
strain HA-est-ls42-14, HM368262
Curtobacterium luteum, X77437
strain HA-est-ls47-14, HM368265
Rhodococcus fascians, X79186
Rhodococcus sp. GWS-TZ-H135, AY332200
Rhodococcus sp. GWS-BW-H95M, AY370623
strain HA-est-ls48-14, HM368266
strain HA-est-ls54-28, HM368271
Rhodococcus globerulus, X81931
strain HA-est-ls-51-14, HM368269
Mycobacterium smegmatis, NR_025311
Actinobacterium GWS-BW-H99, AY332093
Nocardioides marinus, DQ401093
strain HA-est-ls53-14, HM368270
Nocardioides sp. GP-1, AY145529
DGGE band HA-est-B22-14, HM368343
Uncultured actinobacterium, AY370632
Iamia majanohamensis, AB360448
Bacillaceae bacterium, GWS-BW-H68-M, AY370627
Bacillus pumilus, AB271753
Bacillus idriensis, AY904033
strain HA-mar-ls29-14+28, HM368290
Bacillus foraminis, AJ717382
strain HA-mar-ls17-14, HM368289
Bacillus sp. GP-4, AY145532
0.10

Growth of isolates on humic acids and aromatic compounds

Thirteen isolates of *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* were subjected to growth tests with seawater media supplemented with various carbon sources. As we focused on the decomposition of these carbon sources in seawater media our emphasis was mainly on *Alphaproteobacteria*, the dominant bacterial group retrieved from the marine isolates. All isolates were able to grow on the complex MB medium and 11 of 13 isolates on glucose and HA as the sole carbon source (Table 3). Four alphaproteobacterial isolates needed vitamins for growth on the sole carbon sources. Benzoic acid did not support growth of any of the isolates. Phenol as the sole carbon source supported growth of 4 of the 10 alphaproteobacterial isolates and of the 2 gammaproteobacterial and actinobacterial isolates,

Table 3: Growth of various organisms isolated from the flow through cultures on Marine Broth (MB 10%), glucose (1 g L^{-1}), humic acids (HA, 50 mg L^{-1}) and phenol (0.5 g L^{-1}) as the sole carbon source in artificial seawater medium. * supplementation with vitamins.

Isolate ID	Phylogenetic affiliation	MB	Glucose	HA	Phenol	Benzoic acid	Vanillic acid	Salicylic acid	Ferullic acid	Coumaric acid	Cinnamic acid
HA-mar-Is3-14+28	*Alphaproteobacteria* (*Sulfitobacter*)	+	+	+	-	-	+	+	+	+	+
HA-mar-Is13-28	*Alphaproteobacteria* (*Oceanibulbus*)	+	+	+	-	-	+	+	+	-	+
HA-mar-Is4-28	*Alphaproteobacteria* (*Roseovarius*)	+	-	+	+	-	+	+	+	+	+
HA-est-Is34-14	*Alphaproteobacteria* (*Roseovarius*)	+	-	-	-	-	-	-	-	-	-
HA-est-Is38-14	*Alphaproteobacteria* (*Paracoccus*)	+	+	-	+	-	-	-	-	-	-
HA-mar-Is1-28*	*Alphaproteobacteria* (*Ahrensia*)	+	+	+	-	-	-	-	-	-	-
HA-mar-Is2-14+28*	*Alphaproteobacteria* (*Thalassospira*)	+	+	+	-	-	-	-	-	-	-
HA-mar-Is15-14*	*Alphaproteobacteria* (*Sphingopyxis*)	+	+	+	+	-	-	-	-	-	-
HA-est-Is45-14+28	*Alphaproteobacteria* (*Sphingobium*)	+	+	+	-	-	-	-	+	-	+
HA-mar-Is23-14+28*	*Alphaproteobacteria* (*Erythrobacter*)	+	+	+	+	-	-	-	-	-	-
HA-mar-Is6-28	*Gammaproteobacteria* (*Alteromonas*)	+	+	+	+	-	-	-	-	-	-
HA-mar-Is10-28	*Gammaproteobacteria* (*Alteromonas*)	+	+	+	+	-	-	-	-	-	-
HA-mar-Is5-28	*Actinobacteria* (*Micrococcaceae*)	+	+	+	+	-	-	-	-	-	-

respectively. Three of the four isolates of the *Roseobacter* clade were able to grow on almost all tested aromatic acids whereas the one affiliated to *Sulfitobacter* grew only on MB (Table 3). All other isolates were not able to grow on any of the aromatic acids tested except the *Sphingobium* isolate which grew on ferullic and cinnamic acid.

Discussion

The flow through culture experiments showed that the marine and the estuarine bacterial communities were able to decompose HA to >60% of the initial concentration within appr. 60 days. Between 43 and 65% of the HA were decomposed by the marine and between 56.5% and 70.5% by the estuarine bacterial community, respectively, and most of the decomposition took place during the initial 3-4 weeks when the bacterial community exhibited its greatest diversity. In both experiments, the foreign salinity enhanced the decomposition. These findings are in line with observations by Boyd & Osborn (2007) who found that the fluorescence properties of CDOM in the estuarine salinity gradient of San Francisco Bay changed most when it was incubated with a bacterial community of a different salinity. Also Langeneder *et al*. (2003) found a positive effect of changing salinities on the growth rate, but not on the growth efficiency, of bacterial communities of riverine and oligohaline origin growing on riverine DOM when exposed to the foreign salinity. It thus appears that estuarine and marine bacterial communities enhance their decomposition of DOM, including HA and CDOM, when exposed to a different salinity, possibly by an increased community growth rate and accompanied with a change in their community composition and physiological properties. Our treatments with the added sediment particles exhibited higher removal rates than those without sediments. As humic substances readily adsorb to particles and soil (Kaiser & Guggenberger, 2000) we assume that the enhanced removal rate is at least partially due to physico-chemical adsorption of HA to the sediment particles and not a true bacterial decomposition. The sediment particles were only little colonized by bacteria and the number of particle-associated bacteria was always <10%, in the treatments with sediment particles as well as in the other treatments. Hence, DOM decomposition was predominantly mediated by free-living bacteria. Abiotic flocculation of DOM and thus transformation into the particulate phase obviously did not contribute to DOM removal, as in the abiotic controls of the experiments with a marine inoculum DOC concentrations decreased by <10% during the incubation period. Further, DOM decomposition by bacteria growing on the wall of the

incubation vessel presumably was insignificant. We never observed visible bacterial growth on the walls of the 10 L glass bottles.

Estuaries and intertidal flat systems are characterised by intense resuspension of sediment particles (Lunau *et al*., 2006; Bartholomä *et al*., 2009) and mixing of water masses rich in CDOM and of different salinities (Chen *et al*., 2007; Lübben *et al*., 2009) and harbouring distinct bacterial communities (Crump *et al*., 1999; Selje & Simon, 2003). Hence we conclude that these systems and shallow seas with highly structured coastal environments with a water residence time of days to weeks are a prime location for bacterial decomposition and adsorptive removal of HA and presumably other humic components of DOM of terrestrial origin. Kisand *et al*. (2008), in fact, have shown that HA of terrestrially-derived DOM are decomposed by marine as well as estuarine bacterial communities of salinities 15 and 5, whereas other humic fractions, fulvic acids and hydrophilic acids, are decomposed only by marine bacterial communities. In addition, photodegradation may further contribute to the removal of CDOM and subsequent bacterial decomposition in estuaries and the coastal zone (Moran *et al*., 2000; Chen *et al*., 2007). Hence, microbial and non-biological processes thus contribute to the strong reduction of CDOM and refractory DOM with terrestrial and aromatic signatures in estuarine and land-ocean gradients (Cauwet 2002).

Most of the HA decomposition in both experiments took place within the initial 3-4 weeks when the highest number of DGGE bands was detected. Thereafter, HA decomposition slowed down simultaneously with a change in the bacterial community composition and reduction in the number of DGGE bands. These notions indicate that the estuarine and marine bacterial communities used to inoculate the flow through cultures harbour bacteria capable of decomposing substantial proportions of the HA fraction of the terrestrially-derived DOM. As the inocula were 5 µm prefiltered natural samples they presumably did not only contain bacteria but also nanoflagellates. The strong decline of the bacterial numbers after the first peak in all treatments is an indication that grazing occurred during this time and presumably also later, even though fluctuations in bacterial numbers were less pronounced after 2-3 weeks. The molecular composition of the terrestrially-derived HA DOM fraction is very complex and includes diverse aromatic and aliphatic organic compounds. The analysis of the initial HA media and the outflow of the flow-through cultures at the end of the experiments by pyrolysis GC-MS, in fact, showed pronounced differences, mainly a reduction of aliphatic and partially also of aromatic compounds at the end and thus a loss of complexity (Rocker *et al*., 2011). The reduced number of DGGE bands in the course of the flow-through culture experiments thus appears to be a response to the reduced concentration and complexity of the

HA, but presumably also to grazing, at least in the early phase of the experiments, and reflects the adaptation of the bacterial communities to this stale HA-derived DOM in the course of the ~70 day experiments. At the end of the experiments, though, quite a few different bacteria were still present in the various treatments as shown by the phylogenetic analysis of the 16S rRNA gene fragments of the excised DGGE bands and of the isolated strains.

The flow-through cultures were run with a dilution rate of 0.01 d^{-1} which means that after 70 days only 70% of the water in the culture vessels were exchanged and that bacteria of the inoculum could have still been present. The reduced number of DGGE bands thus must not have been a result of washing out of very slowly or non-growing bacteria towards the end of the experiment but possibly also a consequence of the fact that the abundance of several bacteria went below the detection limit of the DGGE method for individual taxa which is 1% of total extractable DNA (Muyzer *et al.*, 1993). The pronounced changes in the bacterial communities in both experiments and the various treatments, however, underscore that the bacterial communities did respond to the feeding with the HA media and possibly to grazing by nanoflagellates.

Despite rather similar HA decomposition patterns in the various marine and estuarine treatments the composition of the bacterial communities derived from the marine and estuarine inocula was quite different at the end of the decomposition experiments. This was evident from the phylogenetic analysis of the phylotypes retrieved from the DGGE bands and isolates. *Alphaproteobacteria* were the most diverse phylogenetic lineage in the flow-through cultures with the marine inoculum and other lineages constituted much lower fractions, as shown by the sequenced 16S rRNA gene fragments and isolates. In contrast, in the flow-through cultures with the estuarine inoculum, a much greater diversity was present, and data of the DGGE analysis and isolates yielded different results. Whereas in the DGGE analysis, *Gammaproteobacteria* clearly dominated, followed by *Alphaproteobacteria* and *Actinobacteria*, the latter lineage constituted almost half of the isolate groups, and *Alphaproteobacteria* and *Gammaproteobacteria* accounted only for roughly 25%. Interestingly, phylotypes affiliated to *Betaproteobacteria*, *Actinobacteria* and *Planktomycetales* and isolates affiliated to *Betaproteobacteria* were only retrieved from the flow-through cultures with the estuarine inoculum.

As these bacteria, detected by DGGE or isolated on HA, persisted until the termination of the experiments we conclude that most of them were involved in decomposing HA, even though presumably to different degrees. Several of these organisms, however, may have also consumed the remains of lysed bacteria or persisted just as starving and inactive cells.

Because HA consist to >21% of aromatic compounds (Hopkinson *et al*., 1998; Gebhardt, 2005) we assume that the ability of the bacteria detected to degrade these compounds is a strong indication that they in fact degraded the HA, at least partly. The growth tests of the 13 isolates showed that 11 were able to grow on HA, 7 also on phenol whereas none grew on benzoic acid. However, 3 isolates affiliated to the *Roseobacter* clade and one closely to *Sphingobium koreensis*, were able to grow on other aromatic acids, i.e. lignin building blocks, as the sole carbon source, thus indicating the potential of these isolates to decompose various aromatic components of HA. In fact, some of the organisms we isolated or detected as phylotypes are closely related to marine bacteria which have been shown to be able to decompose humic substances, lignin-related aromatic compounds and/or polycyclic aromatic hydrocarbons (PAH). The presence of the ring-cleaving dioxygenase gene *pcaH*, a key enzyme in the β-ketoadipate pathway to break down lignin-related aromatic compounds, has been shown in various strains of the genus *Sulfitobacter* of the *Roseobacter* clade (Buchan *et al*., 2001). This and other pathways to degrade aromatic compounds have been detected in the genomes of quite a few organisms of the *Roseobacter* clade including the genera *Sulfitobacter* and *Roseovarius* (Newton *et al*., 2010). The degradation of aromatic compounds and in particular of those derived from vascular plants appears to be a distinct property of many representatives of the *Roseobacter* clade (Buchan *et al*., 2005) thus explaining the presence of members of this clade at the end of our experiments.

Several bands and isolates we retrieved affiliated to *Sphingobium*, *Sphingopyxis* and *Sphingomonas* of *Alphaproteobacteria* (Fig. 2A). Members of these lineages have been shown to be able to degrade humic matter in freshwater (Hutalle-Schmelzer *et al*., 2010) and PAHs in marine environments and soil (e.g. Sohn *et al*., 2004; Hilyard *et al*., 2008; Alonso-Gutiérrez *et al*., 2009; Kertezs & Kawasaki, 2010). Members of *Gammaproteobacteria* we retrieved from the flow through cultures are closely related to organisms of the genera *Alcanivorax* and *Alteromonas* which appear to be particularly capable of degrading PAHs (Fig. 2B; Yakimov *et al*., 1998; Fernández-Martínez *et al*., 2003; Harwati *et al*., 2007).

The dominant group of isolates from the flow-through cultures with the estuarine inoculum were *Actinobacteria*. In addition, two actinobacterial phylotypes were detected in the DGGE bands. *Actinobacteria* have been found also in other studies to be important in degrading humic matter, but mainly in limnetic environments (Haukka *et al*., 2005; Hutalle-Schmelzer & Grossart, 2009; Hutalle-Schmelzer *et al*., 2010). *Actinobacteria* from estuarine and marine environments, however, are known to be capable of degrading various polymeric substances including lignin and PAH (Haider *et al*., 1978; Piza *et al*., 2004; Alonso-Gutiérrez

et al., 2009). Piza and colleagues (2004) found a very diverse community of *Actinobacteria* in a highly polluted Brazilian estuary, in particular at its brackish end. Alonso-Gutiérrez *et al*. (2009) reported that *Actinobacteria*, in addition to *Alphaproteobacteria*, were mainly involved in the degradation of hydrocarbons of an oil spill.

In addition to the bacteria mentioned above, we also found a few phylotypes and isolates affiliated to *Betaproteobacteria*, *Planktomycetales*, *Sphingobacteria* and *Firmicutes* and one phylotype affiliated to a yeast genus, *Sporobolomyces*, known to degrade lignin (Košíková & Sláviková, 2004). We have no direct indication that these organisms were involved in degrading HA in our experiments, but we assume that those affiliated to *Betaproteobacteria* and *Sphingobacteria* were little or not involved. Mou *et al*. (2007) reported that these phylogenetic lineages were underrepresented in mesocosms enriched in two aromatic monomers as model substrates of vascular plant-derived humic matter.

Quite a few of the phylotypes and isolates we retrieved from the flow-through cultures were closely related to phylotypes and isolates detected in the Weser estuary and the Wadden Sea in previous studies and labelled in the phylogenetic trees of Fig. 2 as GWS, AP and HP (Selje & Simon, 2003; Grossart *et al*., 2004; Selje *et al*., 2005; Stevens *et al*., 2005; Stevens *et al*., 2007). This comparison shows that the phylotypes we retrieved are not only selected during our flow through culture experiments on this HA medium under very selective conditions but are, in fact, present in the ambient bacterial communities in the Wadden Sea and Weser estuary. Their presence is consistent with the hypothesis that these bacterial communities are well capable of decomposing HA and presumably other humic substances of terrestrial origin and aliphatic and aromatic hydrocarbons in general entering this highly dynamic intertidal coastal environment. Our results further indicate that HA are rather well decomposed in this environment and that the dynamic changes in salinities favour their decomposition.

Acknowledgements

We are most grateful to A. Schlingloff for technical assistance in sequencing 16S rRNA gene fragments of DGGE bands and isolates, to R. Gahl-Janssen for support in isolating and maintaining isolated strains, to H. Simon for help in sampling and to C.E. Wegner for assistance in phylogenetic analyses. This work was supported by grants Si 360/22-1 and TRR51 from Deutsche Forschungsgemeinschaft (DFG).

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Supplementary Information

On the following pages:

Table S1: Overview on the identification of phylotypes retrieved from 16S rRNA gene fragments of the DGGE gels of samples from the late stage of the flow through cultures inoculated with a marine (mar) or estuarine (est) bacterial community and fed with a humic acid (HA) medium of salinities 14 or 28. Given are band identification (ID), flow through culture origin, accession no., next related validated species, % similarity(simil) and its habitat, habitats of relatives >97% similarity (m: marine, es: estuarine, so: soil, se: sediment, li: limnetic) with accession no. and indication of close relatives degrading aliphatic or aromatic hydrocarbons. na: not available.

Table S2: Overview on the identification of isolated bacteria retrieved from samples of the late stage of the flow through cultures inoculated with a marine (mar) or estuarine (est) bacterial community and fed with a humic acid (HA) medium of salinities 14 or 28. Given are identification of isolate groups and isolates, salinity of the inoculum, accession no., next validated relative, % similarity (% simil) and habitats of next validated relative and habitats of relatives >97% similarity (m: marine, es: estuarine, so: soil, se: sediment, li:limnetic) with accession no. and indication of close relatives degrading aliphatic or aromatic hydrocarbons. na: not available.

Table S1

Band ID	origin	acc. no	next validated relative	% simil	habitat of next val. relative	habitats of next relatives	decomposer of aliphatic or aromatic hydrocarbons
Alphaproteobacteria							
HA-mar-B6-28	1	HM368304	*Sphingopyxis flavimaris* strain SW-151, NR_025814	94	sea water of the Yellow Sea in Korea	na	na
HA-mar-B12-28	2	HM368307	*Agrobacterium stellulatum*, D88525	96	bottom sediments and sea water	na	na
HA-mar-B14-28	2	HM368308	*Erythrobacter citreus* FE35F/1, AF118020	99	Mediterranean Sea	m (AY007680), se (EU360290), so (EF034330), es (DQ153155), li (AY328639)	(EU440970), (AB429067), (AB302352)
HA-mar-B20-28	2	HM368310	*Devosia subaequoris* type strain: HST3-14, AM293857	88	sediment from Hwasun Beach	se(DQ068288)	na
HA-mar-B24-28 HA-mar-B34-28	3,4	HM368311, HM368314	*Thalassospira tepidiphila*, AB265822	99	petroleum-contaminated seawater	m (AB265822), se (DQ659435), es (AF231417)	(AF231419)**, (AF231417)**, (DQ659435)
HA-mar-B25-28	3	HM368312	*Phaeobacter arcticus* strain 20188, DQ514304	98	marine samples collected from the Arctic	se (EU195946), m (AY239003),	na
HA-mar-B35-28	4	HM368315	*Tateyamaria omphalii*, AB193438	95	Top shell *Omphalius pfeifferi pfeifferi*	na	na
HA-mar-B45-14	5	HM368317	*Novosphingobium pentaromativorans* strain US6-1, NR_025248	96	estuarine sediment	se (EF460064), m (EF628499)+, es (AY145559)	na
HA-mar-B54-14	6	HM368320	*Blastomonas ursincola (Erythromonas ursincola)*, AB024289	93	underwater hydrothermal vents of the Bol'shoi River	na	na
HA-mar-B52-14	6	HM368318	*Thalassospira lucentensis* QMT2, NR_028004	98	Mediterranean Sea	m (NR_028004), se (EU595531), es (EF419206),	(AB265822), (AF231417), (EU440969)
HA-mar-B7-28 HA-est-B34-14	1,3	HM368305, HM368325	*Thalassobius mediterraneus*, AJ878874	96	sea water off the western Mediterranean coast	m (AJ878874), es (AY145594), se (AY171343)	(AB330819),
HA-mar-B8-28, HA-mar-B16-28	1,2	HM368306, HM368309	*Sulfitobacter pontiacus* ChLG-10, NR_026418	98	Black Sea	se (AF254101), m (AF007254),	(EU052696) , (EU239922),
HA-mar-B44-14 HA-est-B31-14 HA-est-B55-28 HA-est-B56-28 HA-est-B60a-28	5,3,5	HM368316, HM368324, HM368327, HM368328, HM368329	*Marivita cryptomonadis* strain CL-SK44, EU512919	99	marine phytoplankton *Cryptomonas sp.*	m (EU512919), es (EF471683), se (DQ659449)	(DQ659449), (EU440950)
HA-est-B6-14	1	HM368322	*Marivita cryptomonadis* strain CL-SK44, EU512919	94	marine phytoplankton *Cryptomonas sp.*	na	na
HA-mar-B53-14	6	HM368319	*Roseovarius mucosus* DFL-24, AJ534215	98	*Alexandrium ostenfeldii*	m (EF215730), li (DQ837273), so (EU328076), se (EU140521)	(EU052702), (EU052731)
HA-est-B3-14	1	HM368321	*Rhodobacter ovatus* type strain JA234T, AM690348	99	sediment from polluted pond	li (AB473971), es (EF471652),	(FJ830712)
HA-est-B47-14	4	HM368326	*Altererythrobacter luteolus strain* SW-109, AY739662	99	tidal flat of the Yellow Sea in Korea	m (AY739662), so (AF432339) ,	(AF432339)
HA-est-B10-14	1	HM368323	*Thalassobaculum litoreum*, EF203900	94	coastal seawater (near Gori, Korea).	so (AM936707)	na
Betaproteobacteria							
HA-est-B28-14	3	HM368332	*Oxalicibacterium horti*, AB469786	97	forest soil sample	li (EU642202), so (AB469786), ri (FJ694545),	(DQ145569)

Table S1 (continued)

Band ID	origin	acc. no	next validated relative	% simil	habitat of next val. relative	habitats of next relatives	decomposer of aliphatic or aromatic hydrocarbons
Gammaproteobacteria							
HA-mar-B10-28 HA-mar-B11-28	2	HM368334, HM368335	*Alcanivorax venustensis* strain ISO4, NR_025145	99	Mediterranean Sea	se (DQ659430), m (AY683537)	(GQ153640), (EU239901), (AY683537)
HA-mar-B47-14	5	HM368338	*Legionella impletisoli*, AB233209	90	soils contaminated with industrial wastes in Japan	m (FJ826308) ∞	na
HA-mar-B60-14 HA-est-B74-28	6,6	HM368339 HM368349	*Pseudomonas pachastrellae* KMM 330, AB125366	98	deep-sea sponge	se (FJ169986), m (AB125366),	(EU695223), (EU670645)
HA-mar-B9-28	1	HM368333	*Alcanivorax borkumensis*, Y12579	100	sea water sediment samples (North Sea)	m (Y12579), se (EU195941), so (EU328046)	(GQ408897), (AB257196), (Y12579)
HA-mar-B27-28 HA-mar-B37-28	3,4	HM368336, HM368337	*Alcanivorax jadensis* strain T9, NR_025271	99	intertidal sediment from the German North Sea coast	m(EU438901), se (NR_025271)	(EU438901), (052761)
HA-est-B4-14	1	HM368340	*Marinobacter salicampi* strain ISL-40, EF486354	89	marine solar saltern of the Yellow Sea in Korea	na	na
HA-est-B17-14	2	HM368341	*Alteromonas marina* SW-47, NR_025260	99	sea water of the East Sea in Korea	m (NR_025260), se (NR_025780), es (DQ836766)	(AB302344), (AB302345)
HA-est-B18-14	2	HM368342	*Alkalispirillum mobile* DSM12769, NR_024961	93	Summer lake, Lake County, Oregon, a salt sea	na	na
HA-est-B40-14	4	HM368345	*Rheinheimera aquimaris* SW-353, EF076757	90	isolated from seawater from the East Sea, Korea	na	na
HA-est-B46-14	4	HM368346	*Idiomarina salinarum* ISL-52, EF486355	96	marine solar saltern of the Yellow Sea	es (DQ234155), se (DQ068277)	(EU440967)
HA-est-B60-28 HA-est-B77-28	5,6	HM368348, HM368350	*Legionella pneumophila subsp. Pascullei*, AF122885	98	pathogen from humans	se (EF459912), so (EF663718), li (EU641584),	(AY050582)
HA-est-B51-28	5	HM368347	*Marinobacter sediminum*, AJ609270	91	sediment	na	na
Actinobacteria							
HA-est-B22-14	3	HM368343	*Iamia majanohamensis*, AB360448	88	sea cucumber *Holothuria edulis*	m (AY370632), es (EF471700)	na
HA-est-B23-14	3	HM368344	*Leifsonia poae*, AF116342	95	*Poa annua* root gall	es (EF471631), m (EF659443), li (AJ575527)	na
Bacteroidetes							
HA-est-B48-14	4	HM368330	*Marinicola seohaensis* SW-152, AY739663	98	sea water of the Yellow Sea in Korea	se (EU697080), m (AY739663),	(EU697080)
HA-mar-B38-28	4	HM368331	*Lewinella nigricans*, AB301615	89	beach sand near Lagos, Nigeria	na	na
Planctomycetales							
HA-est-B50-28	5	HM368351	*Phycisphaera mikrensis*, AB474364	87	marine algae	se (EU050874), so (AM935485)	na

