Dioxin-dechlorierende Bakterien in anaeroben Kulturen aus kontaminierten Flußsedimenten

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Inhalt

Zusammenfassung

Über die Identität Dioxin-dehalogenierender Bakterien ist noch wenig bekannt. In dieser Arbeit wurde durch Anwendung von molekularen Methoden der mikrobiellen Ökologie erstmalig ein Hinweis auf die Beteiligung eines *Dehalococcoides*-Stammes an der Dioxin-Dehalogenierung erhalten. In nachfolgenden Versuchen wurde der für die Dehalogenierung von Chlorbenzolen bekannte *Dehalococcoides*-Stamm CBDB1 als erstes Dioxine-dechlorierendes Bakterium identifiziert.

Als Voraussetzung für diese Arbeiten wurden zunächst Anreicherungskulturen mit Sedimenten des stark mit polychlorierten Dioxinen und Furanen (PCDD/F)-kontaminierten Flusses Spittelwasser angesetzt und auf ihre Fähigkeit untersucht, chlorierte Dioxine unter strikt anaeroben Bedingungen zu dechlorieren. In diesem Teil der Arbeit war es zunächst wichtig, die Transformationswege anhand spezieller Modellverbindungen aufzuklären. Die Kulturen aus verschiedenen Schichten zweier Sedimentkerne waren in der Lage, 1,2,3,4-Tetrachlordibenzo-*p*-dioxin (1,2,3,4-TeCDD), 1,2,3-Trichlordibenzo-*p*-dioxin (1,2,3-TrCDD) und 1,2,4-Trichlordibenzo-*p*-dioxin (1,2,4-TrCDD) reduktiv zu dehalogenieren. Dabei wurden unterschiedliche Dechlorierungswege gefunden, die sich ausgehend von den eingesetzten Dioxinen besonders in der Bildung der dichlorierten Kongenere 1,3- und 2,3-Dichlordibenzo-*p*-dioxin (DiCDD) unterschieden. In nachfolgenden Versuchen wurden Anreicherungskulturen von weniger PCDD-belasteten Flußsedimenten angelegt. Wie gezeigt werden konnte, waren diese Kulturen ebenfalls in der Lage chlorierte Dioxine zu dehalogenieren. Für eine erfolgreiche Anreicherung ist die historische Kontamination der Sedimente offenbar keine Voraussetzung.

Zum selektiven Nachweis von Bakterien, die für ihre Fähigkeit bekannt sind, verschiedene halogenierte Verbindungen zu transformieren, wurden PCR-Primerpaare abgeleitet. Mit einem speziellen *nested* PCR-Verfahren konnte das Vorhandensein einiger

dieser Bakterien in den Kulturen gezeigt werden. Besonders interessant war der Befund, dass in allen untersuchten Kulturen Organismen der Gattungen *Desulfitobacterium* und *Dehalococcoides* nachgewiesen werden konnten. Das amplifizierte *Dehalococcoides*-16S rRNA-Genfragment war identisch mit der Sequenz des Stammes CBDB1. Dieser Stamm war zuvor als Tri- bis Hexachlorbenzol-Dechlorierer isoliert und charakterisiert worden (Adrian *et al*. 2000, *Nature* 408: 580-583) und wurde in der vorliegenden Arbeit für Versuche zur Dioxin-Dechlorierung eingesetzt. Der Stamm CBDB1 konnte die getesteten Verbindungen 1,2,3,4-TeCDD, 1,2,3-TrCDD, 1,2,4-TrCDD und 2,3-DiCDD zu niedriger chlorierten Verbindungen dehalogenieren. Die Verbindung 2-Monochlordibenzo-*p*-dioxin war das vorläufige Endprodukt. Bemerkenswert war die Transformation der unter umwelttoxikologischen Aspekten relevanten Verbindung 1,2,3,7,8-Pentachlordibenzo-*p*-dioxin. Während der Dehalogenierung reicherte sich 2,3,7,8-Tetrachlordibenzo-*p*-dioxin an, welches aber durch diese Reinkultur weiter bis zu 2,7- oder 2,8-DiCDD umgesetzt wurde.