** humic substance degrading

Table S2

group	Isolates	salinity of inoculum	acc. no.	next validated relative	% simil	habitat of next validated relative	habitats of next relatives	decomposer of aliphatic or aromatic hydrocarbons
Alphaproteobacteria								
AL-1	HA-mar-Is1-28	28(28)	HM368274	*Agrobacterium kielensis strain* B9, D88524	98	soil (Kiel)	m (AF359524), se (EU528264)	Na
AL-2	HA-mar-Is2-14+28	28+14(28)	HM368275	*Thalassospira lucentensis DSM* 14000T, NR_028004	99	Mediterranean Sea	m (AB265822), se (AY186195, deep sea)	(AB265822), (EU440969), (EU239909)
AL-3	HA-mar-Is3-14+28 HA-mar-Is16-14+28	14+28(28)	HM368276, HM368353	*Sulfitobacter pontiacus* ChLG-10, NR_026418	99	Black Sea	m (AF007257), se (AF254101, deep sea), es (EF471650)	(EU239922), (EU052696), (AF007254)
AL-4	HA-mar-Is4-28	28(28)	HM368277	*Roseovarius mucosus* DFL-24, AJ534215	97	Alexandrium ostenfeldii	m (AJ534215), se (DQ334642), so (EU328076)	(EU052702), (EU328076), (EU239908)
AL-5	HA-mar-Is13-28	28(28)	HM368352	*Sulfitobacter marinus strain* SW-265, DQ683726	99	East Sea (Korea)	m (DQ683726), es (AY145593,)	(EU052734), (NR_026418)
AL-6	HA-mar-Is15-14	14(28)	HM368278	*Sphingopyxis chilensis strain* S37, NR_024631	98	superficial river sediment	m (CP000356), se (NR_024631, river-sed.), so (DQ376581), ri (AB022601), li (NR_024999)	(NR_024631), (DQ376581), (DQ158105)
AL-7	HA-mar-Is18-14	14(28)	HM368279	*Phaeobacter inhibens*, AY177712	96	German Wadden Sea	m (EF215730), se (EU652593)	(EU239924), (AF388307)
AL-8	HA-mar-Is19-14	14(28)	HM368280	*Sphingopyxis flavimaris strain* SW-151, NR_025814	98	sea water of the Yellow Sea in Korea	m (AY515408), so (EU328007), se (EU287342, deep sea), es (EF471499)	(EU239907), (EU328007)
AL-9	HA-mar-Is20-14+28 HA-est-Is45-14+28	14+28(14), 14+28(28)	HM368281, HM368287	*Sphingobium xenophagum strain* BN6, NR_026304	98	River Elbe	m (AB276370), li (AY136097), so (U37346), se (AB047364)	(AY512602), (AB276370), (AB196246)
AL-10	HA-mar-Is23-14+28 HA-est-Is52-14	14+28(28), 14(14)	HM368282, HM368288	*Erythrobacter citreus* FE35F/1, AF118020	98	Mediterranean Sea	m (AB429067), se (FJ607439)	(AB429067), (AB196251), (AB302352)
AL-13	HA-est-Is33-14	14(14)	HM368283	*Sphingomonas koreensis strain* JSS-26, NR_024998	99	Natural mineral water	so (AJ968701), li (NR_024998)	(NR_024685)
AL-14	HA-est-Is34-14	14(14)	HM368284	*Roseovarius pacificus strain* 81-2, DQ120726	96	deep sea sediment	es (AY145563)	na
AL-15	HA-est-Is35-14	14(14)	HM368285	*Oceanicola nanhaiensis strain* SS011B1-20, DQ414420	100	sediments of the South China Sea	m (AM779569) , se (DQ414420)	na
AL-16	HA-est-Is38-14	14(14)	HM368286	*Paracoccus carotinifaciens strain* E-396, NR_024658	100	soils , Japan	m (AY745834), se (DQ357050) , so (NR_024658)	Na.
Betaproteobacteria								
Be-1	HA-est-Is57-28	28(14)	HM368291	*Azoarcus anaerobius strain* DSM 12081, NR_026438	93	anoxic sewage sludge	na	na
Gammaproteobacteria								
G-1	HA-mar-Is6-28 HA-mar-Is10-28 HA-est-Is41-14	28(28),14(14)	HM368292, HM368295, HM368300	*Alteromonas macleodii strain DSM 6062*, Y18228	99	marine	m (AB078014), se (NR_025780), es (DQ836765)	(AB302345), (AB302344),
G-2	HA-mar-Is8-14+28	14+28(28)	HM368293	*Alcanivorax venustensis* strain ISO4, NR_025145	100	Mediterranean Sea	m (AB053126), se (DQ768632)	(EU239901, (EU052751)∞

Table S2 (continued)

group	Isolates	salinity of inoculum	acc. no.	next validated relative	% simil	habitat of next validated relative	habitats of next relatives	decomposer of aliphatic or aromatic hydrocarbons
G-3	HA-mar-Is9-28 HA-mar-Is22-14 HA-est-Is39-14	28(28), 14(28), 14(14)	HM368294, HM368296, HM368298	*Pseudomonas pachastrellae* KMM 330, AB125366	97	deep-sea sponge	m (AB125367), se (DQ356975),	(EU695224)x
G-6	HA-est-Is36-14+28	14+28(14)	HM368297	*Pseudomonas stutzeri*, AF094748	98	distributed in soil and water	se (AM111063), m (AB176955), li (AM905943), so (AY382612)	(EF025737, (AM905943), (AY683884)
G-7	HA-est-Is40-14	14(14)	HM368299	*Marinobacter salicampi strain* ISL-40, EF486354	90	marine solar saltern in Korea	m (AM238590), es (EF471583), li (EU639722)	na.
G-8	HA-est-Is44-14	14(14)	HM368301	*Pseudomonas sabulinigri strain* J64, EU143352	99	black sand	m (EU365599), so (EU143352)	(EU239921), (DQ533960)
G-11	HA-est-Is59-14	14(14)	HM368302	*Erwinia billingiae*, Y13249	98	trees	ri (AM117486), se (AY524555), li (AM992182), so, (DQ520801), m (AM259899)	(FJ422735), (DQ351903), (EF138627)
G-12	HA-est-Is60-14	14(14)	HM368303	*Psychrobacter maritimus strain* PI2-20, NR_027225	98	coastal sea ice	se (FJ613604), m (EF101551), so (EU009757), es (EF471493), ri (EF523607)	na
Actinobacteria								
Act-1	HA-mar-Is5-28	28(28)	HM368261	*Micrococcus luteus* DSM 20030T, AJ536198	99	n.f.	m (AM913979), li (AJ717369), so (AY512635), se (DQ659431), m (AJ441006, saline lake)	(AY512635), (EU440972), (EF061936)
Act-4	HA-est-Is42-14 HA-est-Is43-14	14(14)	HM368262 HM368263	*Curtobacterium flaccumfaciens pv. flaccumfaciens* strain LMG 3645, AJ312209	98	navy beans	li (AY374110), so (EU723164), se (EF592577), m (EU741196), ri(GQ398367)	(DQ238838),
Act-5	HA-est-Is46-14	14(14)	HM368264	*Arthrobacter ardleyensis strain* An25, AJ551163	98	Antarctic lake sediment	m (AY370617), se (AJ715981), es (DQ314547), so (EU282772)	(FJ477042), (EF376011)
Act-6	HA-est-Is47-14 HA-est-Is48-14 HA-est-Is54-28	14(14), 28(14)	HM368265 HM368266 HM368271	*R.fascians* (DSM 20669), X79186	98	Infects plants, leaf	se (EU016150), m (AY745830), so (AF181690), es (AY745831)?, li (AY957906)	(AF181690), (AY512642), (AB070458)
Act-7	HA-est-Is49-14	14(14)	HM368267	*Microbacterium foliorum strain* P333/02, NR_025368	99	grasses and surface litter	so (EU239498), li (AY622232), m (AY370612), se (DQ448707)	(AJ698726), (DQ227343), (AY785738)
Act-8	HA-est-Is50-14	14(14)	HM368268	*Arthrobacter agilis strain* DSM 20550, NR_026198	99	water, soil, and human skin	m (AY612779), se (AF197049), li (EU753660)	n.f
Act-9	HA-est-Is51-14	14(14)	HM368269	*Mycobacterium smegmatis strain* ATCC 19420, NR_025311	98	wounds	so (DQ372729)	(DQ227004), (AY177352)
Act-10	HA-est-Is55-28	28(14)	HM368272	*Promicromonospora flava* type strain CC, AM992980	97	sediment of the Baltic sea	m(EU787013), so (DQ387860), se(DQ448724)	n.f.
Act-11	HA-est-Is56-28	28(14)	HM368273	*Micrococcus endophyticus strain* YIM 56238, EU005372	99	Aquilaria sinensis roots	m (AY370619), li (AJ717369), se (DQ448712)	(AY512635), (AB302333), (EU095950)
Act-12	HA-est-Is53-14	14 (14)	HM368270	*Nocardioides marinus* strain CL-DD14 DQ401093	98	seawater (East Sea, Korea)	m (AB330817), se (NR_025777)	(AB330817), (AB183711)
Firmicutes								
Bac-1	HA-mar-Is17-14	14(28)	HM368289	*Bacillus foraminis type strain* CV53T, AJ717382	98	highly alkaline non-saline groundwater	m (DQ492812), se (AM950294), so (AB245377), es (DQ314538), li (AJ717382)	(FJ772024)
Bac-2	HA-mar-Is29-14+28	14+28(28)	HM368290	*Bacillus idriensis strain SMC* 4352-2,	99	human blood	li (FJ204464), m (EF101988), so (EU134616), es (DQ314544)	(EU908203)

3

Differential decomposition of humic acids by marine and estuarine bacterial communities at varying salinities

Differential decomposition of humic acids by marine and estuarine bacterial communities at varying salinities

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Running head:humic acid decomposition by bacteria

Key words: humic matter, DOC, bacteria, flow-through culture, pyrolysis-GC/MS

Abstract

Humic substances (HS) constitute 50-80% of total dissolved organic matter (DOM) in freshwaters but in the open ocean <3%, indicating that large fractions of DOM and HS are removed in the estuarine and coastal zone. In order to assess the role of bacteria in this removal, we conducted experiments in flow-through cultures to examine the decomposition of peat bog-born humic acids (HA) by marine (salinity 30, Exp1) and estuarine (salinity 10, Exp2) bacterial communities. After ~70 days 40->60% of the HA were decomposed and the highest fractions in treatments in which the bacterial communities were fed by HA media of a foreign salinity, e.g. of 14 in Exp1 and of 28 in Exp2. Some of the HA material was not decomposed but broken down to smaller moieties entering the fulvic acid (FA) fraction. The analysis of the HA media and their residuals after bacterial decomposition by pyrolysis GC/MS showed that individual organic compounds were decomposed. In Exp1, 32 aliphatic and aromatic compounds, including lignin biomarkers, were detected in the HA medium of which mainly aliphatic compounds were decomposed. In Exp2, 49 compounds were detected of which ~40-60% were not detected any more after bacterial decomposition in the HA fraction but still in the FA fraction. The results show that estuarine and marine bacterial communities can decompose large amounts of HA and that this process is important in reducing the amount of terrestrial HS and DOM entering the estuarine and coastal region.

Introduction

Humic substances (HS) are ubiquitous in terrestrial and aquatic environments and an important carbon pool in the global carbon cycle, comprising 50-80% of total dissolved organic matter (DOM) in freshwaters (Thurman 1985; McKnight and Aiken 1998; Dittmar and Kattner 2003). These heterogeneous and complex substances are considered recalcitrant but can at least partially be decomposed by microorganisms (Bano et al. 1997; Bussmann 1999; Donderski and Burkowska 2000; Rosenstock and Simon 2003; Anesio et al. 2005; Rosenstock et al. 2005; Kisand et al. 2008) and are photoreactive due to absorption in the ultraviolet region of the solar spectrum (Kulovaara 1996; Miller and Moran 1997; Obernosterer and Herndl 2000; Mopper and Kieber 2002). Because of these absorption properties HS constitute a major fraction of chromophoric (C)DOM which is often also considered as highly recalcitrant (Boyd and Osburn 2004; Obernosterer and Benner 2004; Benner and Kaiser 2011).

According to differences in solubility at low pH, HS are operationally subdivided into hydrophilic acids with the lowest molecular weight, i.e. <600 Da, fulvic acids (FA) with a molecular weight of 500 to 1200 Da, humic acids (HA) with a molecular weight of >1200 Da, and the non-soluble humins (Thurman and Malcolm 1981; McKnight and Aiken 1998). Sources for aquatic HS can be allochthonous, i.e. terrestrial, and autochthonous organic matter, e.g. phytoplankton residues, often microbially and physico-chemically altered. Terrestrial HS originate from vascular plant residues (lignin and various structural biopolymers) and other detrital organic matter which is transformed and modified by microorganisms and physico-chemical reactions (McKnight and Aiken 1998). Humic substances are transported via rivers and estuaries to the sea. The global annual discharge of DOM through rivers corresponds to about 0.25×10^{15} g C (Cauwet 2002) but HS in the open ocean comprise only a small fraction of total DOM (0.7-2.4%; Opsahl and Benner 1997). This comparison implies that large fractions of DOM and HS are removed in the near-shore area. In fact, present estimates suggest that terrestrial and thus predominantly humic DOM, after mixing with seawater, is degraded rapidly in the coastal zone (Hedges et al. 1997; Cauwet 2002). During the estuarine passage with increasing salinities, DOM and HS undergo alterations like flocculation and adsorption to suspended matter (Sholkovitz 1976; Sholkovitz et al. 1978; Uher et al. 2001), photochemical mineralization and microbial decomposition (Wikner et al. 1999; Moran et al. 2000; Boyd and Osburn 2004; Pullin et al. 2004; Farjalla et al. 2009; Kisand et al. 2008). Our knowledge on the bacterial decomposition of DOM and HS and on concentrations and the significance of HA and FA as microbial substrates along the

estuarine salinity gradient is, however, scarce. In the salinity gradient of the Weser estuary, Germany Wadden Sea, HA are more readily degraded than FA and hydrophilic acids (Kisand et al. 2008). The authors reported that HA comprised ~30% of total HS in a humic-rich stream and Gebhardt (2005) found that HA accounted for 16 to 37% of total HS in a drainage channel discharging into the Wadden Sea, southern North Sea. In both studies FA constituted higher proportions than HA which may reflect that HA are more readily degradable than FA, the fraction with a lower molecular weight. This notion is consistent with the size-reactivity continuum model of Amon and Benner (1996) proposing that the degradability of marine DOM decreases with decreasing molecular weight.

The molecular structure of the complex high-molecular-weight HS is still largely unknown. It has been shown that amino acids and carbohydrates are present in HS (Hubberten et al. 1994; Rosenstock and Simon 2003) and that lignin derivatives are important structural HS components (Schulten and Gleixner 1999; Lehtonen et al. 2000). Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) is a powerful tool to study complex high-molecular-weight organic compounds and therefore useful to characterize the molecular structure of DOM. This technology, particularly in combination with off-line tetramethylammonium hydroxide (TMAH) derivatization, has been applied to elucidate the composition of HS in soil and aquatic systems (Martin et al. 1995; Schulten and Sorge 1995; Schulten and Gleixner 1999; Lehtonen et al. 2000; Frazier et al. 2005; Poerschmann et al. 2007). It allows the identification of individual structural moieties and functional groups of the complex HS after heat-induced breakdown to smaller units. Even though Py-GC/MS does not yield quantitative information on the composition of HS, it enables an insight into the great variety of aromatic and aliphatic building blocks of HS. It can also be applied to identify biomarkers, e.g. of lignin, which enter the pool of allochthonous HS. This technology has not been applied before to investigate structural modifications of HS during the decomposition by aquatic bacterial communities but appears to be a promising tool for such studies.

The Wadden Sea, stretching over 500 km from Den Helder in The Netherlands to Skallingen in Denmark, is the largest tidal flat system on earth and therefore of prime importance as a bioreactor for the degradation of HS in the coastal zone of the North Sea. Humic substances are discharged into the Wadden Sea by large rivers such as Rhine and Elbe but also by a large number of drainage channels (Warnock et al. 1999; Fooken and Liebezeit 2000; Lübben et al. 2009).

The aim of this study was to examine the bacterial decomposition of HA at salinities of 14 and 28 to mimic decomposition conditions in the polyhaline (marine) and oligohaline

(estuarine) zone of the German Wadden Sea and its estuarine tributaries. Therefore, flow-through cultures were set up which were supplied with HA of a humic-rich stream, inoculated by a marine and estuarine bacterial inoculum and incubated for ~70 days at marine and estuarine salinities. The results show that HA were decomposed to >50% and that decomposition was highest at the salinity foreign to that of the inoculum. The complexity, i.e. the number of HA compnents identified by Py-GC/MS, was reduced during decomposition and HA were partly broken down to FA.

Material and methods

Approach

Flow-through systems were used to assess the decomposition of HA by naturally-derived estuarine and marine bacterial communities (Fig. 1). In one set of experiments a marine bacterial community was used as inoculum (Mar1 to Mar 6). It was collected on the day of the start of the experiment in June 2007 from the polyhaline (salinity 30) German Wadden Sea at Neuharlingersiel (53° 43' 67" N, 07° 42' 73" E). In a second set of experiments, started in May 2008, an oligohaline (salinity 10) bacterial community collected near Kleinensiel (Weser estuary, Northwest Germany; 53° 26’ 37’’ N, 8° 29’ 06’’ E) served as inoculum (Est 1 to Est 6). In both experiments the decomposition of HA was examined at salinities of 28 and 14. The HA were extracted from water collected in a freshwater stream rich in HS near Oldenburg (see below). In each set of experiments three different treatments were tested, one with the salinity close to that of the inoculum, either 14 or 28, one with the salinity close to that of the inoculum but amended with combusted sediment particles, and one with an enhanced (28) or reduced (14) salinity relative to that of the inoculum. The sediment particles were added to examine whether flocculation and adsorption of HA to these particles enhances abiotic removal of HA or leads to enhanced decomposition of HA by particle-associated bacteria.

Setup of experiments

Duplicate 10-l glass bottles were filled with 10 l of artificial sea water (ASW) according to Kisand et al. (2008) and modified by adding 34.5 µM KNO_3 and $NaNO_3$, and 3.5 µM K_2HPO_4 and KH_2PO_4, respectively. For a detailed description of the experimental setup see Rocker et al. (2011). Briefly, ASW media of salinities 28 and 14 were supplemented with HA at a final concentration of 6 mg HA l^{-1}. Wadden Sea sediment combusted for 2 h at 500°C

was added to one set of duplicates at a concentration of 30 mg l^{-1}. The HA media, after removal of particles through a 0.22 µm pore-size cartridge filter, were supplied to the glass bottles at a dilution rate of 0.01 d^{-1}. The bottles were continuously aerated by 0.22 µm-prefiltered room air. Two hundred fifty ml of 5.0 µm prefiltered inocula were added to the flow-through systems which were incubated at room temperature for 71 (marine) and 74 (oligohaline) days, respectively. For the first 3 days experiments were run in batch mode to avoid washing out of the bacteria. Subsamples for measurements of DOC, bacterial abundance and biomass production were withdrawn periodically from the outflow. In order to examine possible abiotic removal of DOM by flocculation two sterile controls of a salinity of 28 were run in parallel to the inoculated experiments in 2007, one for 56 and one for 84 days. On the last day of each experiment 10 l of the outflow were collected for the analysis of HA by Py- GC/MS.

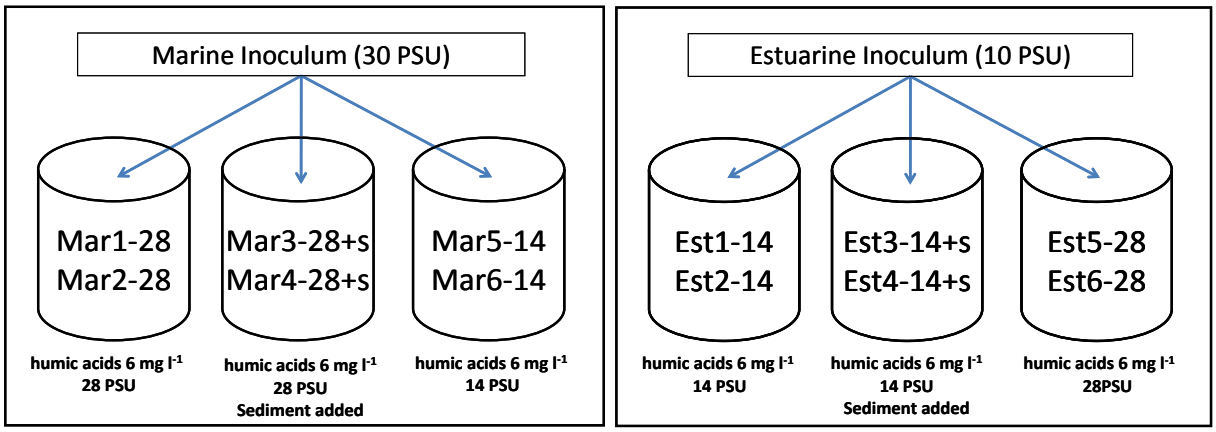

Figure 1: Experimental design of the HA decomposition experiments with a marine (left panel) and estuarine (oligohaline; right panel) inoculum.

Extraction of humic and fulvic acids

Sixty l of water rich in HS were collected in 20-l PE bottles from Wildenloh (peat bog area near Oldenburg, Northwest Germany) in June 2007 and May 2008 for the experiments inoculated with the marine and estuarine bacterial community, respectively, and pre-filtered through a 0.1 µm cartridge using tangential flow ultrafiltration (Amicon-Millipore). The high-molecular-weight DOM was isolated by column chromatography of 10-30 l of the acidified (pH 2.0) filtrate using XAD-8 resins (Sigma-Aldrich, trademark name DAX-8) (Thurman and Malcolm 1981; Malcolm and MacCarthy 1992). Humic substances were eluted with 0.1 N NaOH. The HA fraction was obtained by precipitation at pH 1 (overnight at 4°C), centrifugation at 4,000 g and redissolution of the precipitate in ultra-clean water. HA were re-

concentrated onto the XAD-8 resin and desalted by elution with 0.1 N NaOH. Sodium ions were removed by cation exchange chromatography (Amberlite IR-120(H)). The HA were then vacuum concentrated, and the concentrate was freeze-dried.

To analyse the transformation of HA after microbial degradation 10 l of the outflow were collected at the end of the experiments and subjected to HA extraction as described above. In addition, the FA fraction was obtained as the supernatant of the overnight precipitation at pH 1 and freeze-dried. Both, HA and FA were subjected to Py-GC/MS analysis.

DOM analysis

Ten ml subsamples were immediately filtered through a 0.2 µm polyethersulfon filter, acidified with 40 µl of 6 N HCl and stored at 4°C in the dark until analysis. DOC was determined in five replicate analyses of one sample by high-temperature combustion with a TOC-V CSH analyzer (Shimadzu) and by using potassium hydrogen phthalate as a standard. The standard deviation of the five replicates was always <5%.

Bacterial abundance

Ten ml subsamples, fixed with glutardialdehyde (final concentration 1%) for 45 min, were stored at -80°C until further processing. Enumeration of bacteria was performed with a Becton Dickinson FACS Calibur flow cytometer as described in Rocker et al. (2011). Data were processed and bacterial numbers calculated by WinMDI 2.9 (Windows Multiple Document Interface for Flow Cytometry, http://facs.scripps.edu/software.html).

Bacterial biomass production (BP)

Rates of BP were determined by the incorporation of ^{14}C-leucine (Simon and Azam 1989). Triplicate 5 ml subsamples and a formalin-killed control were incubated with ^{14}C-leucine (10.8 GBq mmol^{-1}) at a final concentration of 60 nM in the dark at room temperature. After 1 h, samples were fixed with formalin (2% final concentration), filtered onto 0.2 µm cellulose nitrate filters and further processed as described in Lunau et al. (2005). Biomass production was calculated according to Simon and Azam (1989), assuming no intracellular isotope dilution and applying a conversion factor of 1.675 kg C (mol leucine)$^{-1}$.

Pyrolysis GC/MS

Py-GC/MS analysis of the HS material after off-line TMAH derivatization was carried out according to Schulten and Sorge (1995), Lehtonen et al. (2000) and modified as follows. The

freeze-dried HS fractions (HA, FA) were transferred into ferromagnetic micro cups (590°C, specs, ~500 µg) and soaked with10 µl of TMAH. This mixture was incubated under nitrogen atmosphere for 1 h at 100°C. The samples were subsequently pyrolyzed for 10 s (Curie-point pyrolyzer, 590°C). The GC injector port (split-mode 1:15) was set at 280°C. The initial column temperature was 40°C, held for 1 minute and ramped at 4°C per minute to 280°C.This temperature was held for another 10 min. During sample run the split injection mode was used with a split ratio 1:15. A DB-5MS (0.25 mm ID x 30 m, film thickness 1.25 µm, J & W Scientific) GC column was used. Helium was used as carrier gas in constant flow mode with 0.8 ml/min. The compounds were detected with an Agilent MSD 5973N mass spectrometer using EI ionization, 70 eV, a mass range of 50-650 u and 2.48 scans s^{-1}. For further details see also Gebhardt (2005). The compounds were identified using the spectral library of the National Institute of Standards and Technology (NIST MS search 2.0), literature data and mass spectral interpretation. In some cases peak broadening or partly resolved peaks occurred and the Automated Mass Spectral Deconvolution and Identification System (AMDIS) was used to distinguish between these coeluting compounds. We analyzed the constitutents of the HA in the feeding media and of the HA and FA fractions collected after microbial degradation at the end of both sets of experiments. The identified compounds were grouped into different classes according to aromaticity, aliphatic nature, saturation and acidic state (Tables S1, S2). Background measurements (wires with TMAH without HS fractions, same procedure as described before) were carried out with regard to possible interferences.

Statistical analysis

To analyze the temporal dynamics in DOC concentration and bacterial abundance, we applied linear regression models with concentration and abundance as response values. The 3 treatment types and the 2 replicates of each experiment were included in dummy coding. To meet the assumption of linearity, the relation between time and the responses was investigated in scatter plots. As a result, all responses were log-transformed, time was log-transformed for the analysis of DOC concentrations and included as a third order polynomial for bacterial abundance. To determine possible differences in the temporal development of the responses, all two-way and three-way interactions were included such that the temporal dynamics were allowed to vary in subgroups of the data. Significance of effects was determined via F-tests on the achieved improvement in terms of explained variance. To correlate the pooled data on bacterial abundance and production of both experiments a Pearson product moment correlation with log transformed data was applied.

Results

Isolation and decomposition of humic acids

The water collected for isolating HA yielded an extract of 250 mg HA l^{-1}. A fraction of this extract was used for the HA media, supplying the experiments in a concentration of 6 mg HA l^{-1} and the rest kept for Py-GC/MS analysis. In both experiments and all treatments HA were decomposed to a substantial extent. Initial DOC concentrations in the experiment with the marine inoculum (Mar1 to Mar6) ranged from 400 to 820 µM DOC and decreased in the course of the experiment until day 62 to ~250 µM DOC (Fig. 2a-c). In the experiment with the estuarine inoculum (Est1 to Est6) initial DOC concentrations were somewhat higher and ranged from ~600 to >900 µM DOC (Fig. 3a-c). They also decreased to ~250 µM DOC towards the end of the experiment at day 74. The DOC decrease in the experiment with the estuarine inoculum in general was somewhat higher than in the experiment with the marine

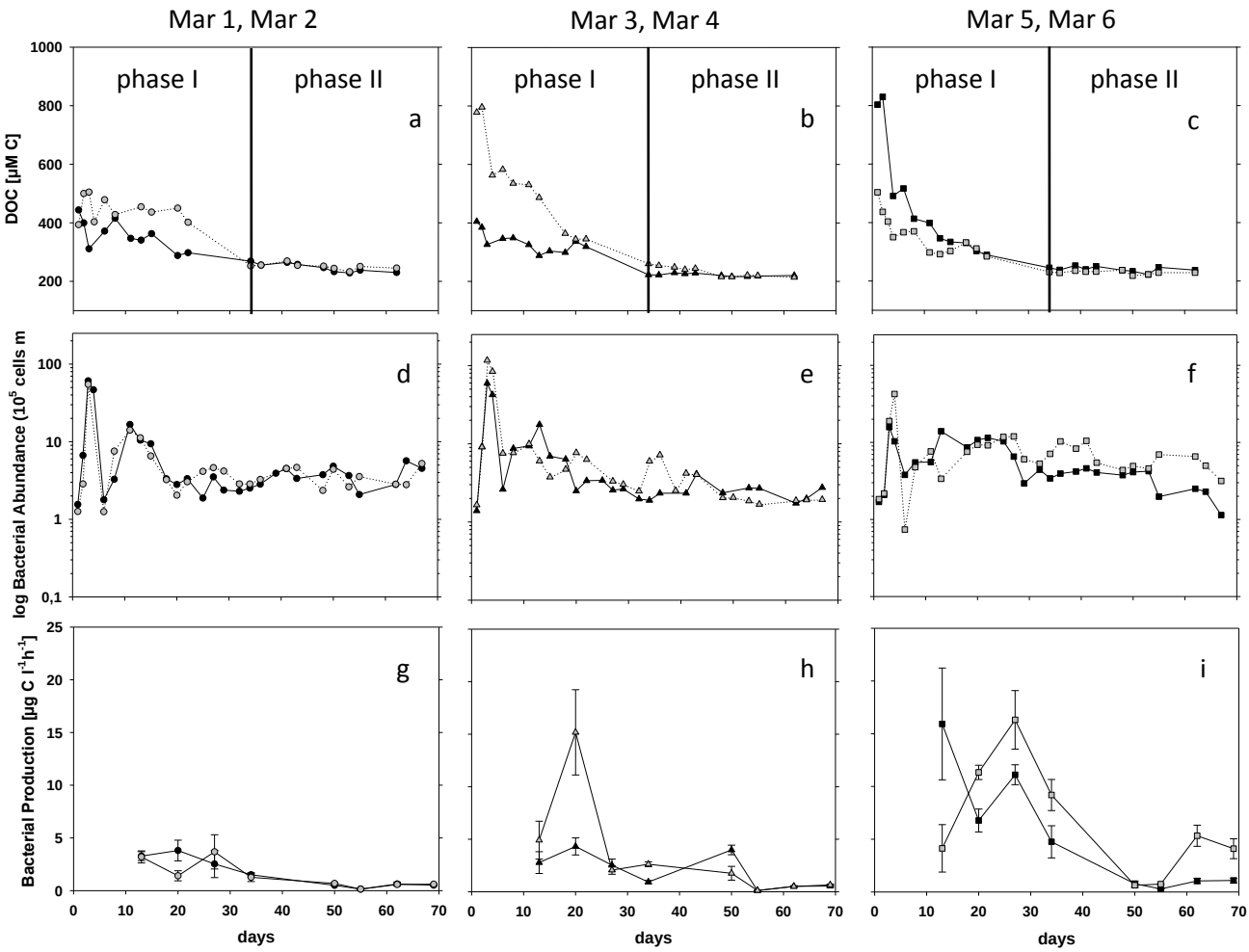

Figure 2: Time course of the HA decomposition flow-through cultures with a marine inoculum: DOC concentrations (a, b, c), bacterial abundance (d, e, f) and bacterial biomass production (g, h, i) of the various replicate treatments. For abbreviations and details of the treatments see Fig. 1.

inoculum (Table 1). Total decomposition varied from 37.8% to 75.6% of the initial concentration in both experiments and the various treatments and >90% of this decomposition occurred in the initial 34 days which were defined as phase 1 (Table 1, Figs. 2a-c, 3a-c).

In the experiment with the marine inoculum, decomposition was lowest in the treatment with the marine HA medium (Mar1, Mar2) as shown by the amount and relative proportion of DOM decomposed during phase 1. The other treatments, that with the marine HA medium and added sediment particles (Mar3, Mar4) and that with the estuarine HA medium (Mar5, Mar6), revealed higher DOM removal. In contrast, in the experiment with the estuarine inoculum, DOM decomposition was highest in the treatment with the marine HA medium (Est5, Est6) and lowest in that with the estuarine HA medium (Est1, Est2). DOM removal in the treatment with added sediment particles (Est3, Est4) was higher than in that without the particles but not as high as that with the marine HA medium. A certain fraction of

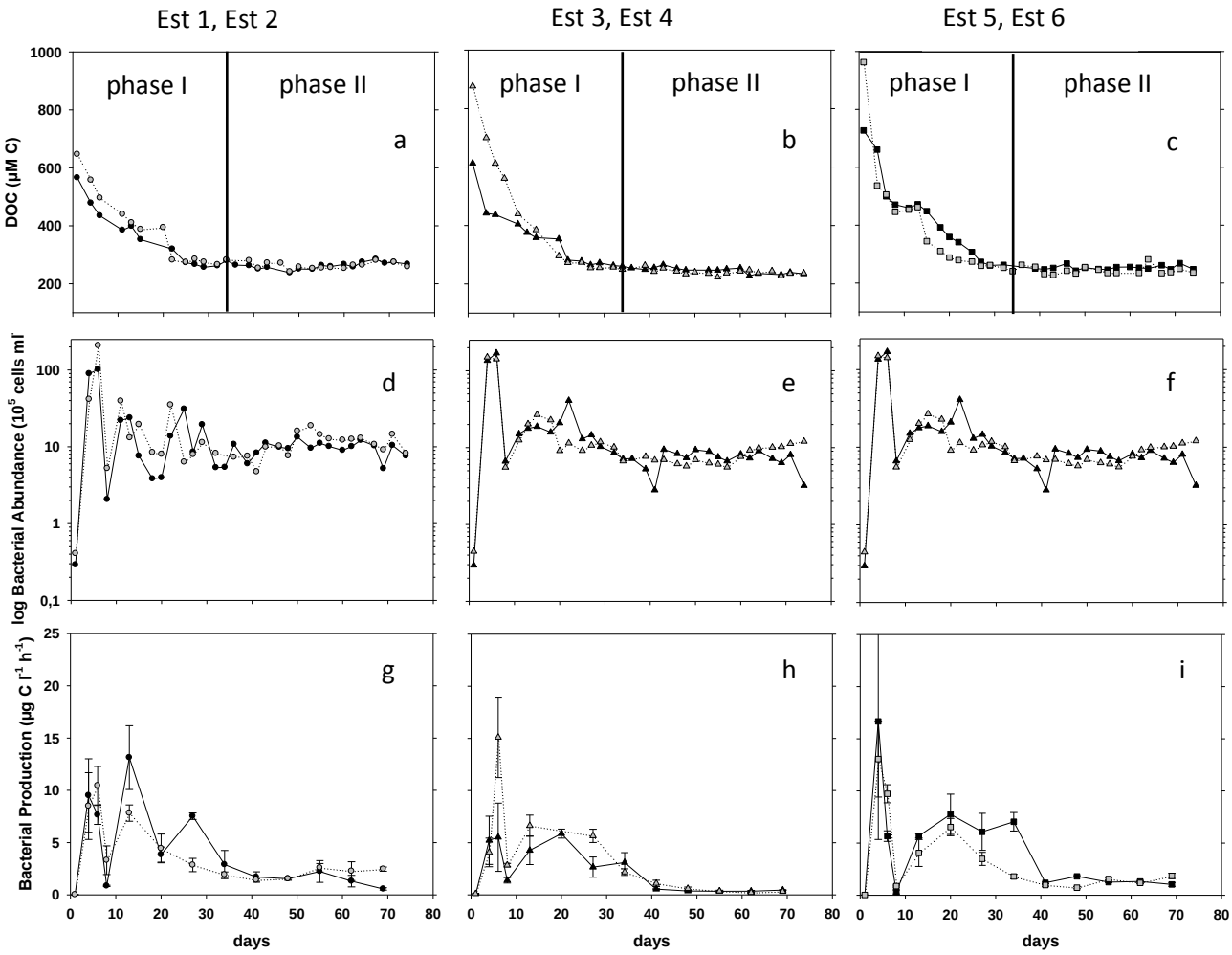

Figure 3: Time course of the HA decomposition flow-through cultures with an estuarine inoculum: DOC concentrations (a, b, c), bacterial abundance (d, e, f) and bacterial biomass production (g, h, i) of the various replicate treatments. For abbreviations and details of the treatments see Fig. 1.

the DOM removal in the treatments with added sediment particles (Mar3, Mar4, Est3, Est4) presumably was due to adsorption of HA to the added particles which partly adhered to the wall of the glass bottles after 14 to 18 days.

In the two sterile control experiments with the marine HA medium and without a bacterial inoculum, DOC concentrations decreased by <9% during the incubation periods of 56 and 84 days, respectively. The duplicates of each treatment in both experiments exhibited significantly different decomposition patterns over time. In the experiment with the marine inoculum they were, however, more similar to each other than in the other treatments. The variability explained by the interaction between log-transformed time and treatment is 0.3078 (p<0.001) with 2 degrees of freedom (DF) as compared to 0.0421 (p<0.07) on one DF for the interaction between duplicate and log-transformed time. For the experiment with the estuarine inoculum, the variability between the duplicates was not much different as compared to the other treatments as shown by the corresponding values of 0.2531 (p<0.001) and 0.1400 (p<0.001), respectively.

Table 1. Decrease of DOC (ΔDOC) for the entire course and phase 1 (day 0-34) and 2 (day 34-end) of the flow-through experiments inoculated with a marine and an estuarine bacterial community and incubated with a humic acid medium of the salinity indicated. Given is ΔDOC in mg and as % of the initial concentration. Combusted sediment particles were added to treatments Mar3, Mar4, Est3 and Est4.

	Marine						Estuarine					
	Mar1	Mar2	Mar3	Mar4	Mar5	Mar6	Est1	Est2	Est3	Est4	Est5	Est6
salinity	28	28	28	28	14	14	14	14	14	14	28	28
Total ΔDOC (mg C l^{-1})	4.8	3.6	4.7	8.7	10.5	7.3	7.5	8.2	8.4	11.3	11.0	14.3
ΔDOC phase 1 (mg C l^{-1})	3.1	2.3	3.3	6.8	8.4	5.2	5.2	6.0	6.0	9.2	8.0	11.3
ΔDOC phase 2 (mg C l^{-1})	1.7	1.3	1.4	1.9	2.1	2.1	2.3	2.2	2.4	2.1	3.0	3.0
Total ΔDOC (%)	48.3	37.8	45.7	72.6	74.6	54.7	54.4	59.1	62.9	71.7	64.9	75.6
ΔDOC phase 1 (%)	39.5	35.7	45.0	66.6	69.5	54.1	50.5	56.3	57.6	71.5	63.9	75.0
ΔDOC phase 2 (%)	8.8	2.1	0.7	6.0	1.1	0.6	3.9	2.8	5.3	0.2	1.0	0.6

Bacterial abundance and production

Bacterial abundance strongly increased from initially ~$1.0x10^5$ cells ml^{-1} to $2\text{-}8x10^6$ cells ml^{-1} in the experiment with the marine inoculum (Fig. 2d-f) and to >$1.0x10^7$ cells ml^{-1} in the experiment with the estuarine inoculum within 2-3 days (Fig. 3d-f). Thereafter, numbers decreased rapidly again but reached a second peak in both experiments and all treatments after 10 to 20 days. Later on, bacterial abundance went down again, fluctuated slightly and finally levelled off around $2\text{-}5x10^5$ cells ml^{-1} in the experiment with the marine inoculum and around $0.8\text{-}1.2x10^6$ cells ml^{-1} in the experiment with the estuarine inoculum. At least 90% of the bacteria were free-living cells, based on microscopic evidence, also in the treatments with added sediment particles. The duplicates of each treatment in both experiments exhibited significantly different patterns of bacterial abundance over time. In the experiment with the marine inoculum, however, they more closely resembled those of the other treatments as shown by the linear model of the variance of DOM decomposition applied to bacterial abundance. It explained a variance of 12.727 (p<0.002) on 6 DF for the interaction between the cubic polynome in time and treatment as compared to the explained variance of 0.833 (p< 0.690) on 3 DF for the interaction between duplicates. For the data of the experiment with the estuarine inoculum, the difference between duplicates and between treatments is of similar magnitude showing that the duplicates were as different as the various treatments (3.204, p<0.779, on 6 DF versus 2.325, p<0.507 on 3 DF).

Rates of BP were measured less frequently than bacterial abundance and DOC concentrations. In the experiment with the marine inoculum, data on BP are only available from day 13 to 69 (Fig. 2g-i). Rates of BP before day 30 were higher than thereafter and values in the treatments with the estuarine HA medium (Mar5, Mar6) were highest, consistent with the highest DOM decomposition in these treatments. The linear variance model was not applied as much fewer BP data than of DOC concentration and bacterial abundance were available and only starting on day 13. Pooling the data of all treatments, temporal dynamics of BP covaried significantly with bacterial abundance (Pearson correlation, n=42, r=0.63, p<0.001).

In the experiment with the estuarine inoculum highest rates of BP were measured within the initial 14 days in all treatments (Fig. 3g-i). Temporal dynamics were pronounced until day 40. As for the other experiment, the linear variance model was not applied. Pooling all BP data, they covaried significantly with bacterial abundance (Pearson correlation, n=65, r=0.79, p<0.001).

Pyrolysis GC/MS analysis of humic and fulvic acids

At the end of the HA decomposition experiments low amounts of HA were still isolated, but in addition to HA also FA, indicating that HA were not only decomposed but partly broken down to FA. Enough HA material from each of the various treatments of both experiments was isolated for Py-GC/MS analysis except from treatments Mar1 and Mar2 of which some material got lost during the extraction procedure. Fulvic acid fractions were obtained from all treatments of both experiments except treatment Mar1. Because of the low amount of HA and FA finally obtained we were unable to make a mass balance of the amount initially added, the fraction decomposed and that bound in both humic fractions.

In the HA medium obtained from the sample collected in June 2007 and used for the experiment inoculated by a marine bacterial community, 32 compounds were identified in the pyrolyzate (Tables 2, S1, S2). They included 15 aliphatic compounds, comprising short-chained saturated and unsaturated mono-, di- and tricarboxylic fatty acids (Table S1), and 17 aromatic compounds such as benzene and benzoic acid derivatives and a cinnamic acid derivative (Table S2). Derivatization with TMAH converted most compounds into methyl esters. At the end of the decomposition experiment between 2 and 19 of these compounds were still identified in the remaining HA fractions. Only two compounds were detected in treatment Mar4 but 15-19 in the other treatments (Tables 2, S1, S2). Aliphatic compounds were decomposed relatively more effectively than aromatic compounds, resulting in an enhanced aromaticity of 80->90% in the HA fraction at the end of the decomposition experiment, based on the pyrolysis products (Fig. 4a)

Several of the aliphatic compounds still present in the FA fraction were not detected in any or found only in one or a few of the treatments in the HA fraction, suggesting that some HA compounds were broken down into smaller units (Table S1). Hence, the aromaticity of the FA fractions was lower than that of the HA fractions and only slightly higher (66-73%) than that of the initial HA fraction (62%; Fig. 4b). Even though the aromaticity of the decomposed HA fraction was higher than that of the HA medium aromatic compounds were also decomposed. This can be inferred from the general decrease of the DOC concentration but is also obvious from the fact that in several treatments a number of compounds such as benzene derivatives were not detected or disappeared completely in the HA fractions of all treatments; examples are benzene tricarboxylic acid derivatives, and 3-(3,4-dimethoxyphenyl)-2-propenoic acid methylester (Table S2). The experiment with the marine inoculum, however, did not yield consistent results in this respect as in some replicates of the

Fraction	HA		FA
	number	%	number
HA medium marine	32	100	0
Mar1	na	na	Na
Mar2	na	na	20
Mar3	19 (1)*	61	30
Mar4	2	6	24
Mar5	18	58	32
Mar6	15	48	21
HA medium estuarine	49	100	0
Est1	22	45	36
Est2	25	51	37
Est3	29	59	36
Est4	16	33	40
Est5	28	57	39 (2)*
Est6	26	53	39

* tentatively identified compounds

Table 2: Number and percentage of compounds identified after PyGC/MS analysis of HA media for the flow-through cultures inoculated with a marine and estuarine bacterial community and in the HA and FA fractions of the various treatments of both experiments collected at the end of the experiments. For details of the treatments see Fig. 1. na: not available

various treatments the relative proportions of some of the aromatic compounds remained constant or even increased relative to the HA medium (Fig. 5a). Changes in aromaticity in the FA fractions relative to the HA medium were less pronounced than in the HA fraction (Fig. 5b).

In the HA medium obtained from the sample collected in May 2008 and used for the experiment inoculated by an estuarine bacterial community 49 compounds were identified in the pyrolysate (Table 2). Fifteen aliphatic compounds were detected, belonging to the same classes of short-chain fatty acids as those of the HA medium of the experiment with the marine inoculum of the previous year (Table S1). However, the composition differed from

that of the previous year. The HA medium of the estuarine experiment contained many more aromatic compounds than that for the marine experiment, 39 in total (Table S2), including four phenol derivatives which were not considered further in the comparatative analyses because they were most probably products of incomplete methylation by TMAH. The greater diversity was mainly due to more than twice as many benzene derivatives and to phenylacetic acid, phenylketone and phenol derivatives which occurred only in the HA medium used for the estuarine experiment.

At the end of this decomposition experiment the number of identified compounds also decreased, i.e. to 36-62% of the initial number in the various treatments (table 2). As in the other experiment, a greater number of compounds was detected in the FA fraction. However, the aromaticity did not systematically increase in the various treatments and replicates (Fig. 4c). In treatments Est1, Est4 and Est6 it increased by 10-20% relative to the initial value of 69% but decreased in treatments Est3 and Est5. The aromaticity of the FA fractions in this

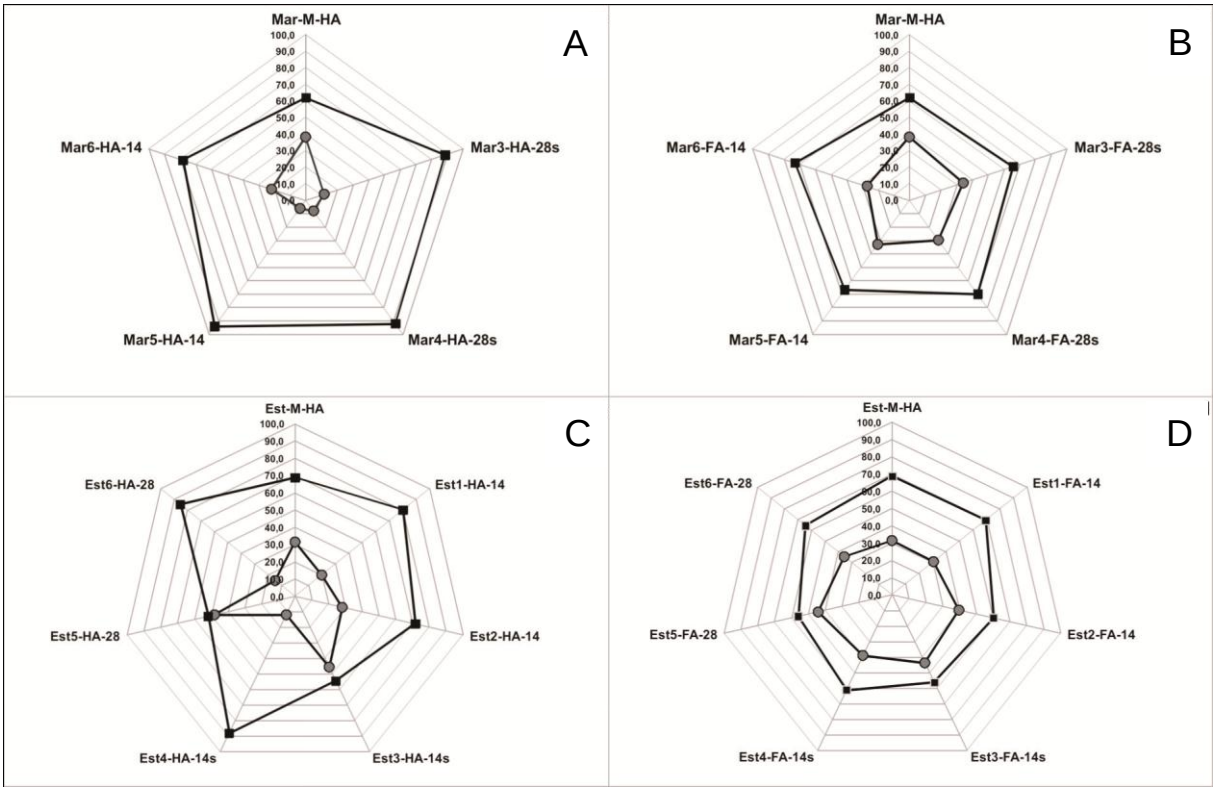

Figure 4: Percentages of aliphatic (inner lines and grey circles) and aromatic compounds (outer lines and black squares) identified in the HA media and HA and FA fractions at the end of the flow-through culture experiments supplied with HA media of salinities 14 and 28. A: Marine HA medium (Mar-M-HA) and HA fractions of treatments Mar3, Mar4, Mar5, Mar6. B: Marine HA medium (Mar-M-HA) and FA fractions of treatments Mar3, Mar4, Mar5, Mar6. C: Estuarine HA medium (Est-M-HA) and HA fractions of treatments Est1, Est2, Est3, Est4, Est5, Est6. D: Estuarine HA medium (Est-M-HA) and FA fractions of treatments Est1, Est2, Est3, Est4, Est5, Est6.

experiment was also different from that of the HA fractions but, in contrast to the experiment with the marine inoculum, remained constant or slightly decreased relative to that of the initial HA fraction (Fig. 4d). Differences in aliphatic compounds between the HA and FA fractions were not that pronounced in the estuarine experiment as compared to the marine experiment.