Ein primäres Problem für die Charakterisierung und Isolierung von Dioxin-dechlorierenden Bakterien ist ihr geringer Anteil in Mischkulturen, der zum Teil durch die geringe Wasserlöslichkeit und Verfügbarkeit der Substrate bedingt ist. Der Anteil der Dioxin-dechlorierenden Population an der Gesamtzellzahl wurde mit etwa 0,01 % bestimmt. Im Rahmen der Arbeit wurde daher versucht, mit alternativen chlorierten Verbindungen eine Anreicherung von Dioxin-Dehalogenierern zu erreichen. 1,2,3-Trichlorbenzol, welches über ein Zweiphasensystem mit Hexadekan zugesetzt wurde, konnte durch die Mischkulturen zu 1,3-Dichlorbenzol dechloriert werden. Dabei nahm die Zahl der Dioxin-dehalogenierenden Bakterien um drei Grössenordnungen zu. Die Änderung der Populationsstruktur während des Anreicherungsschrittes mit Trichlorbenzol wurde mit 16S rDNA-basierten Methoden dokumentiert. Durch Restriktionsfragmentlängen-Analyse von 16S rRNA-Genbanken konnte

die Zunahme von zehn verschiedenen Restriktionsmustern während der 1,2,3-Trichlorbenzol-Dechlorierung gezeigt werden. Abgeleitet aus der starken Zunahme der entsprechenden Sequenzen in der Genbank deutete dies auf die Anreicherung eines mit *Dehalococcoides* sp. CBDB1 verwandten Stammes und eines Bakteriums der *Cytophaga-Flavobacterium-Bacteroides*-Gruppe hin. Mit einer anderen 16S-basierten Methode, *Single-Strand Conformation Polymorphism*, konnte ausserdem die Anreicherung eines *Trichlorobacter thiogenes*-ähnlichen Bakteriums (δ-*Proteobacteria*) dokumentiert werden.

Die Interaktionen zwischen dechlorierenden Bakterien und anderen Populationen innerhalb von Mischkulturen sind wenig untersucht, könnten aber von entscheidender Bedeutung für die Isolierung von Dioxin-dechlorierenden Bakterien sein. Es war interessant, dass in den Anreicherungskulturen, die mit einem Gemisch von organischen Säuren inkubiert wurden, ein Vertreter der Gattung *Syntrophus* identifiziert werden konnte. Seine mögliche Rolle bei der Bereitstellung von Wasserstoff als direktem Elektronendonor für die reduktive Dechlorierung wird diskutiert.

Einführung

Dioxin und Öffentlichkeit

Das Erscheinen von Rachel Carsons "Silent Spring" im Jahr 1962 zeigt auf eindrucksvolle Weise die Folgen des massiven Einsatzes von chlorierten organischen Verbindungen und markiert zweifellos einen Wendepunkt in der Zeit von 1950-1970, als diese "fortschrittlichen" Chemikalien beinahe euphorisch und nahezu uneingeschränkt verwendet wurden. Bei der industriellen Umgestaltung der Landwirtschaft wurde die beachtliche Zunahme der Erträge damit erkauft, dass die Monokulturen ungleich anfälliger gegen Pflanzenschädlinge waren. Es wurde nun versucht, mit einem Cocktail von Bioziden, darunter eine Reihe von chlorierten Verbindungen, dieser ungewollten Entwicklung entgegenzuwirken. Erst viel später erkannte man, zusätzlich zu den unbeabsichtigten Wirkungen und ökologischen Folgen der Persistenz in der Umwelt, dass viele dieser Stoffe "Dioxin" als Verunreinigung enthielten oder es bei der Herstellung freigesetzt wurde. Dioxin gelangte mit dem Unfall in einer Pestizid-Fabrik in Seveso im Jahr 1976 augenblicklich in das Interesse der Öffentlichkeit. Obwohl es bei einer Reihe von industriellen Prozessen ständig gebildet wird, beschränkt sich die öffentliche Wahrnehmung seither fast ausschliesslich auf derartige Ereignisse etwa in Form von Chemieunfällen oder der Kontamination von Lebensmitteln.