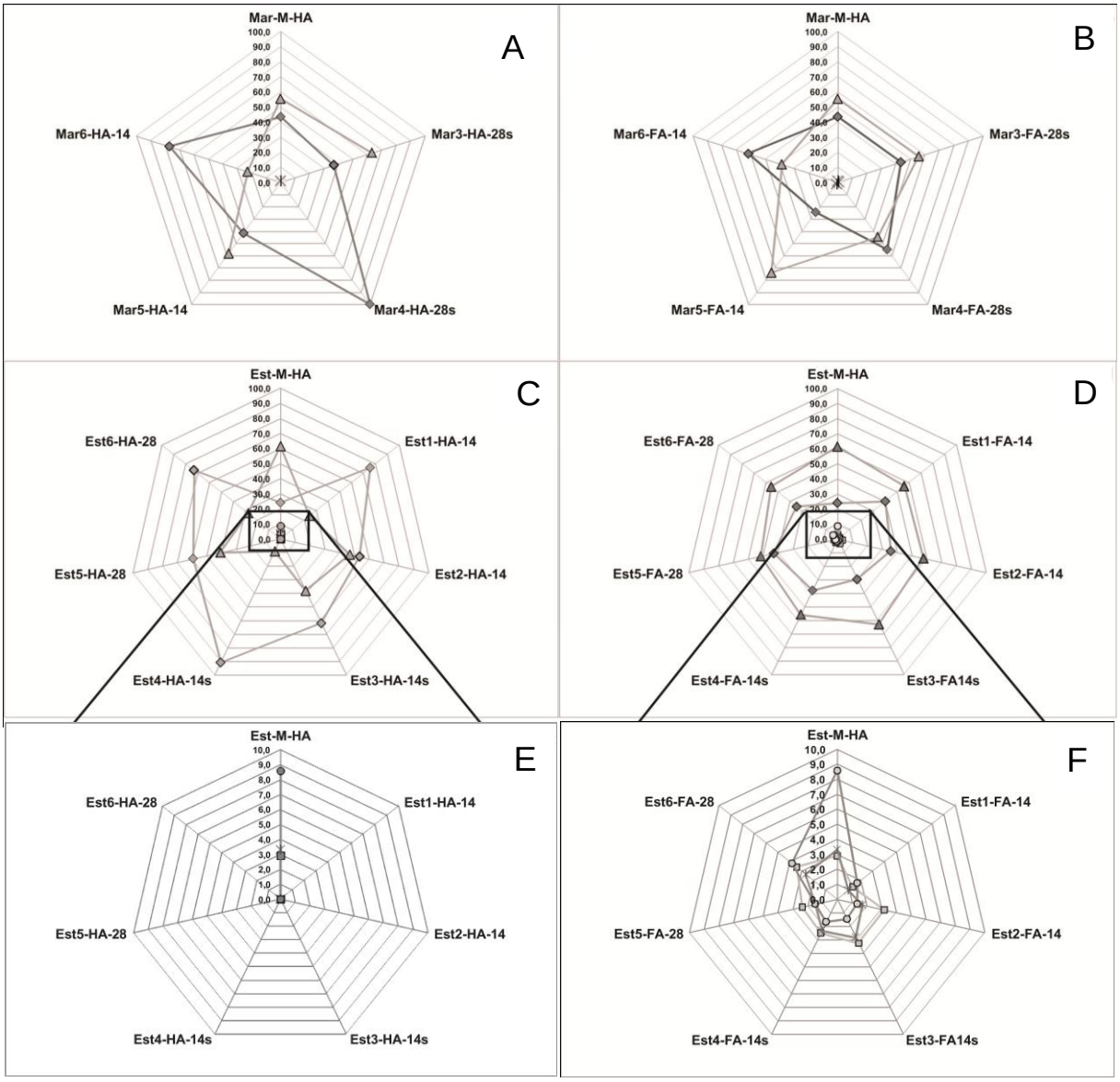

Figure 5: Percentages of monoaromatic acids (Δ), monoaromatic benzenes (◊), cinnamic acid derivates (+), phenylketones (O, only panel e and f), and phenyl acetic acids (□, only panel e and f) identified in the HA media and HA and FA fractions at the end of the flow-through culture experiments supplied with HA media of salinities 14 and 28. A: Marine HA medium (Mar-M-HA) and HA fractions of treatments Mar3, Mar4, Mar5, Mar6. B: Marine HA medium (Mar-M-HA) and FA fractions of treatments Mar3, Mar4, Mar5, Mar6. C: Estuarine HA medium (Est-M-HA) and HA fractions of treatments Est1, Est2, Est3, Est4, Est5, Est6. D: Estuarine HA medium (Est-M-HA) and FA fractions of treatments Est1, Est2, Est3, Est4, Est5, Est6. E, F: Close up of panels C and D with phenylketones (O), and phenyl acetic acids (□).

Only two of the saturated fatty acid derivatives detected in the FA fractions were not detected in the HA fractions (Table S1). Of the unsaturated and dicarboxylic fatty acid derivatives only few compounds were not detected in several replicates of the various treatments. Greater differences between both humic fractions occurred with respect to aromatic compounds. Several benzene and all cinnamic and phenylacetic acid as well as phenylketone derivatives were not detected in the HA fractions of all or several treatments but were present in the FA fractions (Table S2).

Discussion

The marine and the estuarine bacterial communities were able to decompose HA to ~40->60% of the initial concentration within approximately 70 days and most of the decomposition took place during the initial 21 to 28 days. A smaller proportion was decomposed in both experiments when the inoculum was supplied with the HA medium of the salinity similar to that of the inoculum. Higher fractions were removed in the treatments with added sediment particles but also in those in which the inoculum was supplied with a HA medium of the foreign salinity, i.e. a salinity of 28 in the experiment with the estuarine inoculum and of 14 in that with the marine inoculum. There was quite some variability of the temporal dynamics of the DOM decrease wirthin the replicates and only in the experiment with the marine inoculum was this variability significantly lower than that among the three different treatments. However, the proportion of the DOM decomposed was consistently greater in both experiments in the treatments with the added sediment particles and the HA medium with the foreign salinity as compared to that with the salinity of the inoculum.

We can not completely rule out the possibility that the HA media of some of the replicates and treatments got contaminated by non-HA DOM as the initial concentrations in the various treatments and between replicates showed some variability. However, the main reason for this variability was the fact that the freeze-dried HA were redissolved to different degrees in the various media. Flocculation did not contribute to HA removal as shown by the abiotic controls but adsorption of HA to the added sediment particles and adhesion of the latter to the wall of the incubation bottles obviously caused some abiotic HA removal. It had been shown that HS readily adsorb to particles and soil (Kaiser and Guggenberger 2000). Therefore, we assume that the enhanced removal rate in these treatments was at least partially due to physico-chemical adsorption of HA to the sediment particles and not a true bacterial decomposition.

Hence, considering only microbial decomposition, the highest fraction of HA was decomposed in both experiments when the inoculum was exposed to the HA medium of the foreign salinity. This result appears puzzling as there was no consistent effect of salinity on the decomposition and thus on a possible physico-chemical modification of the HA. It rather appears that the microbial communities enhanced their decomposition properties when they were exposed to HA at foreign salinity but we have no clear-cut explanation for this effect. Interestingly, there is one report consistent with our findings of this salinity effect. Boyd and Osborn (2004) found that the fluorescence properties of CDOM and its humic constituents in the estuarine salinity gradient of San Francisco Bay (0 to 33) changed most significantly when the CDOM of a given salinity was incubated at a different salinity with a bacterial community of this salinity. This effect was not observed in the Chesapeake Bay. The authors speculate that changes in the conformational structure may have occurred when in particular the humic DOM was exposed to a different salinity. Newly activated ligands and functional groups may have become available inducing new enzyme activities and possibly the activity of other bacteria such that the decomposition was enhanced. As our observations are consistent with those of Boyd and Osburn (2004) it is conceivable that similar interactions between the HA and the bacterial communities at varying salinities may explain our findings.

Estuaries and intertidal flat systems are characterised by intense resuspension of sediment particles (Lunau et al. 2006; Bartholomä et al. 2009), mixing of water masses of different salinities and are rich in HS and CDOM (Fooken and Liebezeit 2000; Chen et al. 2007; Lübben et al. 2009). Hence we conclude that these systems and shallow seas with highly structured coastal environments with a water residence time of days to weeks are a prime location for bacterial decomposition and adsorptive removal of HA and presumably other humic DOM components of terrestrial origin. It was shown that HA of terrestrially-derived DOM are decomposed by marine as well as estuarine bacterial communities at salinities 15 and 5, whereas other humic fractions, FA and hydrophilic acids, were decomposed only by marine bacterial communities (Kisand et al. 2008). The results of our study confirm these findings and extend them by showing by means of Py-GC/MS that specific aliphatic and aromatic compounds of the HA fraction are decomposed and that HA are partly broken down to smaller units entering the FA fraction. Humic acids appear to be better degradable than FA, thus their removal may explain to a substantial extent the disappearance of humic matter and CDOM in the estuarine mixing zone and the costal sea. Anesio et al. (2005) also showed that HA are better degradable than FA by limnetic bacterial communities, however, only after irradiation with UV light. Our findings are consistent with

the size reactivity continuum model proposed by Amon and Benner (1996) and with other reports, that FA and low-molecular-weight (<1000 Da) DOM in the sea are more diagenetically altered and thus less biodegradable than HA and high-molecular-weight (>1000 Da) DOM (Benner 2002; Loh et al. 2004) and shows that this model also applies to the microbial decomposition of HA.

It is well known that flocculation and adsorption to suspended matter (Sholkovitz 1976; Sholkovitz et al. 1978; Uher et al. 2001) and photochemical mineralization and subsequent microbial decomposition (Moran et al. 2000; Boyd and Osburn 2004; Pullin et al. 2004; Chen et al. 2007, Farjalla et al. 2009) are important processes in the removal of humic DOM in estuaries. The specific role of bacterial decomposition of humic DOM was mainly studied with irradiated DOM showing that this effect favors the bacterial decomposition of CDOM. Our study, together with that of Kisand et al (2008), demonstrates that estuarine and marine bacterial communities are well capable of decomposing humic DOM and specifically HA also without any irradiation. As the water in estuaries and intertidal flat systems is very turbid photodegradation presumably is much less important than further out in the coastal zone and possibly overestimated. Our results and those of Kisand et (2008) suggest that bacterial communities can decompose large fractions of HA and other refractory DOM in estuaries and that this process has been underestimated relative to photodegradation, flocculation and adsorption.

We applied flow-through cultures with a dilution rate of 0.01 d^{-1} to assess the HA decomposition by estuarine and marine bacterial communities and found that >60% of the HA were decomposed within ~70 days. During this time 70% of the medium in the incubation bottles was replaced, allowing a permanent supply not only of HA but also of inorganic nutrients. Other studies examining the decomposition of HA or CDOM and yielding lower fractions of DOC decomposed applied batch cultures and mesocosms, presumably leading more easily to limiting conditions by other factors than supply of DOC (Moran et al. 2000; Obernosterer and Benner 2004; Anesio et al. 2005, Benner and Kaiser 2011). Hence, we suggest that flow-through cultures are more appropriate for such studies, mimicking the ambient conditions in a more appropriate way than mesocosms.

Free-living bacteria dominated to >90% in both experiments and in all treatments including those with the added sediment particles. However, as the inocula of our experiments were 5 µm prefiltered natural samples they presumably did not only contain bacteria but also nanoflagellates. The strong decline in bacterial numbers after the first peak in all treatments is an indication that grazing occurred during this time and presumably also later, even though

fluctuations in bacterial numbers decreased after 2-3 weeks. Hence, grazing by nanoflagellates also contributed to the decomposition of HA, presumably not directly, but indirectly by recycling of bacterial biomass and factors limiting bacterial growth and by affecting the composition of the bacterial communities. The composition of the bacterial communities in both experiments exhibited pronounced differences. As shown by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments the number of bands decreased over time and changes were most pronounced during the main decomposition phase of the initial 21 to 28 days (Rocker et al. 2011).

The marine bacterial community was largely dominated by *Alphaproteobacteria* whereas the estuarine community mainly consisted of *Gammaproteobacteria*. The isolation of *Alphaproteobacteria* capable of growing on HA and lignin building blocks such as cinnamic, coumaric, ferrulic, salicylic and vannilic acid at the end of the experiment with the marine inoculum demonstrated that some members of this community were truly able to decompose the supplied HA medium and obviously also the lignin biomarkers.

The Py-GC/MS analysis after TMAH derivatization allowed us to elucidate the HA composition in the media and their modification after bacterial decomposition in the various treatments of both experiments to a certain extent. This technology had been applied before to elucidate the composition of HS in soil and aquatic systems (Martin et al. 1995; Schulten and Sorge 1995; Lehtonen et al. 2000; Frazier et al. 2005; Poerschmann et al. 2007) but not yet of HS or HA during bacterial decomposition and alteration. We were able to identify 32 and 45 aliphatic and aromatic compounds in the marine and estuarine HA medium, respectively (Table 2). Our analysis showed changes in composition of the HA used for both media even though the HA were obtained from the same location at roughly the same season but in two consecutive years. Humic acids of the later year used for the medium of the experiment with the estuarine inoculum contained more aromatic compounds than HA of the previous year. Less than 60% of the compounds detected initially in the HA media were neither detectable in the HA nor FA fraction of the outflow at the end of both experiments. This suggests that these compounds were decomposed even though it can not be excluded that pyrolysis of the HA and FA moieties isolated from the outflow were too small to generate these pyrolysis products from both fractions.

We are aware of the fact that only a small proportion of the isolated organic material can be characterized by Py-GC/MS. Yields of volatile products are estimated between 5 and 10% (van de Meent et al. 1980) or 7 and 15 wt% of total organic carbon (Poerschmann et al. 1997, respectively. Overall, the DOM is much more complex as the most sensitive analytical

technologies such as electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS; Koch et al. 2005) have demonstrated. Nevertheless, Py-GC/MS appears to be a valuable tool to demonstrate qualitative changes of DOM and specific fractions such as HA during microbial decomposition. Both, aliphatic and aromatic compounds disappeared during decomposition completely in either experiment or only in the HA fraction, suggesting that some moieties of the complex HA containing these compounds were completely decomposed or broken down to FA. In the experiment with the marine inoculum, relatively more aliphatic compounds were decomposed in the HA fraction as the aromaticity of this fraction was higher at the end of the experiment relative to the HA medium at the beginning (Fig. 4). The aromaticity of the FA fraction, however, exhibited only little changes as compared to the HA medium, suggesting that those moieties of the HA fraction broken down to FA were altered relatively little by bacterial enzymatic attack. There was, however, no systematic difference among the three treatments, indicating that the specific bacterial enzymatic attack was not affected by the different conditions in the treatments. In the experiment with the estuarine inoculum changes in aromaticity were less consistent.

Tracking individual compounds by Py-GC/MS showed that several aromatic compounds disappeared completely or only in the HA fraction and/or in several treatments also in the FA fraction (Table S2). Of particular interest were aromatic compounds with biomarker properties of lignin such as 1,2,3-trimethoxybenzene, methoxybenzoic acid methyl esters, cinnamic acid and phenylacetic acid derivatives (Martin et al. 1995; Hatcher and Minnard 1996; Lehtonen et al. 2000; Page et al. 2001; Benner and Kaiser 2011). 1,2,3-Trimethoxybenzene (AW) disappeared completely in the experiment with the estuarine inoculum and 3-(3,4-dimethoxyphenyl)-2-propenoic acid methyl ester in the experiment with the marine inoculum in the HA fraction and in one of the replicates of the treatments of which enough material for Py-GC/MS analysis could still be isolated (Mar3-4, Mar5-6; Table S2). Other compounds, like the benzene and phenylacetic acid derivatives, exhibited little changes during the bacterial decomposition or disappeared only in the HA fraction in one or both experiments. These observations suggest that only some lignin biomarkers were bacterially decomposed whereas most of them were resistant to bacterial decomposition and transferred from the HA to the FA fraction, presumably by the break down to smaller moieties with similar functionality. This is consistent with other reports that the bacterial degradation of lignin-derived aromatic compounds is rather limited and only greatly enhanced after photochemical oxidation (Moran et al. 2000; Obernosterer and Benner 2002; Benner and Kaiser 2011).

In conclusion, we have shown that marine and estuarine bacterial communities decompose HA by ~40->60% and that the decomposition is strongest when marine bacteria decompose HA under estuarine conditions and estuarine bacteria under marine conditions. Part of the HA were not completely decomposed but broken down to FA which is consistent with the size reactivity continuum model of Amon and Benner (1996). Py-GC/MS proved to be a valuable tool to elucidate to a certain extent the composition of HA and to identify aliphatic and aromatic compounds and whether they were decomposed. Our results, together with those of Kisand et al. (2008), indicate that the bacterial decomposition of HA and of other humic DOM in the estuarine transition zone and in intertidal flat systems is an important sink for terrestrial DOM.

Acknowledgements We are most grateful to A. Schlingloff for DOC analyses. To B. Kuerzel for help with the flow cytometric analyses of bacterial numbers and to H. Simon for assistance in sample collection. This work was supported by grant Si 360/22-1 from Deutsche Forschungsgemeinschaft (DFG). V. Kisand was supported by the European Regional Development Fund through the Center of Excellence in Chemical Biology, Estonia.

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Supplementary Information

Table S1: Aliphatic compounds detected by the pyrolysis GC/MS analysis of the HA media used in the experiments with a marine (Mar-M) and estuarine (Est-M) inoculum and in the HA and FA fractions of the various treatments collected after microbial decomposition. Samples for the marine medium (Mar-M) were collected in June 2007 and for the estuarine medium in May 2008. nd: not detected

peak		Mar-M	HA-Mar	FA-Mar	Est-M	HA-Est	FA-Est
	saturated fatty acid methyl ester (FAME)						
B	Propanoic acid, methyl ester	X	Mar3, Mar4, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
F	Propanoic acid, 2-methyl-, methyl ester	X	Mar5	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5,
H	Acetic acid, hydroxy-, methyl ester				X	nd	Est3
K	Acetic acid, methoxy-, methyl ester	X	nd	Mar2, Mar3, Mar4, Mar5			
N	Butanoic acid, 2-methyl-, methyl ester	X	nd	Mar2, Mar3, Mar5			
O	Propanoic acid, 2-methoxy-, methyl ester	X	Mar3	Mar2, Mar3, Mar4, Mar5			
U	Butanoic acid, 3-methyl-2-oxo-, methyl ester	X	nd	Mar3, Mar4, Mar5			
Z	Pentanoic acid, 4-oxo-, methyl ester				X	nd	Est2
AT	Nonanoic acid, methyl ester				X	nd	nd
BT	Pentadecanoic acid, 14-methyl-, methyl ester	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6			
BU	Octadecanoic acid, methyl ester	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5			
	unsaturated fatty acid methyl ester (FAME)						
A	2-Propenoic acid, methyl ester	X	Mar6	Mar2, Mar3, Mar5, Mar6	X	Est2, Est3, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
G	2-Propenoic acid, 2-methyl-, methyl ester	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
I	2-Butenoic acid, methyl ester, (Z)-				X	Est1	Est1, Est2, Est3, Est4, Est5, Est6
L	2-Butenoic acid, methyl ester, (E)-				X	Est1	Est1, Est3, Est4, Est5, Est6

Table S1 (continued):

peak		Mar-M	HA-Mar	FA-Mar	Est-M	HA-Mar	FA-Mar
	saturated dicarboxylic acids						
AD	Butanedioic acid, dimethyl ester	X	Mar3	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AE	Butanedioic acid, methyl-, dimethyl ester	X	Mar3,	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AI	Butanedioic acid, 2,2-dimethyl-, dimethyl ester	X	Mar3,	Mar3, Mar4, Mar5, Mar6			
AN	Pentanedioic acid, dimethyl ester				X	Est3, Est5,	Est1, Est2, Est3, Est5, Est6
	unsaturated dicarboxylic acids						
AC	2-Butenedioic acid (E)-, dimethyl ester	X	Mar3,	Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AK	Butanedioic acid, methylene-, dimethyl ester				X	Est3, Est5,	Est1, Est2, Est3, Est4, Est5, Est6
	tricarboxylic acids						
BE	Propane tricarboxylic acid 1,2,3-trimethylester	X	nd	Mar3, Mar5,	X	Est2, Est3, Est5,	Est1, Est2, Est3, Est4, Est5, Est6

Table S2: Aromatic compounds detected by the pyrolysis GC/MS analysis of the HA media used in the experiments with a marine (Mar-M) and estuarine (Est-M) inoculum and in the HA and FA fractions of the various treatments collected after microbial decomposition. Samples for the marine medium (Mar-M) were collected in June 2007 and for the estuarine medium in May 2008.

peak		Mar-M	HA-Mar	FA-Mar	Est-M	HA-Mar	FA-Mar
	benzene derivates						
E	Benzene				X	nd	nd
M	Toluene				X	Est2, Est3, Est5,	Est4, Est5,
Q	Ethylbenzene				X	nd	nd
S	Benzene, 1,2-dimethyl-				X	nd	Est4, Est5,
T	Benzene, 1,3-dimethyl-				X	nd	Est5, Est6
W	Benzene, methoxy-	X	Mar5, Mar6	Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AA	Benzene, 1-methoxy-2-methyl-	X	Mar5, Mar6	Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est5, Est6	Est1, Est3, Est4, Est5, Est6
AB	Benzene, 1-methoxy-4-methyl-	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AM	Benzene, 1-ethyl-4-methoxy-				X	nd	nd
AO	Benzene, 1,2-dimethoxy-	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est3, Est6	Est1, Est2, Est4, Est5, Est6
AP	Benzene, 1-ethenyl-4-methoxy-				X	Est6	Est1, Est2, Est3, Est4, Est5, Est6
AQ	Benzene, 1,4-dimethoxy-	X	Mar3, Mar5,	Mar2, Mar3, Mar4, Mar5,	X	Est1, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AR	Benzene, 1,3-dimethoxy-	X	Mar3, Mar4, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AU	2,4-Dimethoxytoluene				X	Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AW	1,2,3-Trimethoxybenzene				X	nd	nd
BA	1,2,4-Trimethoxybenzene				X	nd	Est2, Est4, Est5,
BD	Benzene, 1,3,5-trimethoxy-	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2,	Est4, Est6

Table S2 (continued)

peak		Mar-M	HA-Mar	FA-Mar	Est-M	HA-Mar	FA-Mar
	benzene dicarboxylic acids						
BI	1,4-Benzenedicarboxylic acid, dimethyl ester				X	Est2, Est3, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BJ	1,3-Benzenedicarboxylic acid, dimethyl ester				X	Est2, Est3, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
	benzene tricarboxylic acids						
BQ	1,2,4-Benzenetricarboxylic acid, trimethyl ester	X	nd	Mar5,	X	nd	nd
BS	1,3,5-Benzenetricarboxylic acid, trimethyl ester	X	nd	Mar5,			
	benzoic acid derivates/benzaldehyde derivates						
AJ	Benzoic acid, methyl ester	X	Mar3, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
AX	Benzoic acid, 3-methoxy-, methyl ester	X	Mar3, Mar5,	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BB	Benzoic acid, 4-methoxy-, methyl ester	X	Mar5,	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BF	Benzoic acid, 3-methoxy, 4-methyl, methyl ester				X	Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BH	Benzaldehyde, 3,4-dimethoxy-	X	Mar5,	Mar2, Mar3, Mar5,	X	Est2, Est3, Est5, Est6	Est1, Est2, Est4, Est5, Est6
BL	Benzoic acid, 3,5-dimethoxy-, methyl ester	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BM	Benzoic acid, 3,4-dimethoxy-, methyl ester	X	Mar3, Mar5, Mar6	Mar2, Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
BP	Benzoic acid, 3,4,5-trimethoxy-, methyl ester	X	Mar3, Mar5, Mar6	Mar3, Mar4, Mar5, Mar6	X	Est1, Est2, Est3, Est4, Est5, Est6	Est1, Est2, Est3, Est4, Est5, Est6
	cinnamic acid derivates						
BO	2-Propenoic acid, 3-(4-methoxyphenyl)-, methyl ester				X	nd	Est1, Est2, Est3, Est4, Est5, Est6
BR	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	X	nd	Mar3, Mar5,	X	nd	Est1, Est2, Est3, Est4, Est5, Est6

Table S2 (continued)

peak		Mar-M	HA-Mar	FA-Mar	Est-M	HA-Mar	FA-Mar
	phenylacetic acid						
BG	Benzeneacetic acid, 4-methoxy-, methyl ester				X	nd	Est1, Est2, Est3, Est4, Est5, Est6
BN	Benzeneacetic acid, 3,4-dimethoxy-, methyl ester				X	nd	Est3, Est4, Est5, Est6
	phenylketone						
AY	Ethanone, 1-(3-methoxyphenyl)-				X	nd	Est1, Est2, Est3, Est4, Est5,
BC	2-Propanone, 1-(4-methoxyphenyl)-				X	nd	nd
BK	Ethanone, 1-(3,4-dimethoxyphenyl)-				X	nd	Est1, Est2, Est3, Est4, Est5,
	phenoles						
Y	Phenol				X	nd	nd
AG	Phenol, 4-methyl-				X	nd	nd
AH	Phenol, 2-methoxy-				X	nd	nd
AS	Phenol, 2-methoxy-4-methyl-				X	nd	nd
	not classified						
P	N,N-Dimethyl-2-ethoxyethylamine	X	nd	nd			
AL	2,5-Dimethylanisole	X	nd	nd			

Fig. S1

Pyrolysis-GC/MS chromatogram of the pyrolyzate used for media in the dilution cultures inoculated with a marine bacterial community (Mar-M). Identified peaks are marked A to AH. For the name of the compounds identified see Tables S1 and S2.

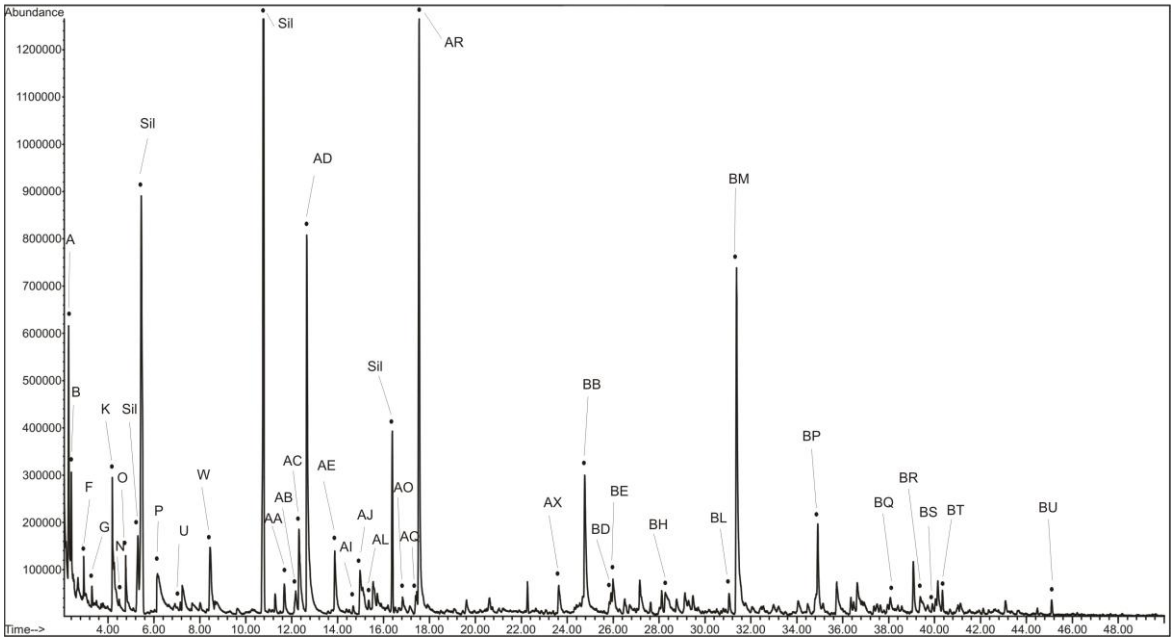
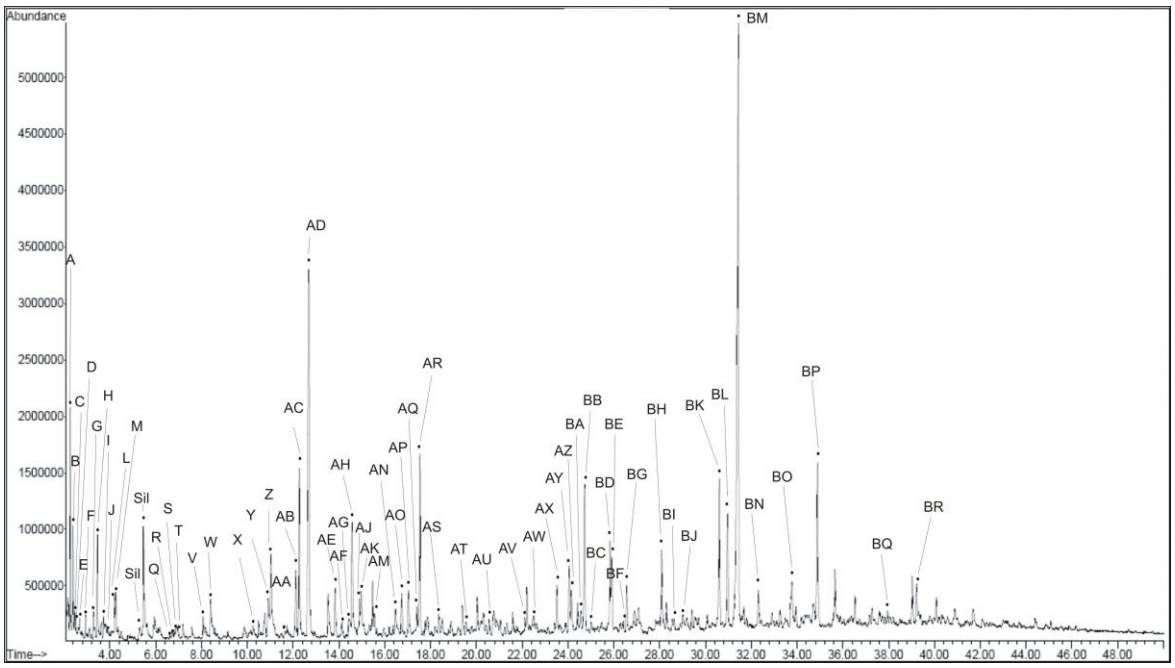


Fig. S2

Pyrolysis-GC/MS chromatogram of the pyrolyzate used for media in the dilution cultures inoculated with estuarine bacteria (Est-M). Identified peaks are marked A-BJ. For the name of the compounds identified see Tables S1 and S2.



4

Significant decomposition of riverine humic-rich DOC by marine but not estuarine bacteria assessed in sequential chemostat experiments

Significant decomposition of riverine humic-rich DOC by marine but not estuarine bacteria assessed in sequential chemostat experiments

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Key words: humic substances, DOC, bacteria, chemostats, estuary, marine

Vol. 53: 151–160, 2008
doi: 10.3354/ame01240

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online September 29, 2008

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ABSTRACT: Humic substances (HS) are the most abundant natural organic compounds in aquatic and terrestrial environments. However, the bacterial degradation of HS in the estuarine salinity gradient and in coastal regions as a sink for HS, entering the open sea, is not well understood. Therefore, we studied the bacterial degradation of humic-rich dissolved organic carbon (DOC) at increasing salinities between 1 and 30 in an estuary of the southern North Sea (Weser). Three-stage chemostats, inoculated with natural brackish (salinity 5 and 15) and coastal marine (salinity 30) bacterial communities fed 0.1 µm filtered natural humic-rich freshwater adjusted to the respective salinity, were run for up to 51 d at dilution rates of 0.1 and 0.15 d^{-1}. DOC concentrations, bacterial numbers, and production were assessed in each stage over the incubation time. In addition, the fulvic acid (FA), humic acid (HA), and hydrophilic acid (HPA) fractions of the HS were determined by XAD fractionation. At a salinity of 30, the humic-rich DOC was decomposed to more than 60%, whereas in the other 2 experiments at salinities of 15 and 5, no detectable decomposition occurred. At the highest salinity condition, all 3 HS fractions were reduced substantially, whereas at a salinity of 5, only the HA and HPA fractions decreased, and at 15, only the HA fraction decreased. In the latter experiments, concentrations of the FA and HPA fractions in some chemostat stages increased despite unchanged DOC concentration. Our study provides evidence that the main bacterial decomposition, i.e. conversion into bacterial biomass and remineralization, of humic-rich DOC occurs in the polyhaline estuarine region and that at lower salinities, only some transformation of the humic fractions takes place, modifying the adsorptive properties of the HS, but no bacterial DOC decomposition occurs. The sequential chemostats were a useful approach to study the complex process of bacterial HS decomposition.

KEY WORDS: Humic substances · DOC · Bacteria · Chemostat · Estuary · Marine

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INTRODUCTION

Humic substances (HS) are the most abundant natural organic compounds in aquatic and terrestrial environments. They represent up to 25% of total organic carbon on earth and up to 50% of organic carbon in freshwaters (Aiken et al. 1985). Despite considerable discharge of river water into the sea, the terrigenous dissolved organic matter (DOM) comprises only a small fraction (0.7 to 2.4%) of the total DOM in the ocean (Opsahl & Benner 1997).

HS are usually defined as organic compounds of different solubility at low pH or adsorbed to various types of hydrophobic macroporous resins at low pH (e.g. XAD-2, XAD-4, XAD-8) and eluted by alkaline solutions and hydrophobic eluants (e.g. Thurman & Malcolm 1981, McKnight & Aiken 1998). The major compounds isolated by these procedures include the 2 hydrophobic fractions: fulvic acids (FA), with a molecular weight range of 500 to 1200 Da, and humic acids (HA), which represent the high molecular weight fraction. The third fraction, hydrophilic acids (HPA), has

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the lowest molecular weight, i.e. <600 Da. Riverine inputs of dissolved organic carbon (DOC), of which HS comprise a substantial fraction, constitute the major flux of such compounds into the coastal zone.

Flocculation at increasing salinity is one important process of DOC removal during the estuarine passage (Sholkovitz 1976, Sholkovitz et al. 1978, Kerner et al. 2003), but photochemical and bacterial degradation also contribute to this removal (see below). Present estimates imply that terrestrial and thus humic DOC, after mixing with seawater, is degraded rapidly in the coastal zone (Hedges et al. 1997, Cauwet 2002, Mannino & Harvey 2004). Hence, estuaries, the transition zone between freshwater and coastal marine environments, presumably are of prime importance for the degradation and thus for losses of riverine and terrestrial DOC.

Degradation, formation, and transformation of HS are complex processes. Heterotrophic bacteria are the major decomposers of HS in aquatic ecosystems, converting them into biomass and remineralizing them to inorganic carbon. However, heterotrophic bacteria may also produce refractory HS, especially in the marine environment (e.g. Ishiwatari 1992, Tranvik 1998, Ogawa et al. 2001). Physico-chemical processes, particularly flocculation and photochemical reactions, also play a role in the formation of recalcitrant DOC (Obernosterer et al. 1999, 2001, Obernosterer & Herndl 2000, Kerner et al. 2003). On the other hand, photochemical transformation is an important pathway to decompose recalcitrant DOC (Moran & Zepp 1997, Obernosterer et al. 2001).

For soil HS, a steady state of formation and degradation can be assumed. This can also be largely applied to freshwater HS, despite increased UV radiation levels at the surface waters in lacustrine environments compared to soil, presumably enhancing the degradation of HS. When reaching the estuarine transition zone between saline and freshwater, HS are exposed to new conditions. Salinity plays an important role in enhancing the decomposition rates of highly refractory HS (e.g. Wikner et al. 1999). When HS enter brackish and marine ecosystems, the increased salinity and ionic strength of the saline environment may enhance the degradation rate of HS dramatically. However, the role of HS degradation in estuarine gradients and in coastal regions as a sink for DOC/HS, entering the open sea, is not well understood (Cauwet 2002, Søndergaard et al. 2003, McKenna 2004). Most data indicate that loss rates of riverine DOC during initial mixing in the estuarine salinity gradient cannot explain the rapid disappearance of terrigenous DOC during the passage through this transition zone. Only a small fraction of riverine DOC appears to be degraded during the estuarine mixing, and this degree of degradation increases with the residence time of the DOC. It is likely that a substantial part of the degradation occurs in more extended coastal regions, bringing terrigenous DOC concentrations eventually to trace levels in the open sea. However, detailed studies of the bacterial degradation of humic-rich DOC under these conditions are still scarce.

The present challenge is to understand the general observation that terrestrial HS is largely missing from the marine ecosystem, and to apply refined analytical tools for the identification of mechanisms transforming and/or mineralizing the allochthonous riverine HS. One way to shed light on the complex mechanisms such as degradation of terrestrial HS in estuaries and the coastal zone is to design laboratory experiments in which several key parameters are controlled and their influence on the degradation of HS and changes of the bacterial community composition can be reliably measured. Traditional batch cultures do not mimic the conditions met in the natural environment in an appropriate way. Therefore, we developed an approach using sequential chemostat cultures to simulate stepwise degradation of the HS under well controlled conditions. These cultures were used to study the degradation of freshwater-borne HS by estuarine bacterial communities covering the salinity range from the oligohaline (salinity 5), mesohaline (salinity 15) to the polyhaline region (salinity ~30).

The main aims of this study were to assess the degradation of HS and its constituents and the efficiency of the bacterial decomposition, i.e. relative proportions of biomass production and mineralization, of HS at increasing salinity in sequential chemostats. We postulate that the humic-rich DOC is decomposed differently at the various salinities and that different degrees of HS decomposition occur in different stages of these cultures, i.e. degradation of more refractory material in the 'later' stage with a lower growth efficiency.

MATERIALS AND METHODS

Approach. Simple, laboratory-built chemostats were used to grow estuarine bacteria on HS-rich freshwater adjusted to various estuarine salinities. The bacterial inocula originated from the oligo- to mesohaline section (salinities of 5 and 15) of the Weser estuary, northwestern Germany (53° 21′ 44″ N, 08° 29′ 41″ E; 53° 26′ 40″ N, 08° 29′ 07″ E), and from the polyhaline (salinity 30) Wadden Sea at Neuharlingersiel, northwestern Germany (53° 43′ 20″ N, 07° 43′ 20″ E). The HS-rich water was collected from 2 humic-rich streams in the hinterland of the Wadden Sea (Esens region, northwestern Germany; total DOC up to 5.2 mM C) on 11 May and 2 December 2003.

Set up of the chemostats. Three experiments were carried out. Expt 1, with the bacterial inoculum of salinity 30 (Wad30) from the Wadden Sea, was run in duplicate chemostats at 20°C. The 2 other experiments, inoculated with bacteria from regions with salinities of 5 (Wes5) and 15 (Wes15) of the Weser estuary, were run in 1 replicate at 15°C. The bacterial inocula were pre-filtered through 0.8 µm isopore filters to avoid growth of bacterial grazers in the chemostat cultures, and 1000 ml of the inoculum (1×10^6 bacteria ml^{-1}) were added to the first chemostat prior to pumping in the medium. Chemostat Stages II and III were subsequently filled by the overflow of the previous stage such that Stage II started to run 5 (Wes5, Wes15) and 8 d (Wad30), and Stage III 18 (Wes5, Wes15) and 24 d (Wad30), after the start of Stage I. Grazers were never observed in the cultures.

The chemostats were built from pre-combusted (2 h 500°C) 12 l glass bottles, acid-washed silicon stoppers, and silicon tubes, and were set up in a 3 stage sequence (Fig. 1). Peristaltic pumps (8 channel Ismatec MC-MS/CA8) were used to pump the medium into and out of the chemostats, which contained 10 l of medium. The pumps were operated at a dilution rate of 0.1 d^{-1} (Wad30) or 0.15 d^{-1} (Wes5 and Wes15). The chemostats were continuously aerated using room air (filtered through 0.22 µm filters). The outflow from the last chemostat was filtered through 0.22 µm capsule filters (Mini capsule filters HT Tuffryn, Pall Life Sciences) and collected into acid-washed plastic (PE) containers (Fig. 1). They were stored at 4°C until XAD extraction (see 'Extraction of HS using XAD resins' below). Subsamples of 150 ml for further analyses were withdrawn from each chemostat stage at the outflow every second day, but analyses were only performed on selected subsamples.

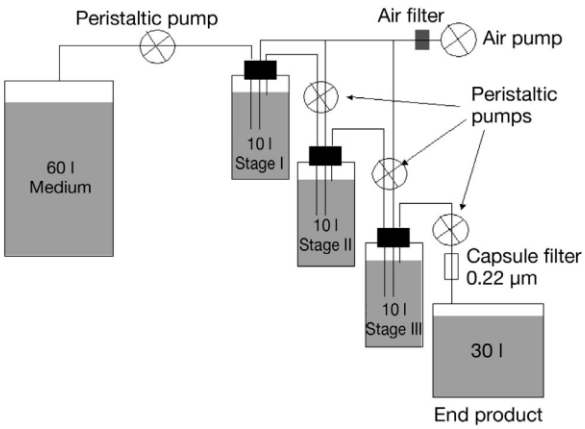

Fig. 1. Set up of the chemostat experiments. 60 l Medium: 0.8 µm-filtered freshwater adjusted to the higher salinity in the plastic jar; Peristaltic pumps: pumps to move water through the chemostats; 10 l Stage I, II, III: glass bottles of 3 chemostats; Air filter and Air pump for aeration of chemostats with sterile room air; Capsule filter: filter for sterilizing the end product; End product: plastic container for collecting water after the last chemostat

HS medium. For the medium, natural freshwater samples (~2 × 60 l) were collected into acid-washed (0.2 M HCl) PE containers (20 l each) from 2 humic-rich streams of the same drainage system in the hinterland of the Wadden Sea (Esens region, northwestern Germany, total DOC up to 5.2 mM C) on 11 May and 2 December 2003. The first sample was used for the Wad30 experiment and the second sample for the inocula of the Wes5 and Wes15 experiments. To remove the freshwater bacteria, the water was passed through a 0.1 µm cartridge using tangential ultrafiltration (Amicon-Millipore) on the same day. The filtered bulk water was used as the main component of the growth medium for the chemostats but was amended with 20 µM of NH_4Cl and 5 µM of HNa_2PO_4 and a vitamin solution (1 ml l^{-1}). The vitamin solution in 100 ml of distilled water consisted of 4 g 4-aminobenzoe acid, 1 mg D(+)-biotin, 10 mg nicotine acid, 5 mg Ca-D(+) panthotenate, 15 mg pyridoxine × HCl, 10 mg thiamine × 2 HCl, and 5 mg vitamin B12. The salinity of the medium was adjusted to the *in situ* salinity level from where the bacterial inoculum was taken, adding 1.5 l (for salinity 5) or 4.5 l (for salinity 15) of artificial seawater medium (ASW) of a salinity of 200 (1 l ASW salinity of 200, solution A [500 ml]: NaCl, 135.93 g; Na_2SO_4, 22.74 g; KCl, 3.84 g; $NaHCO_3$, 1.11 g; KBr, 0.55 g; H_3BO_3, 0.15 g; NaF, 0.02 g and solution B [500 ml]: $MgCl_2 \times 6H_2O$, 61.44 g; $CaCl_2 \times 2H_2O$, 8.61 g; $SrCl \times 6H_2O$, 0.14 g) to 60 l of the filtered freshwater medium (Parsons et al. 1984). For adjusting salinity to 30, 6 l of 300 ASW were added to 60 l of the filtered freshwater medium. DOC concentrations before and after the salinity adjustment were similar, indicating that no flocculation and thus abiotic DOC removal occurred. The medium was stored in 60 l acid-washed plastic jars (total volume of 120 l) and sterilized silicon tubing was used to pump the medium into the chemostats.

Abiotic DOC removal. A control experiment to examine whether processes other than bacterial decomposition, e.g. abiotic flocculation, contributed to DOC removal was carried out in June 2007. Therefore, a batch experiment with HS-rich stream water, pre-filtered through a 0.1 µm cartridge using tangential ultrafiltration and adjusted to salinities of 1, 15, and 30, was set up and incubated for 7 d at 15°C in the dark but without a bacterial inoculum. Periodically, subsamples were withdrawn to analyze concentrations of DOC and total organic carbon (TOC).

DOC. For DOC measurements, 15 ml subsamples were filtered (0.2 µm Gelman Acrodisc HT Tuffryn), and analysis was performed by combustion at 850°C and IR-detection with a multi N/C 3000 analyzer (Analytik Jena) or with a Shimadzu TOC-VCPH (abiotic control experiment). Samples for TOC analysis were not pre-filtered.

The samples were acidified prior to analysis with 50 µl of 50 % H_3PO_4. Calibration was done using a K-hydrogenphthalate solution as a standard in the range from 0.5 to 10 mM of carbon (CV 0.8 to 3.5 %). All glassware used was acid-washed and pre-combusted at 500°C for 2 h.

Extraction of HS using XAD resins. Water samples, acidified to pH 2.0, were pre-filtered through a 0.1 µm cartridge using tangential flow ultrafiltration (Amicon-Millipore). The high-molecular-weight material of the DOC was isolated by column chromatography of 10 to 30 l of filtered sample using a series of XAD-8 and XAD-4 resins (DAX-4 and DAX-8, Sigma-Aldrich) (Thurman & Malcolm 1981, Malcolm & MacCarthy 1992). HS were eluted from the XAD-8 resin with 0.1 N NaOH. The separation with the XAD-8 resin provides HA and FA, which are hydrophobic, whereas XAD-4 resin separates HPA. The eluate from the XAD-8 resin was separated into FA and HA fractions at pH 1 (overnight at 4°C, followed by centrifugation at 4000 × *g*). All samples of HA, FA and HPA were reconcentrated separately onto the resin on which they were isolated, desalted by elution with 0.1 N NaOH on cation exchange resins, vacuum concentrated, and freeze-dried. For rough mass balance calculations, the differences in DOC were measured before the sample was applied on XAD-8, after XAD-8/before XAD-4, and after XAD-4. The yield of HA, FA, and HPA was measured by weighing the material after freeze-drying.

Total number of bacteria (TNB) and bacterial production (BP). Bacteria were fixed with formaldehyde (final concentration 1 %) and stained with SYBR Green I as described by Lunau et al. (2005). Briefly, sub-samples (usually 1 to 3 ml) were filtered onto 0.2 µm black isopore filters (Whatman) and washed with 1 ml of 1× TAE buffer (pH 8). The filter was placed on a glass slide into the embedding solution containing SYBR Green I and covered by a cover slip. About 250 to 300 cells were counted using a Zeiss Axiolab microscope at 1000× magnification (lamp: HBO 50, filter set: Zeiss, excitation 450–490, FT 510, LP 515). BP was determined using the standard ^{14}C-leucine incorporation method (Simon & Azam 1989). Briefly, 60 nM (final concentration) of ^{14}C leucine (specific activity 10.8 GBq mmol^{-1}, Hartmann Analytic), ensuring saturation of the uptake systems, was added to triplicate 5 ml samples and duplicate formaldehyde-killed controls and incubated for 1 h in the dark. Incubation was stopped by adding formaldehyde (final concentration 1 %), and protein was extracted using 5 % ice-cold trichloroacetic acid (TCA) for 5 min. Thereafter, samples were filtered onto 0.45 µm nitrocellulose filters and rinsed 6 times with ice-cold 5 % TCA. Dried filters were dissolved with ethylacetate and radioassayed by liquid scintillation counting. Biomass production was calculated according to Simon & Azam (1989).

RESULTS

The initial DOC concentration in the medium of the Wad30 experiment was 3.8 mM C, 0.9 mM lower than that in the Wes5 and Wes15 experiments, and the accumulated fractions of HS in the Wad30 experiment amounted to 84 % of DOC compared to 74 % in the latter (see Tables 1 & 2, Fig. 2). The chemostat experiments resulted in distinct decomposition steps of the DOC in the various stages of the continuous cultures (Table 1). There were 2 controlling parameters: the dilution rate of the chemostat (0.1 d^{-1} for Wad30 and 0.15 d^{-1} for Wes5 and Wes15), and the substrate concentration in the medium. Both controlling parameters were kept constant during the course of 1 experiment in order to reach steady state conditions between the decomposition of substrates and the renewal of the substrate pool by fresh HS.