Spektakuläre Fälle wie in Seveso haben teilweise zum Umdenken im Umgang mit industriellen Problemstoffen geführt. Dioxine und andere POPs (persistent organic pollutants) gehören mittlerweile zu den gut untersuchten und kontrollierten Umweltgiften. Im Jahr 2001 wurde die "Stockholm Convention" (98) unterzeichnet, in der verbindlich geregelt wird, die Produktion und Freisetzung von persistenten Stoffen zu minimieren oder möglichst vollständig zu

unterbinden. Zum "dreckigen Dutzend" gehören ausschließlich chlorierte organische Verbindungen wie Dieldrin, DDT, Hexachlorbenzol und PCBs.

Ein mikrobieller Abbau von chlorierten organischen Verbindungen kann die Folgen von jahrzehntelanger Produktion oder Freisetzung insbesondere von Dioxinen nicht rückgängig machen. Allerdings gibt es mittlerweile keinen Zweifel mehr, dass Dioxine und andere halogenierte "Xenobiotika" zu den Naturstoffen gehören und so einem natürlichen turnover unterliegen. Die Persistenz und Toxizität dieser Verbindungen ist aber offensichtlich zu einem ernsten Problem geworden, seitdem der Mensch in großem Maßstab zu ihrer Entstehung und Freisetzung beigetragen und so die Balance von Bildung und Abbau verändert hat.

Polychlorierte Dibenzo-*p*-dioxine und -dibenzofurane: Physikochemische Eigenschaften und Toxizität

Polychlorierte Dibenzo-*p*-dioxine und -dibenzofurane (PCDD und PCDF) sind trizyklische organische Verbindungen, die sich aufgrund des Grades der Chlorierung und der Position der Chlorsubstituenten in ihren physikochemischen Daten unterscheiden. Theoretisch sind 75 verschiedene Einzelverbindungen für die Gruppe der PCDD möglich (Abb. 1), die als Kongenere bezeichnet werden. Kongenere mit gleicher Zahl von Chlorsubstituenten (Isomere) werden in Homologengruppen zusammengefasst. Bedingt durch die unterschiedliche Substitution lassen sich ebenfalls 135 verschiedene PCDF ableiten (Abb. 1).

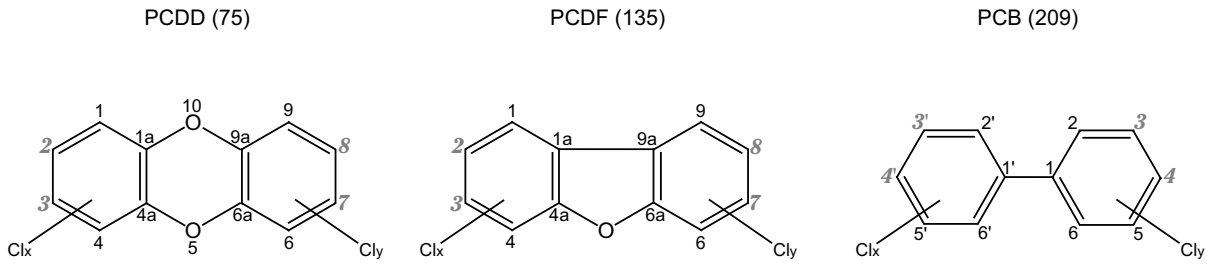

Abbildung 1 Chemische Struktur von polychlorierten Dibenzo-*p*-dioxinen (PCDD), polychlorierten Dibenzofuranen (PCDF) und polychlorierten Biphenylen (PCB). Die Anzahl der möglichen Kongenere ist in Klammern angegeben. PCDD/F: Substitutionen in Pos. 1, 4, 6 und 9 = peripher (*peri*); Pos. 2, 3, 7 und 8 = lateral; PCB: Pos. 2, 6 , 2' und 6' = *ortho*, Pos. 3, 5, 3' und 5' = *meta*, Pos. 4 und 4' = *para*. Die mit der Toxizität verknüpfte laterale Struktur für PCDD und PCDF bzw. die 3,3',4,4'-Struktur für PCB ist hervorgehoben.