Decomposition of DOC

In the Wad30 experiment, the decomposition of DOC was substantial. The major decomposition took place from filling up the chemostat on Day 8 to Day 17 in Stage I. Later, DOC was further decomposed only very little

Table 1. Concentrations and decomposition of DOC (ΔDOC%) and growth efficiency (GE) in the 3 stages of the 3 continuous culture experiments (Wad30: Wadden Sea inoculum at salinity 30; Wes15, Wes5: Weser estuary inocula at salinities 15 and 5, respectively). ΔDOC% was determined as the difference of DOC concentrations at the inflow and outflow of the chemostat stages relative to that of the previous stage and as that of Stage III relative to the medium (total). GE was calculated as the ratio of bacterial production over ΔDOC in the chemostat stages (see Figs. 2 & 3). nd: not determined

Experiment	DOC (mM C)	ΔDOC (%)	GE (%)
Wad30			
Medium	3.80		
Stage I	2.05	46.4	11.9
Stage II	1.88	9.7	43.1
Stage III	1.33	26.4	22.4
Total		64.4	
Wes15			
Medium	4.73		
Stage I	4.85	0.0	nd
Stage II	4.72	2.6	67.0
Stage III	4.5	4.7	38.9
Total		4.8	
Wes5			
Medium	4.73		
Stage I	4.71	0.5	5.78
Stage II	4.71	0.0	nd
Stage III	4.7	0.2	12.98
Total		0.7	

(Figs. 2 & 3). DOC decomposition in Stage III was higher than in Stage II, as shown by the substantially lower DOC concentrations. DOC mean values between Days 17 and 51 in chemostat Stages I and II and in Stage III between Days 32 and 51 were significantly lower than that in the medium (*t*-test, $p < 0.01$, Fig. 2). DOC decomposition in the duplicates was not significantly different (*t*-test, $p = 0.91$). The highest decomposition occurred in Stage I (ΔDOC: 1.76 mM C), whereas in Stages II and III, an additional 0.2 and 0.5 mM C were decomposed. The cumulative decomposition over the 3 stages added up to 64.4 % of the DOC in the growth medium, from 3.8 ± 0.3 to 1.38 ± 0.49 mM C in the outflow. In the chemostats with lower salinities and a higher dilution rate (0.15 d^{-1}; Wes15 and Wes5) no significant DOC decomposition occurred (Figs. 2 & 3). A decrease in DOC was noticeable in the Wes15 experiment, from 4.73 ± 0.02 to 4.50 ± 0.24 mM C as a cumulative decrease, but was non-significant (ANOVA, $p = 0.108$). The decrease in DOC was also non-significant in the Wes5 experiment, as DOC concentrations in the medium and the 3 chemostat stages remained similar, between 4.73 ± 0.02 and 4.70 ± 0.18 mM C (ANOVA, $p = 0.99$).

The batch experiment without a bacterial inoculum, the abiotic control, did not yield any significant change in concentration of TOC or DOC in any of the salinity treatments over the incubation period of 7 d, despite DOC concentrations twice as high as for the chemostat experiments (Fig. 4). Concentrations of TOC and DOC were similar in the salinity 1 and 30 treatments, whereas TOC concentrations were slightly higher than DOC concentrations in the salinity 15 treatment.

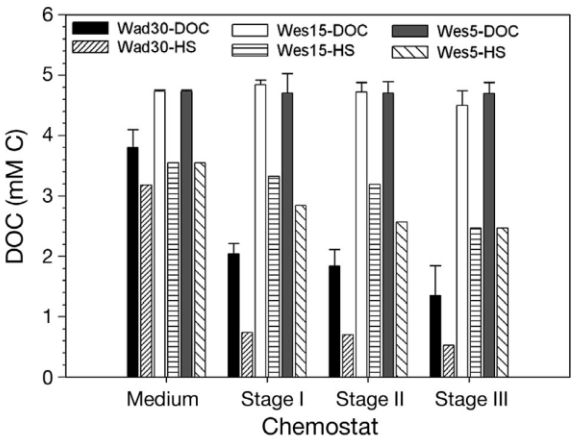

Fig. 2. Mean concentration of dissolved organic carbon (DOC) and of organic carbon bound in humic substances (HS) of the media, and chemostat Stages I, II, and III of the Wad30 (Wadden Sea inoculum at salinity 30), Wes15 and Wes5 (Weser extuary inocula at salinities 15 and 5, respectively) experiments. Given are mean values ± SD (only for DOC) of DOC subsamples collected over the incubation period after all 3 stages. See Fig. 3 for the incubation periods of the stages of the 3 experiments. The Wad30 experiment was run in duplicate, and the Wes15 and Wes5 experiments were run in 1 replicate

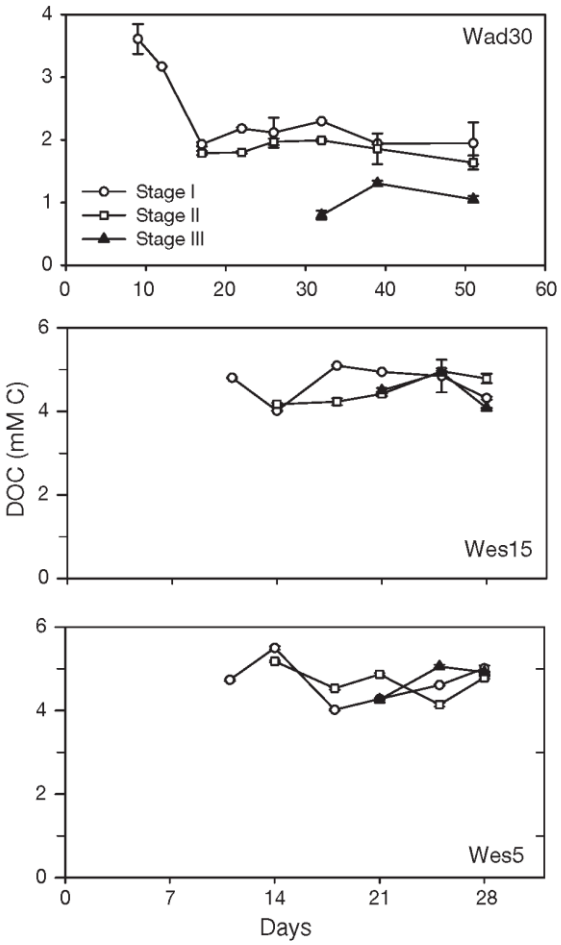

Fig. 3. Time course of DOC concentrations in the various chemostat stages of the Wes5, Wes15, and Wad30 experiments. Stage II started to run 5 d (Wes5, Wes15) and 8 d (Wad30), and Stage III 18 (Wes5, Wes15) and 24 d (Wad30) after the start of Stage I. The initial samples of Stage I in the 3 experiments analyzed were from Day 9 (Wad30) and Day 11 (Wes15, Wes5). Earlier samples than shown from Stages II and III were not available. Note the different scales

HS fractions

HS constituted 49 % of DOC in Wad30, 61 % in Wes5, and 67 % in Wes15 experiments, respectively. These percentages were calculated by comparing the DOC concentration in the initial sample (before extraction on XAD resins) and the sample after extraction on both XAD columns.

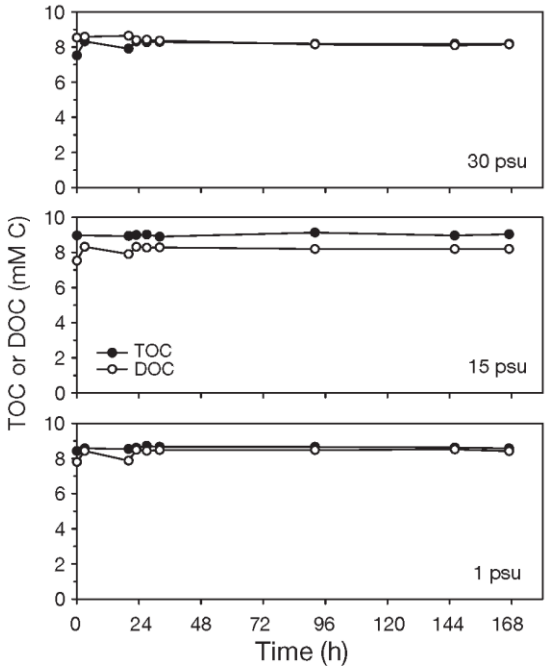

Fig. 4. Concentrations of TOC and DOC during a batch experiment in which 0.1 µm pre-filtered humic-rich water adjusted to salinities 1, 15, and 30 was incubated abiotically for 7 d at 15°C

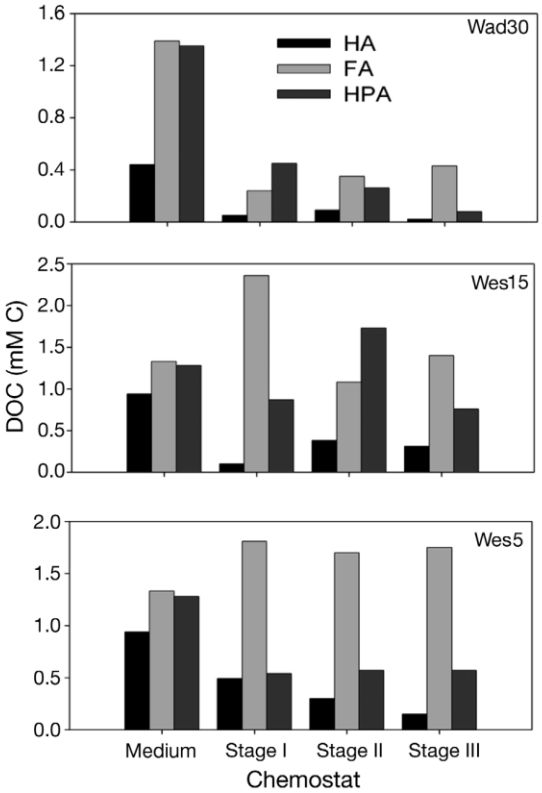

Fig. 5. Concentrations of humic acids (HA), fulvic acids (FA), and hydrophilic acids (HPA) in media of chemostat Stages I, II, and III of the Wad30, Wes15, and Wes5 experiments

The HA fraction was the lowest in all experiments and chemostat stages and generally showed a decreasing trend from the medium to the outlet of chemostat Stage III with the highest decrease in Stage I (Fig. 5). This fraction constituted 13 to 26% of DOC in the medium, but only 3.0 to 12.4% in chemostat Stage III. The FA and HPA fractions were substantially larger than that of HA in all experiments. The FA fraction decreased substantially in chemostat Stage I of the Wad30 experiment, but increased in the following stages and also in the chemostats of the Wes5 experiment relative to the medium. In the Wes15 experiment, the FA fractions exhibited large variations among the various stages (Fig. 5). The HPA fraction systematically decreased in the chemostats of the Wad30 and Wes5 experiments, mainly in Stage I, and exhibited inverse patterns to the dynamics of the FA fractions. In the Wes15 experiment, large but inconsistent fluctuations of this fraction occurred.

The accumulated HS fractions in the 3 experiments accounted for 74 to 84% of total DOC in the medium (Table 2). During the sequential decomposition, their concentrations continuously decreased (Fig. 2), to 15% of the initial HS concentration in the Wad30 experiment and to 69% in the other 2 experiments. The HS fractions as percentages of total DOC also decreased during the experiments, to 41% in the Wad30 experiment and to 54% in the other 2 experiments (Table 2).

Abundance and growth parameters of bacteria

TNB was highest in chemostat Stage I of the Wad30 experiment and decreased to significantly lower numbers in the later stages (Fig. 6). Numbers were not significantly different between the duplicates of the Wad30 experiment (*t*-test, $p = 0.18$). In the Wes15 and

Table 2. Proportions of humic substances (HS) as the sum of the fractions fulvic acids (FA), humic acids (HA), and hydrophilic acids (HPA) as a percentage of total DOC in the 3 experiments, media and chemostat stages as shown in Fig. 2

	Wad30	Wes15	Wes5
Medium	84	74	75
Stage I	36	69	69
Stage II	38	69	63
Stage III	41	55	53

Wes5 experiments, TNB fluctuated without a clear trend among the various stages.

Dynamics of BP in the chemostat stages of the Wad30 experiment (duplicates did not differ, *t*-test, $p = 0.52$) were similar to TNB, also with highest rates in Stage I (Fig. 6). Differences to the later stages were significant (ANOVA, $p < 0.01$). In the Wes15 experiment, similar patterns were observed but with lower values in Stage I (ANOVA, $p = 0.035$). In the Wes5 experiment, BP rates remained similar among the 3 stages.

Bacterial growth efficiency (GE), calculated as the ratio of BP over ΔDOC in the stages of the chemostats, varied from 6 to 67 % without clear differences among the 3 experiments and chemostat stages (Table 1). Hence bacterial DOC decomposition resulted in conversion to bacterial biomass and remineralization to inorganic carbon to varying extents. Relatively low GE occurred in Stage I of the Wad30 and Wes5 experiments and relatively high GE in Stage II of the Wad30 and Wes15 experiments.

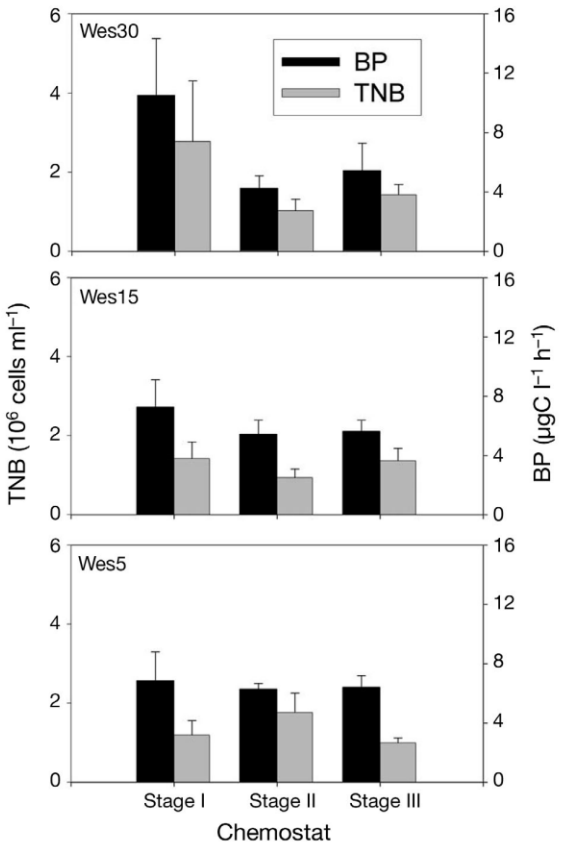

Fig. 6. Total numbers of bacteria (TNB) and bacterial production (BP) in chemostat Stages I, II, and III of the Wad30 (n = 8, 6, 3), Wes15 (n = 7, 6, 4), and Wes5 (n = 7, 6, 4) experiments. Given are mean values ± SD of subsamples collected over the incubation period since all 3 stages started to run, i.e. from Days 25 to 51 in Wad30, and from Days 18 to 51 in Wes5 and Wes15

Concentrations of labile DOC (dissolved amino acids, dissolved free neutral monosaccharides) never exceeded 0.03 % of DOC (data not shown).

DISCUSSION

The humic-rich DOC was decomposed to more than 60 % in the Wad30 experiment, and mainly in chemostat Stage I from filling up the chemostat on Day 8 until Day 17, whereas in the other 2 experiments at salinities of 15 and 5 (Wes15, Wes5), no detectable decomposition occurred. These differences were also reflected by the higher bacterial numbers and production rates in the Wad30 chemostat. At the high salinity condition, all 3 HS fractions were reduced substantially, whereas at a salinity of 5, only the HA and HPA fractions decreased, and at 15 only the HA fraction decreased. At a salinity of 5, the FA fraction increased. However, HS in total were also reduced at the lower salinities, although by <30 %. These findings indicate that at a salinity typical of the polyhaline estuarine zone and close to that occurring in the coastal sea, HS are substantially decomposed, whereas at lower salinities, very little, if any, bacterial decomposition of humic-rich DOC occurs, even though the bacteria do transform the various humic fractions.

Our analyses and the abiotic control experiment gave evidence that the decrease in DOC concentrations in the course of the chemostat experiments was predominantly mediated by the decomposition activities of the bacteria added as inoculum. Abiotic processes such as flocculation did not occur after adjusting the freshwater medium to salinities of 1, 15, and 30. In addition, no DOC losses occurred during a batch experiment with humic-rich freshwater DOC adjusted to higher salinities and without a bacterial inoculum (Fig. 4).

The major differences among the 3 experiments were the bacterial inocula, suggesting that the bacterial community originating from the Wadden Sea (Wad30) was most competent in degrading the humic-rich DOC. It has been shown that the bacterial communities from the various locations of different salinities are phylogenetically different (Selje & Simon 2003, Stevens et al. 2005). However, functional differences and in particular the major players in the decomposition of humic-rich DOC in these communities are still unknown. In addition to the inocula, the high ionic strength may have facilitated the decomposition by the polyhaline bacterial community. Wikner et al. (1999) showed that increasing ionic strength enhances the bacterial decomposition of allochthonous DOC even at

a salinity <5, although another study did not find enhanced bacterial decomposition of humic-rich DOC at increased ionic strength (Søndergaard et al. 2003). From our results, we can conclude that the polyhaline bacterial community was most effective in decomposing the humic-rich DOC, but we do not know whether and how much the high ionic strength under the polyhaline conditions facilitated the enhanced bacterial decomposition. These findings of enhanced bacterial decomposition of humic-rich DOC by a polyhaline bacterial community at high salinities relative to that by oligohaline bacterial communities of the upper sections of the Weser estuary are consistent with reports of a generally conservative mixing of DOC in these estuarine regions. Such enhanced bacterial decomposition provides an explanation for the rapid disappearance of DOC in the outer estuarine regions at high salinities (Cauwet 2002, McKenna 2004, Servais & Garnier 2006).

Other differences were the 0.1 d^{-1} dilution rate and incubation at 20°C of the Wad30 experiment compared to a dilution rate of 0.15 d^{-1} and incubation at 15°C of the Wes15 and Wes5 experiments. The higher dilution rate was intended to shift the DOC decomposition from Stage I toward Stages II and III, and the lower temperature was intended to account for the lower ambient temperature in December compared to May. The higher dilution rates and lower temperature in the Wes15 and Wes5 experiments could have slowed down or even prevented the establishment of a well-adapted bacterial community in the various stages, thus preventing any decomposition of the DOC. However, we assume that this was not the case and that the different decomposition and transformation patterns were a result of the different metabolic properties of the bacterial communities. The higher dilution rate in these experiments would have shifted the decomposition from Stage I to Stages II or III, but a significant DOC decomposition should have been detected, despite the higher dilution rate.

The lower incubation temperature of the Wes5 and Wes15 experiments may have reduced bacterial DOC decomposition. Assuming that bacterial growth at 15°C compared to 20°C is lower by 30% (Shiah & Ducklow 1994), the difference of 5°C would also have reduced the DOC decomposition in the Wes5 and Wes15 experiments by 30% compared to the Wad30 experiment. However, the difference we found was far greater, suggesting that other factors led to the low DOC decomposition in the former 2 experiments.

Other factors that could have affected the different DOC decomposition rates between the Wad30 and Wes5 and Wes15 experiments were nitrogen limitation and qualitative differences of the HS collected in May (Wad30) and December (Wes5 and Wes15). We added 20 µM nitrogen to the DOC medium, which had an ambient concentration of 30 to 50 µM N (S. Koelsch & H. Brumsack unpubl. data). Therefore, N-limitation appeared rather improbable and would have affected all 3 experiments similarly. Concentrations of HS in December were higher than in May, but their relative proportions as % of DOC were lower (Fig. 2). Concentrations of FA and HPA were similar in both months, and only the concentration of HA was lower in May compared to December. The latter fraction was the only one that was decomposed consistently in all 3 experiments, whereas the other fractions were only consistently decomposed in the Wad30 experiment. We assume that the composition of the FA and HPA fractions was similar in May and December and that the low DOC decomposition of the Wes5 and Wes15 experiments was not due to a more refractory nature of these compounds in December compared to May. However, we cannot completely rule out this possibility because we did not examine the molecular structure of these HS fractions by more refined methods than XAD extraction.

Our results also indicate that the bacterial GE can vary largely among different bacterial communities and different decomposition stages. However, we could not identify consistent patterns of GE in relation to the growth stages and/or salinity ranges.

Thus far, decomposition of humic-rich DOC by bacterial communities at varying salinities has been studied very little despite the fact that the DOC reduction in the estuarine transition zone and coastal sea is not well understood. Our study was the first to apply chemostats, whereas previous studies have used batch cultures (Wikner & Hagström 1999, Søndergaard et al. 2003). Applying batch cultures does not allow us to distinguish different degradation stages. Labile substrates are used first and select for a specific bacterial community and may introduce unfavorable conditions for a succeeding community, potentially degrading the more refractory material. Further, batch cultures are limited by the small substrate volume and the fact that no steady state is established. Sequential chemostats such as those we applied do not suffer from the above-mentioned shortcomings and appear to be much more suitable for these investigations. For degradation experiments, these chemostats better meet the multi-G (G: discrete substrate pool) model of a continuum of discrete DOC substrate pools (Westrich & Berner 1984, Raymond & Bauer 2000). The 3 stages we set up enabled us to distinguish among the various levels of DOC degradation and enabled the bacterial communities to adapt to the various growth conditions. The continuous supply of fresh material ensured an unchanged substrate pool during the entire experiment. In fact, this approach showed that the highest decomposition

occurred in the Wad30 experiment in the early phase of Stage I, from filling of the chemostat until Day 17, after the end of the initial phase, and in Stage III. Hence, our finding of a surprisingly high decomposition of humic-rich DOC of a polyhaline bacterial community in sequential chemostats may provide a more realistic scenario of the role of heterotrophic bacteria in the estuarine turnover and loss of DOC than results obtained from batch culture experiments.

HS constituted 36 to 84% of total DOC, and proportions decreased from the medium to the last chemostat stage. These proportions are in the same range as reported in other studies (e.g. McKnight & Aiken 1998, McKenna 2004). We observed some variability among the 3 HS fractions during the experiments; the FA fraction increased in the Wad30 experiment from Stage I to Stage III and in the Wes5 experiment from the medium to Stage I. These naturally occurring macromolecular substances are most complex and heterogeneous and cannot be well characterized chemically. They are defined operationally according to their solubility at low pH or adsorbed to hydrophobic macroporous resins such as XAD (Thurman & Malcolm 1981).

The non-HS fraction, which is not adsorbed at all to or eluted by methanol from XAD resins, includes hydrophilic low molecular weight (LMW, <1.2 kDa) and hydrophobic components (McKnight & Aiken 1998). We did not intend to fully characterize the non-HS fraction, but the dissolved amino acids and dissolved free carbohydrates constituted only minor proportions (data not shown). Due to the large volume needed for the fractionation and extraction procedure, we could analyze only 1 replicate. Even though we must consider some analytical variability of the results, the budget of all 3 fractions shows that the total amount of HS decreased in all 3 experiments, indicating that the shifts among the fractions were accommodated. We assume that our findings show that bacterial decomposition modifies the adsorptive properties of the HS and renders substantial amounts of HS non-humic.

Our findings also provide some evidence that bacterial decomposition at first transforms HA and HPA to FA, a LMW fraction. This notion implies that the distinction between HS and non-HS does not refer specifically to their bacterial degradability but strictly to their adsorptive properties. Further, this notion is consistent with the size-reactivity continuum model proposed by Amon & Benner (1996), hypothesizing that the reactivity of DOC is inversely related to the molecular weight. In Amon & Benner (1994, 1996) the authors showed that bacteria decompose preferentially high molecular weigh (HMW) DOC (>1 kDa) and that LMW DOC is much more recalcitrant. Hence, we assume that the loss of HS in our experiments was mainly due to the preferred bacterial decomposition of the HMW DOC, which consisted of high proportions of HS, and left behind recalcitrant LMW DOC with a significantly lower proportion of HS.

Our findings complement previous notions of the transformation and losses of riverine and humic-rich DOC during its estuarine passage to the coastal sea. They provide the framework for the following scenario of DOC removal during this passage. In the lower salinity range, flocculation and photochemical reactions (Sholkovitz 1976, Sholkovitz et al. 1978, Moran & Zepp 1997, Obernosterer & Herndl 2000, Obernosterer et al. 2001, Kerner et al. 2003) appear to be the most important processes of DOC transformation, leading, however, only to minor DOC degradation. This implies that the DOC is largely recalcitrant and explains its conservative behavior of mixing with seawater, i.e. dilution without significant bacterial decomposition (Cauwet 2002, Servais & Garnier 2006). If non-conservative mixing occurs, it is mainly the result of production of labile DOC in the estuarine region and a function of the residence time of the DOC (McKenna 2004). Removal of the recalcitrant and humic-rich DOC occurs predominantly in the polyhaline estuarine region and possibly in the adjacent coastal sea and is mediated by its bacterial decomposition and breakdown to more recalcitrant LMW compounds. The effectiveness of the bacterial decomposition is a function of environmental conditions such as temperature, but also of the residence time. Hence, bacterial DOC degradation in extended and tidally affected shallow coastal ecosystems such as the Wadden Sea would be higher than in regions of riverine discharge directly into deeper coastal regions and in river plumes in delta regions and in permanently cold regions like the Arctic Ocean (Opsahl et al. 1999).

Acknowledgements. This work was supported by a Marie Curie Fellowship of the European Commission's Environment and Sustainable Development Program (EVK3-CT-2002-50020 to V.K.) and by a grant from the Deutsche Forschungsgemeinschaft (Si 360/22-1). We appreciate valuable suggestions of 3 anonymous reviewers on an earlier version of this manuscript.

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Editorial responsibility: Craig Carlson, Santa Barbara, California, USA

Submitted: July 12, 2007; Accepted: August 6, 2008
Proofs received from author(s): September 12, 2008

5

Abschlussbetrachtung und Ausblick

5 Abschlussbetrachtung und Ausblick

Über den bakteriellen Abbau von Huminsäuren ohne gleichzeitigen Einfluss der Photooxidation im Allgemeinen und speziell im Bereich des Weserästuars und des Deutschen Wattenmeeres, liegen kaum Daten vor. Aufgrund dessen wurden am Institut für Chemie und Biologie des Meeres (ICBM) der Universität Oldenburg in den vergangenen Jahren verschiedene Untersuchungen zum Abbau terrestrischer Huminsäuren durch Bakterien durchgeführt. Im Folgenden werden Ergebnisse der aus dieser Arbeit resultierenden Veröffentlichungen und Manuskripte zusammengefasst und diskutiert. Abschließend wird noch ein Ausblick für eine mögliche Fortführung der Forschungsarbeit gegeben.

Die in dieser Arbeit gewonnen Erkenntnisse liefern wichtige Hinweise auf den bakteriellen Abbau von Huminsäuren (HA), speziell im Wattenmeer und im Weserästuar. Sie ergänzen aber auch bisherige Untersuchungen und tragen allgemein zum Verständnis des Huminstoffabbaus bei. Oft wurde in Studien entweder die Molekülzusammensetzung von Huminstoffen (HS, z.B. Schulten und Leinweber, 1996; Piccolo, 2001), die Zusammensetzung der HS-abbauenden Bakteriengemeinschaften (z.B. Esham et al., 2000; Hutalle-Schmelzer et al., 2009, 2010), die Beteiligung unterschiedlicher Enzyme am HS-Abbau (z.B. Dehorter und Blondeau, 1992; Claus und Filip, 1998; Steffen et al., 2002), das Vorkommen und der Abbau von HS in verschiedenen Habitaten (z.B. Meyers-Schulte und Hedges, 1986; Kisand et al., 2008) oder der Einfluss bestimmter Parameter wie UV-Bestrahlung und Salinität auf den mikrobiellen Abbau von HS (z.B. Kulovaara et al., 1996; Kisand et al., 2008) untersucht. In dieser Arbeit hingegen sind die durchgeführten Inkubationsversuche mit Huminsäuren anhand sehr unterschiedlicher Parameter, von molekularbiologischen bis zu strukturell-analytischen, verfolgt worden.

In Durchflusskulturen wurden Bakteriengemeinschaften aus Bereichen unterschiedlicher Salinität, dem ostfriesischen Wattenmeer und dem Weserästuar, für einen Zeitraum von 71 bis 74 Tagen mit Huminsäuren als einziger Kohlenstoffquelle inkubiert. Da Huminsäuren als refraktär gelten, ist abweichend von regulären Chemostat-Aufbauten eine sehr geringe Verdünnungsrate gewählt worden (0,01 d^{-1}), wodurch in dem Versuchszeitraum nur ca. 70% des Kulturmediums ausgetauscht wurden. Es konnte erstmals gezeigt werden, dass Bakteriengemeinschaften aus dem ostfriesischen Wattenmeer bis zu 74,6%, die aus dem Weserästuar bis zu 75,6% der Huminsäuren im Medium abbauen konnten. Der größte Abbau an Huminsäuren vollzog sich jeweils in Ansätzen mit veränderter Salinität im Vergleich mit der aus welcher das Inokulum stammte. Innerhalb der ersten 21-28 Tage der Inkubationsexperimente ist die Hauptmasse der Huminsäuren im Kulturmedium abgebaut

worden, nachfolgend zumeist nur noch das einströmende Medium, was eine Trennung des bakteriellen Huminsäureabbaus in zwei unterschiedliche Phasen zeigt. Zusammen mit erstmalig durchgeführten Vorher-Nachher-Vergleichen der Huminsäuremoleküle mittels Pyrolyse-GC/MS ist das ein wichtiger Hinweis darauf, dass Huminsäuremoleküle aus schwer abbaubaren Kernstrukturen, umgeben von leichter abbaubaren, zumeist aliphatischen Strukturen bestehen, was bereits in vorangegangenen Studien vermutet wurde (z.B. Haworth, 1971, Piccolo, 2001).

Neben der Abnahme der DOC(dissolved organic carbon)-Konzentration war in den ersten 3-4 Wochen auch eine hohe Dynamik der Bakteriengemeinschaften in beiden Versuchsreihen erkennbar, sowohl in den DGGE-Profilen als auch bei den Bakterienzellzahlen und der Biomasseproduktion. Nach der ersten aktiven Phase nahm die Dynamik auch dieser Parameter deutlich ab. Bezüglich der Bakteriengemeinschaften wurde erstmalig deutlich, dass sich die huminstoffabbauenden Bakteriengemeinschaften im ostfriesischem Wattenmeer und dem oligohalinen Weserästuar deutlich voneinander unterscheiden. Vergleiche der jeweiligen Bakteriengemeinschaften vor und nach der Inkubation hingegen wiesen nur geringe Unterschiede auf, was ein wichtiger Hinweis auf eine Anpassung der dort vorhandenen Bakteriengemeinschaften an terrestrisch eingetragene Substanzen ist.

5.1 Zusammensetzung von huminstoffabbauenden Bakterien-gemeinschaften

In bisherigen Studien sind die Bakteriengemeinschaften des Deutschen Wattenmeeres (Stevens et al., 2005 a, b, 2007; Rink et al., 2007, 2008) und des Weserästuars (Selje und Simon, 2003; Selje et al., 2005) grundlegend untersucht worden. Die hier vorliegenden Ergebnisse ergänzen diese Untersuchungen und liefern erstmalig einen Überblick über einzelne Bakterien und Bakteriengemeinschaften des ostfriesischen Wattenmeeres und des Weserästuars, die in der Lage sind, Huminsäuren, also hochmolekulare Anteile des gelösten organischen Materials, abzubauen.

Bereits in der Arbeit von Kisand et al. (2008) wurde gezeigt, dass Bakterien des Wattenmeeres Huminstoffe unter marinen, nicht aber unter ästuarinen (Salinität 15‰) Bedingungen abbauen (Kap. 4, Abb. 2, 3). Huminsäuren hingegen, eine hochmolekulare Fraktion der Huminstoffe, wurden unter marinen wie auch ästuarinen Bedingungen abgebaut (Kap. 4, Abb. 5). Die Zusammensetzung der am Abbau beteiligten Bakterien wurde von

Kisand et al. (2008) allerdings nicht untersucht. In den nachfolgenden Studien, die in dieser Arbeit präsentiert werden, konnte ebenfalls nachgewiesen werden, dass sowohl marine wie auch ästuarine Bakterien auf terrestrischen Huminsäuren wachsen. Dieses Ergebnis zeigt, dass Bakteriengemeinschaften aus dem gesamten Bereich des Weserästuars und des Wattenmeeres unter großen Schwankungen der Salinität allochthone, über Flüsse und das Grundwasser eingetragene terrestrische Huminsäuren (und vermutlich auch andere Huminstoffe) abbauen können.

Die Bakteriengemeinschaften, die sich aus dem marinen und ästuarinen Inokulum in den Durchflusskulturen entwickelt haben, zeigten deutliche Unterschiede. In den Kulturen, die mit Bakterien aus dem Wattenmeer inokuliert wurden, dominierten Isolate der *Proteobacteria*, mehrheitlich der *Alphaproteobacteria*, vor den deutlich weniger vertretenen *Gammaproteobacteria*, *Firmicutes* und *Actinobacteria*. Diese starke Dominanz einer Bakteriengruppe war in den Ansätzen mit ästuarinem Inokulum nicht zu finden. Die Mehrzahl dieser Isolate sind zwar ebenfalls *Proteobacteria*, hier aber insbesondere *Gammaproteobacteria*, gefolgt von deutlich weniger *Alphaproteobacteria* und einem *Betaproteobacteria*-Isolat (Kap. 2, Abb. 2 a-c, 3 b, d). Die *Actinobacteria* bilden nach den *Gammaproteobacteria* die zweitstärkste Fraktion, und mehrere Isolate sind sehr nah verwandt mit Vertretern, die z.B. aus einem industriell verschmutzten Hafengebiet (Harwati et al., 2007) oder aus einem nach einem Öltankerunfall kontaminierten Areal (Alonso-Gutiérrez et al., 2009) isoliert wurden.

Diese Ergebnisse aus den beiden Inkubationsversuchen sind vermutlich auf zwei Faktoren zurückzuführen, die Zusammensetzung der Bakteriengemeinschaft im Inokulum und die physiologische Vielfalt der unterschiedlichen Bakterienarten. Das Inokulum, welches einem Habitat entnommen wird, spiegelt dessen bakterielle Diversität wider. Bakterien, die schon in großer Zahl im Inokulum vorhanden sind, haben eine größere Chance, sich im Verlauf der Inkubationsexperimente durchzusetzen als die mit sehr viel geringerer Anzahl, sofern die Substrat- und Kulturbedingungen sich nicht allzu sehr von denen des ursprünglichen Habitats unterscheiden. Erkennbar wurde dieses im Vergleich der Bakteriengemeinschaft aus dem ostfriesischen Wattenmeer (bzw. dem Weserästuar) mit der entsprechenden Gemeinschaft, die sich im Verlauf der Inkubation entwickelt hat. Am deutlichsten zeigt sich das in der hohen Diversität der *Alphaproteobacteria*, insbesondere der *Roseobacter*-Gruppe, die sowohl im ostfriesischen Wattenmeer (z.B. Stevens et al., 2005 a, b; Rink et al., 2007) als auch auch in den durchgeführten Experimenten mit marinem Inokulum auftrat. Auch *Gammaproteobacteria* und *Firmicutes*, von denen fünf bzw. zwei Isolate

gewonnen wurden, sind im Wattenmeer vertreten, wobei die *Gammaproteobacteria* eine höhere Diversität aufweisen als die *Firmicutes* (Stevens et al., 2005 a, b, 2009). Eine deutliche Dominanz einer Bakteriengruppe wie bei den marinen Ansätzen trat in den Experimenten mit ästuarinem Inokulum nicht auf. Die hier gefundenen Bakterienarten sind ebenfalls in den vorangegangenen Studien, in diesem Fall denen des Weserästuars, gefunden worden. Auch dort, in den Bereichen des oligohalinen Ästuars, trat keine starke Dominanz einer Bakterienart auf (Selje und Simon, 2003; Selje et al., 2005). Ergänzend zum Einfluss des Inokulums auf die Entwicklung einer Bakteriengemeinschaft ist anzumerken, dass am Ende der Inkubationsexperimente das Kulturmedium nur zu 70% ausgetauscht gewesen ist und somit in den verbleibenden 30% Kulturinhalt noch Bakterien des ursprünglichen Inokulums vorhanden waren.

Für die Entwicklung und Sukzession der Bakteriengemeinschaft bei den HA-Abbauversuchen in Durchflusskulturen ist ebenfalls das physiologische Potential einzelner Bakterienarten bzw. der gesamten Bakteriengemeinschaft ausschlaggebend. Die physiologisch sehr vielfältigen *Alphaproteobacteria* dominierten die marinen Durchflusskulturen, und viele nahe Verwandte der gefundenen Isolate sind bekannt für den Abbau von aromatischen Verbindungen und refraktären Naturstoffen (z.B. Buchan et al., 2001; Harwati et al., 2007; Hilyard et al., 2008; Kertesz und Kawasaki, 2010). So können z.B. verschiedene *Sphingomonadales,* die aus den Durchflusskulturen isoliert wurden (insbesondere *Sphingobium* und *Sphingopyxis),* unterschiedlichste aliphatische und aromatische Kohlenwasserstoffe abbauen (Kertesz und Kawasaki, 2010). Die *Roseobacter*-Gruppe, welche fast die Hälfte der hier gewonnenen *Alphaproteobacteria*-Isolate umfasst, ist physiologisch sehr divers (Buchan et al., 2005; Brinkhoff et al., 2008), und verschiedene Vertreter daraus können Huminstoffe und viele andere aromatische Verbindungen abbauen (Buchan et al., 2001).

Die *Gammaproteobacteria* waren in den Versuchen mit ästuarinem Inokulum wichtiger Bestandteil der Bakteriengemeinschaft. Viele Vertreter der *Gammaproteobacteria* sind in der Lage, auf Öl und schwer abbaubaren Kohlenwasserstoffen zu wachsen, z.B. *Alcanivorax borkumensis* (Yakimov et al., 1998; Schneiker et al., 2006) und *Alcanivorax jadensis* (Bruns und Berthe-Corti, 1999; Fernández-Martínez, et al. 2003), die in der Nordsee isoliert wurden. Auch die recht hohe Zahl von *Actinobacteria* mit Isolaten, deren nahe Verwandte aus einem industriell verschmutzten Hafengebiet (Harwati et al., 2007) oder einem Areal nach einem Öltankerunfall (Alonso-Gutiérrez et al., 2009) stammen, zeigen den Vorteil physiologischer Vielfältigkeit, wenn nur schwer abbaubares Material wie polyaromatische

Kohlenwasserstoffe zur Verfügung stehen. Auch im Zusammenhang mit dem Abbau von Huminstoffen in Gewässern wurden *Actinobacteria* nachgewiesen, z.B. in huminstoffreichen Seen (Hutalle-Schmelzer et al., 2009). *Betaproteobacteria,* die in diesen limnischen Habitaten dominant sind (Burkert et al., 2003; Hutalle-Schmelzer et al., 2009, 2010), traten auch hier beim Abbau von Huminstoffen auf. Sie spielten allerdings nach den hier vorliegenden Ergebnissen sowohl in marinen wie auch in ästuarinen Bereichen keine oder nur eine untergeordnete Rolle beim Huminstoff-Abbau, da insgesamt nur ein Isolat gefunden wurde. Physiologische Vielfältigkeit ist also ein Vorteil, insbesondere wenn hauptsächlich refraktäres Material als Nahrungsquelle zur Verfügung steht. Dies trifft auch auf die anderen gefunden Isolatgruppen aus den Versuchen mit marinem wie ästuarinem Inokulum zu.

Bei der Analyse von Bakteriengemeinschaften anhand von Isolaten sollte allerdings berücksichtigt werden, dass nur ein geringer Anteil aller Bakterien in einer Probe, maximal 5%, meist unter 1%, isoliert werden kann (Meyer-Reil, 2005). Weiterhin ist die Verteilung der verschiedenen Phylotypen beeinflusst durch die Art der Isolierung - hier auf ausschließlich festem und nährstofflimitiertem Medium, was das Wachstum bestimmter Bakterienarten begünstigen kann (Eilers et al., 2001). Weiteren Einfluss auf die Entwicklung der Bakteriengemeinschaft während der Inkubationen kann aber auch eine Freisetzung von leichter abbaubarem DOM, verursacht z.B. durch Grazing von Protozoen, haben.

Ein weiterer wichtiger Aspekt ist der Vergleich der Isolate mit den Phylotypen aus den Banden der DGGE-Analyse. Der Vergleich von Isolaten und DGGE-Banden in den Ansätzen mit marinem Inokulum erbrachte geringe Unterschiede. Der Großteil der untersuchten Banden aus den Durchflusskulturen mit marinem Inokulum gehörte auch hier zu den *Alphaproteobacteria*, eine deutlich geringere Anzahl zu den *Gammaproteobacteria* und lediglich zwei Banden zu den *Bacteroidetes*. Die Durchflusskulturen mit ästuarinem Inokulum hingegen zeigten zwar größere Unterschiede zwischen Isolaten und DGGE-Banden, trotzdem wurden viele Bakteriengruppen durch beide Verfahren nachgewiesen. Bei der Interpretation der Sequenzierungsergebnisse der DGGE-Banden, insbesondere beim Vergleich mit den Isolaten, müssen verschiedene Aspekte berücksichtigt werden. Zum Einen wurde durch die Sequenzierung lediglich der prominenten Banden bereits eine „Vorauswahl“ getroffen und somit nicht die komplette Diversität erfasst. Ein weiterer Punkt ist, dass durch Sequenzüberlagerungen in den DGGE-Gelen eine Vielzahl der Banden kloniert werden musste und jeweils nur ein Klon pro Bande sequenziert wurde. Zusammengenommen führt dies zu einer Reduktion an Sequenzierungsergebnissen und folglich zu reduzierter Diversität. Da aber davon ausgegangen werden kann, dass sehr prominente Banden auch eine hohe

Abundanz der entsprechenden Bakterien in den Proben reflektieren, wurden dennoch die wesentlichen Vertreter der Bakteriengemeinschaft mittels DGGE identifiziert. Weitere Unterschiede zwischen den Ergebnissen der Isolierungen und Sequenzierung der DGGE-Banden sind Folge der bereits erwähnten unterschiedlichen Kultivierbarkeit von Bakterien. Da auch physiologische Untersuchungen mit den erhaltenen Isolaten ein Teilaspekt dieser Studie waren, wurden bevorzugt die Bakterien isoliert, die ein gut sichtbares und schnelles Wachstum zeigten. Die Untersuchung anhand der DGGE hingegen zeigt die vorhandene Bakteriengemeinschaft unabhängig von ihrer Kultivierbarkeit. Somit sind die unterschiedlichen Phylotypen aus DGGE im Vergleich zur Isolierung zu erwarten gewesen.

Die Tatsache, dass mehrere Phylotypen aus den Durchflusskulturen mit Huminsäuren eng verwandt sind mit Phylotypen aus früheren Studien des ostfriesischen Wattenmeeres und des Weserästuars, deutet darauf hin, dass die in beiden Habitaten natürlich vorkommenden Bakterien in der Lage sind, Huminsäuren und auch andere terrestrische Huminstoffe abbauen zu können. Weiterhin sind die gefundenen Phylotypen oftmals mit Bakterien verwandt, die bereits für den Abbau von HS und anderer refraktärer Substanzen und polyaromatischer Kohlenwasserstoffe bekannt sind. Dieses deutet darauf hin, dass spezielle physiologische Fähigkeiten zum Abbau dieser Substanzen innerhalb bestimmter Bakteriengruppen weit verbreitet sind. Dieses betrifft die metabolische Vielfalt der *Alphaproteobacteria*, insbesondere der *Roseobacter*-Gruppe, in den Versuchen mit marinem Inokulum, aber auch die der *Gammaproteobacteria* und der *Actinobacteria* in den Versuchen mit ästuarinem Inokulum. Die Vielfalt stellt einen wichtigen ökologischen Vorteil dar, der zu einer starken Präsenz dieser Isolate nach der Inkubation mit Huminsäuren beigetragen hat. Welche physiologischen Abbauwege dabei eine Rolle spielen, müsste in weiterführenden Arbeiten näher untersucht werden.

5.2 Bakterieller Abbau von Huminstoffen in Abhängigkeit von der Salinität

Es wurde in den beiden Versuchen mit unterschiedlichen Ansätzen gezeigt, dass Bakterien aus Habitaten mit verschiedenen Salinitäten in der Lage sind, ausschließlich mit Huminsäuren als einziger Kohlenstoffquelle zu wachsen.

Kisand et al. (2008) zeigten unter Verwendung dreistufiger Chemostaten, dass huminstoffreicher DOM (dissolved organic matter) unter marinen Bedingungen und mit marinem Inokulum abgebaut wird (Kap. 4, Abb.2, 5). Unter ästuarinen und limnischen Bedingungen mit Inokulaten aus den entsprechenden Habitaten wurden unter den

verschiedenen Bedingungen fast ausschließlich Huminsäuren, also eine hochmolekulare Huminstofffraktion, abgebaut bei gleichzeitigem Anstieg von Fulvinsäuren, also einer Huminstofffraktion niedrigeren Molekulargewichts. Ein bevorzugter Abbau von höher-molekularem DOM wurde bereits von Amon und Benner (1994, 1996) gezeigt, die experimentell einen erheblich stärkeren wie auch schnelleren Abbau von hochmolekularem DOM (HMW-DOM, >1000 Da) gegenüber niedrig molekularem DOM (LMW-DOM, <1000 Da) zeigen konnten. Anhand dieser und anderer Studien stellten sie eine Beziehung zwischen der Größe des Moleküls und dessen Reaktivität her (Size-reactivity continuum model). Mit sinkender Molekülgröße während des fortschreitenden Abbauprozesses sinkt auch die Reaktivität des Moleküls. Die Entstehung biologisch schwer abbaubarer wie auch ungewöhnlicher Substanzen z.T. auch durch abiotische Prozesse während des Abbaus von HMW-DOM ist eine mögliche Folge (Amon und Benner, 1994). Dieses zeigen auch die Ergebnisse von Kisand et al. (2008), wo unter ästuarinen und limnischen Bedingungen die Huminsäuren abnahmen, die Fulvinsäuren hingegen zunahmen. Allerdings bestand das Kulturmedium aus huminstoffreichem Wasser, daher ist eine Differenzierung von bereits vorhandenen und später gebildeten Fulvinsäuren schwierig. Nach Abschluss der Inkubationen mit isolierten Huminsäuren konnten ebenfalls Fulvinsäuren isoliert werden, was die Folge des bakteriellen, aber auch physiko-chemischen Abbaus ist. Allerdings zeigen insbesondere die hohen Werte der bakteriellen Produktion (Kap. 3, Abb.2, 3), dass hauptsächlich Bakterien und weniger diese physikalischen Einflüsse den Abbau der Huminsäuren bewirkt haben. Zudem ist auch die Dynamik der Bakteriengemeinschaft (Kap. 2, Abb.1) und der Bakterienzellzahlen (Kap. 3, Abb.2, 3) sowie die Vielzahl und Vielfalt an huminstoffabbauenden Isolaten ein deutliches Indiz für den Abbau von Huminsäuren primär durch Bakterien.

Eine weitere Erklärung der erhaltenen Ergebnisse liegt in der Zusammensetzung der HA-Moleküle. Nach Strukturvorschlägen von z.B. Haworth (1971) bestehen Huminstoffe aus aliphatischen und aromatischen Verbindungen, wobei letztere zumeist einen schwer angreifbaren Molekül“kern“ bilden und erstere als leicht abbaubare Bestandteile im äußeren Molekülbereich exponiert sind. Der massive Anstieg der Bakterienzahlen v.a. in den ersten Tagen gefolgt von dem beinahe äquivalenten Abfall deutet auf den mikrobiellen Abbau der labilen Komponenten in den HA-Molekülen hin. Diese Vermutung wird gestützt durch die sehr hohe bakterielle Biomasseproduktion vom zweiten bis ca. 6. Tag in den Ansätzen mit ästuarinem Inokulum (Kap. 3, Abb. 2, 3, für marine Ansätze sind die Daten nicht verfügbar) und der ebenfalls zeitlich korrelierten Phase des verstärkten DOM-Abbaus (Kap. 3, Abb. 2, 3). In der ersten Phase der Inkubation bauen die Bakterien nicht nur die einfließenden HA im

Medium ab, sondern auch die HA, die bereits in den Kulturgefäßen vorhanden sind. Nach ca. 3-4 Wochen hingegen wurde nur noch das hineinströmende Medium abgebaut (Kap.3 Fig. 2, 3). Diese Resultate lassen darauf schließen, dass die Bakterien in den Chemostaten die HA bis zu einem bestimmten Grad abbauen konnten und der restliche DOM kaum noch bakteriell abgebaut wurde.

Neben der molekularen Struktur und Größe des eingesetzten Substrates ist die Salinität des Mediums wie auch der Ursprung der Bakteriengemeinschaften (Kap. 5.1) von entscheidender Bedeutung beim Abbau der HA. In den einstufigen Durchfluss-kulturversuchen, wie zuvor in den dreistufigen Chemostaten von Kisand et al. (2008), konnten die eingesetzten Bakterien HA unter marinen wie ästuarinen Bedingungen abbauen (Kap.3, Abb. 2, 3, Kap.4, Abb. 2, 3). Wurde das Inkubationsmedium in ergänzenden Versuchen mit einstufigen Durchflusskulturen auf eine vom Inokulum abweichende Salinität eingestellt (Inokulum marin mit oligohalinem Medium bzw. umgekehrt), war der DOM-Abbau höher als bei gleicher Salinität von Inokulum und Medium (Kap. 3, Abb.2, 3). Ein verstärkter DOM-Abbau durch ästuarine Bakterien in Folge einer Salinitätsänderung wurde bereits von Langenheder et al. (2003) beschrieben. Wie ebenfalls in der Studie dargestellt, sind für solche Effekte mehrere Mechanismen denkbar. Einerseits kann der Salinitätswechsel eine Veränderung der Bakteriengemeinschaft bewirken, was bereits zuvor diskutiert wurde (Kap. 5.1). Eine Veränderung der Molekularstruktur der HA-Moleküle bzw. der am Abbau beteiligten Enzyme durch die geänderte Ionenstärke (Langenheder et al., 2003; Boyd und Osburn, 2004) ist zwar theoretisch möglich, konnte während der durchgeführten Inkubationen aber nicht untersucht werden.

Andererseits ist auch die Beeinflussung der bakteriellen Physiologie durch die Salinität ein möglicher Faktor. Es ist zu vermuten, dass die veränderte Ionenkonzentration zum Abbau beiträgt, sich also auf den bakteriellen Metabolismus auswirkt. Bereits in früheren Studien wurde gezeigt, dass die Salinität Einfluss auf Enzymtätigkeiten hat und dass Enzyme unterschiedliche Salinitätsoptima aufweisen (Stepanauskas et al., 1999). Es wird einerseits vermutet, dass eine verstärkte Ionenkonzentration die Exponierung von aktiven Zentren der Enzyme bewirkt und/oder eine bessere Zugänglichkeit der abzubauenden funktionellen Gruppen zur Folge hat (Langenheder et al., 2003). Andererseits können bestimmte Ionen wie z.B. Mg^{2+} notwendig sein für aktive Zentren bestimmter Enzyme, so dass Salinitätsänderungen Auswirkungen auf die Aktivitäten einzelner Enzyme haben (Stepanauskas et al., 1999)

Dass vermutlich viele unterschiedliche Enzyme, abhängig von Bakterienart, Salinität und Phase der Inkubation, am Abbau der Huminsäuren beteiligt waren, ist anhand der Komplexität der HA-Moleküle sehr wahrscheinlich und wurde anhand der Substrattests mit einzelnen Isolaten deutlich. Es zeigte sich, dass von den als C-Quelle angebotenen Aromaten zumeist nur Phenol abgebaut werden konnte. Allerdings wiesen Isolate der *Roseobacter*-Gruppe ein deutlich breiteres Substratspektrum auf (Kap. 2, Abb. 5). Dass insbesondere diese Bakteriengruppe refraktäres Material wie Lignin und HS abbauen kann, wurde bereits von Buchan et al. (2001, 2004, 2005) gezeigt. Diese Autoren hoben insbesondere die Bedeutung des β-Ketoadipatweges hervor, über welchen, auch von anderen Bakterienarten, verschiedene aromatische, v.a. Lignin-assoziierte Substanzen, abgebaut werden können. Allerdings wurden nur 3 Isolate, die nicht den *Alphaproteobacteria* angehören, den Substrattests unterzogen. Unabhängig von der Bakterienspezies kann eine Vielzahl weiterer Enzyme (Filip et al., 1998; Münster und de Haan, 1998) am Abbau beteiligt sein, was anhand der bereits dargestellten Komplexität der Huminsäuremoleküle nicht überraschend ist. Über welche physiologischen Stoffwechselwege Huminsäuren von den hier untersuchten Bakteriengemeinschaften bzw. den Einzelisolaten abgebaut werden können, sollte anhand fortführender Studien weiter untersucht werden.

5.3 Strukturelle Modifikation von Huminstoffen durch Bakterien

Bisher sind viele Untersuchungen über die strukturelle Zusammensetzung von Huminstoffen veröffentlicht worden (z.B. Hempfling und Schulten, 1991; Ishiwatari, 1992; Martin et al., 1995; Lehtonen, 2000a, b; Piccolo, 2001; Guignard et al., 2005). Studien über den bakteriellen Abbau dieser als refraktär geltenden Substanzen mittels Vorher-Nachher-Vergleich hingegen gibt es bisher nicht.

In den durchgeführten Untersuchungen wurden sowohl das unveränderte Substrat, also die eingesetzten Huminsäurefraktionen (Mar-M, Est-M, Kap. 3, Abb.4, 5, Tab. 2), als auch die erhaltenen Huminstofffraktionen am Ende der Versuche mittels Pyrolyse-GC/MS (Py-GC/MS) analysiert. Es zeigte sich, dass am Ende der Versuche sowohl Huminsäuren als auch Fulvinsäuren aus dem Inkubationsmedium isoliert werden konnten. Der Vergleich von Huminsäure-Ausgangssubstrat mit den isolierten Fulvinsäure-Fraktionen nach den Inkubationen erbrachte nur geringe Unterschiede im Bezug auf das Verhältnis von aromatischen zu aliphatischen Verbindungen (Aromaten: Aliphaten, Kap. 3, Abb. 4 b, d). Dieses Ergebnis zeigt, dass die größeren Huminsäuremoleküle in kleinere Einheiten, die den größeren in ihrer Zusammensetzung sehr ähneln, gespalten wurden. Dieses kann sowohl

Folge eines bakteriell-enzymatischen Abbaus als auch eines rein physikalischen Prozesses sein. In den meisten Modellen von Huminstoffmolekülen (Haworth, 1971; Schulten und Leinweber, 1996; Piccolo, 2001; Dittmar und Kattner, 2003) werden aromatische „Kernbereiche“ über aliphatische Ketten oder andere schwache Bindungen miteinander verbunden. Die Isolierung von Fulvinsäuren nach den hier durchgeführten Inkubationsexperimenten war unter Berücksichtigung der Building-block-Hypothese einerseits eine Folge des bevorzugten Abbaus aliphatischer Verbindungen. Andererseits kann nicht ausgeschlossen werden, dass ein Teil der Huminsäuren bereits beim Lösen in dem ASW-Medium vor der Zugabe der Bakterien in die Kulturgefäße in kleinere Moleküle, also Fulvinsäuren zerfallen ist. Dieses könnte eine Folge der Isolierungsprozedur mit XAD-Harzen gewesen sein, welche mit starken pH-Änderungen, großen Druckunterschieden im Rotationsverdampfer und einer abschließenden Vakuumtrocknung einher ging. Welcher der angesprochenen Mechanismen zu welchem Anteil Ursache des Auftretens der Fulvinsäurefraktionen ist, konnte anhand der vorliegenden Daten nicht geklärt werden. Wie bereits zuvor dargestellt, werden Huminsäuren primär durch Bakterien zu Fulvinsäuren abgebaut, was durch die Betrachtung der Dynamik der Bakterienzellzahlen und der Bakteriengemeinschaft, der bakterielle Produktion und weiterer Parameter deutlich wird.

Ein direkter Vergleich der isolierten Huminstofffraktionen in den marinen und ästuarinen Ausgangsmedien mit den Fraktionen aus den Kulturmedien nach den Inkubationen mit marinen bzw. ästuarinen Bakterien zeigte in allen Fraktionen eine deutliche Abnahme an identifizierbaren Substanzen (Kap. 3, Tab. 2). Die Ergebnisse lassen erkennen, dass im Verlauf der Inkubationen bestimmte Substanzgruppen wie Fettsäuremethylester, Zimtsäuren und Phenylketone vollständig bzw. fast vollständig bakteriell abgebaut wurden, während andere weiterhin nachweisbar blieben. Dieses macht deutlich, dass bestimmte Substrate bakteriell verwertbar sind, während andere von Bakterien nicht verwertet werden können. Benner und Kaiser (2011) zeigten einen bakteriellen Abbau bevorzugt von Aminosäure-Bestandteilen, wohingegen z.B. Ligninphenole primär durch UV-Licht und kaum bakteriell abgebaut werden. Weitere Studien zeigten ebenfalls, wie speziell das Substratspektrum verschiedener aquatischer Bakterien sein kann, insbesondere bei aromatischen Verbindungen (z.B. Esham et al., 2000; Harwati et al., 2007). Auch Untersuchungen über den Abbau anderer Mischsubstrate (z.B. in Böden) zeigten, dass bestimmte Komponenten bevorzugt, andere hingegen nicht abgebaut wurden (z.B. Larsson et al., 1988; Kästner und Mahro, 1996; Hernández et al., 2001; Grötzschel et al., 2002). Die beobachtete Bevorzugung einzelner Substanzgruppen ist vermutlich auf die physiologischen Eigenschaften der beteiligten

Bakterien zurückzuführen. Für den Abbau von Lignin-verwandten Substanzen, die HS ebenfalls strukturell ähneln, konnte z.B. der ß-Ketoadipatweg nachgewiesen werden (Buchan et al., 2001).

Ein weiteres Ergebnis der Untersuchungen ist eine Zunahme der Aromatizität im Vergleich der Ausgangsmedien und der Medien am Ende der Versuche. Daraus ist ein bevorzugter Abbau der aliphatischen Verbindungen (Kap. 3, Abb.4 a, c) in HA-Molekülen durch Bakterien erkennbar. Die ermittelten Verhältnisse von Aliphaten zu Aromaten bei den isolierten HA-Fraktionen am Ende der Versuche weisen zusätzlich darauf hin, dass viele aromatische Verbindungen trotz deutlicher Abnahmen einzelner aromatischer Verbindungen bakteriell schwerer angreifbar sind als aliphatische (Kap. 3 Abb. 5c, d). Bestimmte Aromaten, insbesondere Benzol und seine Derivate, wurden im Versuch mit dem ästuarinen Inokulum unabhängig von der eingestellten Salinität bevorzugt abgebaut (Kap. 3, Abb. 5c, d). In den Substrattests der Bakterienisolate auf verschiedenen Aromaten wurde bereits deutlich, dass zwar fast alle Isolate HA abbauen können, allerdings nur eine begrenzte Auswahl weitere aromatische Verbindungen verwerten kann (Kap. 2, Tab. 3). Hier zeigten wiederum die *Alphaproteobacteria* ein größeres Substratspektrum als alle anderen Bakteriengruppen, was ein deutlicher Hinweis auf deren physiologische Vielfalt hinsichtlich des Aromatenabbaus ist. Allerdings ist bei der Auswahl der untersuchten Isolate primär die Wachstumsgeschwindigkeit und weniger die phylogenetische Zugehörigkeit ausschlaggebend gewesen, weshalb nur drei Isolate anderer Gruppen getestet wurden. Die Ergebnisse aus den Substrattests mit Einzelisolaten (Kap. 2, Tab. 3) zeigten, dass kein Isolat alle angebotenen Aromaten abbauen konnte und nur wenige Isolate mehr als eine aromatische Verbindung. Dieses kann zum Einen darauf zurückzuführen sein, dass andere als die untersuchten Bakterienisolate z.B. die Benzolderivate abgebaut haben. Ebenfalls ist es möglich, dass die untersuchten Bakterien die unterschiedlichen Aromaten nicht allein, sondern nur in Kooperation mit anderen Bakterien abbauen können, also synthroph durch Kometabolismus. Dieses wäre in weitergehenden Versuchen zu prüfen.

Typische ligninähnliche Substanzen wie die Zimtsäurederivate traten in den Ausgangsmedien auf, waren aber in kaum einer Fraktion nach den Inkubationen noch vorhanden (Kap. 3, Abb. 5e, f). Dies lässt darauf schließen, dass nicht nur terrestrische Bakterien (z.B. Gramss et al., 1998; Tikhonov et al., 2010), sondern auch ästuarine und marine Bakterien Lignin-ähnliche Substanzen aus terrestrischen Huminsäuren abbauen können, wie zuvor bereits z.B. von Moran und Hodson (1989, 1994), Esham et al. (2000) und Benner und Kaiser (2011) in anderen Gewässern gezeigt wurde. Aquatische Mikroorganismen

haben sich also physiologisch angepasst, wodurch sie eingetragene terrigene und somit habitatfremde Substanzen abbauen können. Auch hier sind weitergehende Untersuchungen und Vergleiche bezüglich der enzymatischen Zusammenhänge sinnvoll.

Der Vergleich beider Ausgangsmedien, Mar-M und Est-M, zeigte, dass die Anzahl der identifizierbaren aliphatischen Verbindungen gleich war, die der aromatischen Verbindungen allerdings unterschiedlich. Auch die Verteilung der Einzelkomponenten auf die verschiedenen Substanzgruppen unterschied sich (Kap. 3, Tab. S1, S2). Diese deutlichen Unterschiede in den beiden Medien sind in erster Linie auf die verschiedenen Isolierungszeitpunkte zurückzuführen. Die Huminsäuren für das ästuarine Medium sind im Frühjahr 2007 und die für das marine Medium hingegen im Frühsommer 2008 isoliert worden. Die Huminstoffgenese hängt von verschiedensten Faktoren ab, wie in der Einleitung beschrieben. Zwischen den Isolierungen der beiden Huminstoffe liegt ein Zeitraum von ca. einem Jahr, so dass sich vermutlich äußere Faktoren wie z.B. das organische Ausgangsmaterial und Witterung (insbesondere Niederschläge), Temperaturunterschiede, forstwirtschaftliche Aktivitäten etc. auf die Huminstoffbildung ausgewirkt haben. Mehrere Verbindungen aus den beiden Medien sind zuvor auch in Untersuchungen von Kisand (unveröffentlichte Daten) und Gebhardt (2005) gefunden worden. Dieser Vergleich wie auch der Vergleich beider Medien zeigte, dass die Huminstoffgenese trotz des gleichen Isolierungsgebiets sehr vielfältig ist und unterschiedliche Huminstoffmoleküle hervorbringt. Allerdings sind mehrere Verbindungen, welche in den Ausgangsmedien auftraten, in anderen Untersuchungen von Huminstoffen gefunden worden (Hatcher et al., 2001, Song et al., 2005). Dies zeigt wiederum, dass Huminstoffmoleküle unabhängig von den Habitaten mehrere ähnliche bzw. gleiche Strukturen aufweisen können.

Insgesamt zeigte sich anhand des Vorher-Nachher-Vergleichs, dass sowohl marine, als auch ästuarine Bakterien in der Lage sind, terrestrische Huminsäuren partiell abzubauen und somit die ursprünglichen Huminsäure-Moleküle zu verändern. Hierbei wurde, unabhängig von den einzelnen Ansätzen mit unterschiedlichen Bedingungen (Inokulum aus angrenzenden Habitaten, veränderte Salinität, Sedimentzugabe) eine Präferenz zum Abbau aliphatischer Verbindungen festgestellt. Weiterhin wurden aber auch wenige ausgewählte Aromaten vollständig oder weitgehend abgebaut. Zusammengenommen weisen diese Ergebnisse darauf hin, dass vermutlich viele der hier betrachteten Bakterien über spezielle physiologische Eigenschaften (spezielle Enzymausstattung) verfügen, um HA abbauen zu können.

5.4 Ausblick

Die Ergebnisse der vorliegenden Arbeit beantworteten eine Reihe grundlegender Fragen bezüglich des bakteriellen Abbaus von Huminsäuren im ostfriesischen Wattenmeer und im Weserästuar. Zudem sind, resultierend aus den erhaltenen Ergebnissen, neue interessante Fragestellungen aufgetreten.

In dieser Arbeit wurde der Focus auf den Abbau hochmolekularer Huminstoffe gesetzt. Neben den hochmolekularen Huminsäuren gehören auch die niedermolekularen Fulvinsäuren zu den Huminstoffen. Demnach wäre es interessant, in weiterführenden Untersuchungen Inkubationsexperimente parallel mit Huminsäuren und Fulvinsäuren, also Huminstoffen unterschiedlichen Molekulargewichts, durchzuführen. Eine wichtige Fragestellung hierbei wäre, ob die verschiedenen Huminstofffraktionen durch Bakteriengemeinschaften in gleichem oder unterschiedlichem Ausmaß abgebaut werden können. Die Arbeit von Kisand et al. (2008) gibt einen Hinweis darauf, dass Fulvinsäuren nur unter bestimmten Salinitätsbedingungen und mit Bakteriengemeinschaften aus den jeweils korrespondierenden Habitaten abgebaut werden können. Folglich sollte in weiteren Experimenten sowohl der Salzgehalt des Mediums, als auch der Ursprung des Inokulums, also Ästuar oder Wattenmeer verändert werden. Interessant wären hierbei der direkte Vergleich der Bakteriengemeinschaften und deren enzymatische Aktivität (z.B. durch Methoden der Transkriptom- oder Proteomanalyse). Einerseits sollte bei gleichem Inokulum verglichen werden, ob Unterschiede nach Inkubation mit Huminsäuren bzw. Fulvinsäuren auftreten. Andererseits ist auch die Frage, welche Auswirkungen ein Salinitätswechsel in Anlehnung an die hier durchgeführten Experimente auf eine fulvinsäureabbauende Bakteriengemeinschaft hat, wenn z.B. ein Inokulum aus einem ästuarinen Bereich in marinem Medium inkubiert wird und umgekehrt. Auch die Einbeziehung von Bakteriengemeinschaften aus dem Brackwasserbereich der Weser und entsprechende Anpassung der Inkubationsmedien würde das Verständnis von Salinität im Huminstoffabbau erweitern. Verschiedene Methoden wie DGGE und Isolierung könnten hier ein umfassendes Bild einer fulvinsäureabbauenden im Vergleich zur huminsäureabbauenden Bakteriengemeinschaft aufzeigen.

Neben der Betrachtung der gesamten Bakteriengemeinschaft sollte ein weiterer Focus auf der Untersuchung einzelner Isolate liegen. In dieser Arbeit wurden erste Inkubationsversuche mit Isolaten durchgeführt. In weiteren Versuchen sollten nicht nur die Substratspektren der Isolate ermittelt, sondern auch die Beteiligung einzelner Stoffwechselwege oder Enzyme näher untersucht werden. Das Transkriptom oder Proteom von Reinkulturen (z.B. nach Wachstum auf unterschiedlichen Substraten oder bei veränderter

Salinität) könnte hierzu wertvolle Hinweise liefern. Auch in diesen Experimenten sollten der Einfluss der Salinität z.B. auf die Expression bestimmter Enzyme als auch die Unterschiede von hoch- und niedermolekularem Substrat untersucht werden. Der Vergleich der am Huminstoffabbau beteiligten Enzyme in Bakterien verschiedener Gattungen oder auch unterschiedlicher Phyla wäre ebenfalls eine wichtige Ergänzung zum Verständnis des Huminstoffabbaus.

Die Ergebnisse aus den Vorher-Nachher-Analysen mittels Pyrolyse-GC/MS zeigten, dass in den Huminsäurefraktionen insbesondere aliphatische, aber auch sehr spezielle aromatische Verbindungen abgebaut wurden. Entsprechende Untersuchungen sollten auch bei Experimenten bezüglich des Abbaus von Fulvinsäuren stattfinden. Durch einen Vorher-Nachher-Vergleich würde erkennbar, ob einzelne Verbindungen wie z.B. diejenigen, die nach Inkubation mit Huminsäuren nicht mehr nachweisbar waren, auch hier bakteriell abgebaut werden.

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Danksagung

Zu Beginn möchte ich mich bei Prof. Dr. Meinhard Simon für die Möglichkeit zur Promotion in der Arbeitsgruppe „Biologie geologischer Prozesse“ bedanken. Insbesondere danke ich Dir für viele anregende Gespräche und Ratschläge, gerade in den letzten Monaten. Prof. Dr. Jürgen Rullkötter danke ich für die unkomplizierte Übernahme des Zweitgutachtens.

Weiterhin möcht ich mich bei Dr. Veljo Kisand für seine Hilfe, kam sie von nah oder fern, bedanken, für die Begleitung und Hilfe währen der vergangenen Jahre und die vielen hilfreichen Emails aus Estland. Ein großes Dankeschön gilt meinem Bürogefährten PD Dr. Thorsten Brinkhoff. Für die vielen fachlichen und nicht-fachlichen Gespräche, die Geduld bei der Einführung in *arb*, die Unmengen an Schokolade und Muffins und die große Tasse schwarze Tee im richtigen Moment danke ich Dir sehr. Frau Dr. Barbara Scholz-Böttcher möchte ich mich herzlich für die große Hilfe mit der Pyrolyse GC/MS bedanken. Deine geduldige Art und enormes Wissen über die unendlichen Tiefen der Pyrolyse haben mir sehr geholfen! Ebenfalls danken möchte ich Dr. Bert Engelen. Neben den Tips zu Bioedit werden mir Deine Serientips, der Einblick in skurile TV-Serien, und die vielen Tips zu Hörbüchern und Büchern in Erinnerung bleiben. Prof. Dr. Thomas Kneib danke ich sehr für die Hilfe bei der statistischen Auswertung meiner Ergebnisse.

Renate Gahl-Janssen danke ich sehr herzlich für für ihre unkomplizierte Hilfe und viele tolle Tips und Tricks. Auch danke ich Dir für viele, sehr persönlichen Gespräche und dass Du mich auf ungeplanten und nicht ganz freiwilligen Fahrten ins nächste Krankenhaus begleitet hast. Bei Andrea Schlingloff bedanke ich mich sehr für die große Hilfe beim Sequenzieren, beim TOC und natürlich für die Gartentips! Ein großes Dankeschön gilt auch Birgit Kürzel. Unsere Fahrten ans AWI haben mir sehr viel Spaß gemacht, wie auch die Diskussionen am Flow Cytometer. Rolfo, ich danke Dir für Deine immerwährende gute Laune, die einfach ansteckend ist. Dir, Marco, mein Ex-Praktikant, Ex-Diplomand und noch Mit-Doktorand danke ich für Deine Ausdauer und flexible Hilfe, vor allem wenn es mal wieder schnell gehen musste. Auch meinen anderen Praktikanten, v.a. Mascha und Thomas danke ich für die Unterstützung! Ein großes Dankeschön an meine ultrafleissigen Mit-Doktoranden (incl. Ehemaligen) für die große Unterstützung insbesondere in der letzten Zeit meines Labordaseins. Martine danke ich sehr für die Hilfe mit dem Klonieren und viele lustige, ernste, nachdenkliche und vor allem offene Gespräche. Auch Dir Heike möchte ich für Deine immer lustige und mitreissende Art danken, die einen einfach in gute Laune versetzt! Helge und Lemmi danke ich für die Tips beim FlowCytometer und im

Isotpoenlabor, die immerwährenden Laborneuigkeiten und das unkomplizierte Leben im Labor! Auch Beate und Mirko möchte ich für viele Tips zu Beginn meiner Arbeit danken.

Der AG Cypionka danke ich für das nette Miteinander und die Hilfe bei sehr vielen kleinen Dingen des Laboralltags, insbesondere Michael für die lustigen Gespräche am frühen Morgen, Sonja für die Hilfe der Suche nach seltsamen Untensilien und Katja für die Hilfe bei der neuen DGGE. Auch Annette werde ich nie vergessen, mit der ich viele Gespräche, mal ernste, meist aber sehr lustige, führen konnte.

Bei der AG Rullkötter möchte ich mich insbesondere für die große Geduld beim stundenlangen Tropfen der großen Säulen im Labor während der wochenlangen Huminstoffextraktionen bedanken und bei Kerstin für die Hilfestellung bei der Pyrolyse.

Der ehemaligen AG Berthe-Corti danke herzlich ich für die Hilfe bei den ersten Schritten mit großen Kulturgefäßen und für die Überlassung verschiedener Bakterienstämme.

Ein besonderes Dankeschön gilt Peter Kanje für die unkomplizierte Hilfe im Isotopenlabor und die interessanten Gespräche.

Der AG Medlin am Alfred-Wegener-Institut danke ich für die Nutzung des Flow-Cytometers, die mir viel manuelle Zählarbeit erspart hat.

Ein großes Dankeschön gilt Mitarbeitern der Verwaltung, v.a Elke und Gudrun für die unkomplizierte Hilfe in allen Lebenslagen.

Kirstin Glaser danke ich für die schnelle Bearbeitung von Blitzaufträgen, wenn mal wieder die Glasfritten gesprungen sind. Für die Blitzbestellungen und die ergibigen Uni-News bedanke ich mich auch bei Matthias Nolte.

Mein persönlicher Dank gilt in erster Linie meinen Eltern Karla und Hinrich Rocker, die mich bis zum heutigen Tag auf ihre eigene, unbeschreibliche Art unterstützt haben. Für Eure Liebe und Euer Verständnis danke ich Euch von Herzen. Auch bei allen meinen Geschwistern, sowie deren Lebensgefährten/Ehepartnern, möchte ich mich bedanken für viele aufmunternde und verständnisvolle Gespräche und einfach fürs Dasein zu jeder Tages- und Nachtzeit. Ihr seid einfach toll! Meinen Freunden, insbesondere Petra, Silke, Conny, Steffi und Heike danke ich für die große Geduld in den letzten Jahren und das Verständnis, wenn ich mich mal eine längere Zeit nicht gemeldet habe. Ab jetzt bin ich auch wieder per Telefon erreichbar!

Dir Michael danke ich für Deine große Unterstützung in den letzten Jahren. Danke für Deinen Zuspruch, die vielen Umarmungen, die Unmengen an Taschentüchern, Deine Geduld und die stete Versorgung mit frischem Tee, wenn ich am Schreibtisch gesessen habe. Ohne Dich wäre ich nicht hier.

Lebenslauf

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Erklärung

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

Edewecht, den 26.02.2011