Aufgrund einer ähnlichen chemischen Struktur zählt man zu den *dioxin-like compounds* auch die bromierten Dioxine (PBDD) und Dibenzofurane (PBDF) sowie polychlorierte Biphenyle (PCB). Für sieben PCDD- und sieben PBDD-Kongenere, zehn PCDF und zehn PBDF mit Halogensubstituenten in 2,3,7,8-Position sowie 13 der 209 möglichen PCB (coplanare PCB mit der Substitution 3,3',4,4') geht man von Dioxin-ähnlichen toxischen Wirkungen aus. Die

bekannteste und gleichzeitig toxischste Verbindung ist 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (2,3,7,8-TeCDD), welches im Sprachgebrauch oft als "Dioxin" bezeichnet wird. Dioxine und Dibenzofurane treten jedoch fast immer in komplexen Mischungen auf. Um vergleichbare Werte für die Toxizität angeben zu können, hat man - ausgehend vom Faktor 1 für die Verbindung 2,3,7,8-TeCDD - weitere *order of magnitude*-Toxizitätsäquivalente (TEF = *toxic equivalency factors*) für andere 2,3,7,8-substituierte PCDD/F eingeführt. International gebräuchlich sind die Toxizitätsäquivalente der U.S. Environmental Protection Agency (U.S. EPA, I-TEQ) (102) und der WHO (104). Die 2,3,7,8-penta- bis octachlorierten Kongenere werden nach dem Modell der U.S. EPA mit Toxizitätsfaktoren von 0,001 (für Octachlordibenzo-*p*-dioxin und -furan) bis 0,5 für 1,2,3,7,8-Pentachlordibenzo-*p*-dioxin berücksichtigt (102).

Aufgrund ihrer Hydrophobizität, die sich abhängig vom Grad der Halogensubstitution unter anderem in log K_{ow} (Verteilungskoeffizient Octanol/Wasser) -Werten von 4.7 bis 11.16 und in einer Löslichkeit in Wasser bei 25°C von 417 bis 7.4 x 10^{-5} μg/l äußert (93), sind chlorierte Dioxine mit organischen Partikeln in der Umwelt assoziiert. Die lipophilen Eigenschaften bedingen gleichzeitig die hohe Affinität zu biologischen Geweben (*bioaccumulation*) und die Tendenz, sich in Nahrungsketten anzureichern (*biomagnification*). Toxizitätsäquivalente sind geeignete Werkzeuge zur Risikoabschätzung der (akuten) Toxizität, obwohl die Daten aus Tierversuchen nicht zweifelsfrei auf die Wirkung im Menschen übertragen werden können. Außerdem wird das Langzeitverhalten, die Verteilung und Persistenz in biologischem Gewebe nicht berücksichtigt.

Die meisten toxischen Effekte der 2,3,7,8-substituierten Kongenere werden durch die Wechselwirkung mit dem *aryl hydrocarbon receptor* (Ah)-Rezeptor und *aryl hydrocarbon nuclear translocator* (Arnt) vermittelt. So führt die Bindung von Dioxinen an den Ah-Rezeptor

und die Interaktion mit Arnt zu einem transkriptionell aktiven Komplex, der an *dioxin response elements* in der DNA bindet und dadurch eine Reihe von Stoffwechselreaktionen auslöst. Ohtake *et al*. (80) konnten erst vor kurzem molekulare Mechanismen der östrogenen Wirkung von Dioxinen in Zellkulturen zeigen, in denen bei Abwesenheit von Östrogen nach Aktivierung des Ah-Rezeptors durch Dioxin eine Assoziation des AhR/Arnt-Heterodimers mit den Östrogenrezeptoren ER-α and ER-β erfolgt, was eine Kaskade molekularer und zellulärer Antworten auslöst. Umgekehrt bindet der AhR/Arnt-Komplex auch in Anwesenheit von Östrogen an die Rezeptoren, was die seit längerem bekannte anti-östrogene Wirkung von PCDD/F erklären könnte.

Die U.S. EPA schlägt vor, polychlorierte Dioxine und Dibenzofurane als bekannte Karzinogene einzustufen, was bedeutet, dass von diesen Verbindungen ein Risiko selbst bei sehr geringen Konzentrationen ausgeht (102). Die Bewertung von Toxizität und karzinogenen Effekten ist ein Feld heftiger Auseinandersetzungen (23, 64), einerseits bedingt durch den Mangel an humantoxikologischen (*in vivo*) Daten, die fast ausschliesslich in epidemiologischen Studien an expositionsbedingt höher belasteten Kohorten und Kontrollgruppen erbracht wurden. Demgegenüber ist die Bewertung der karzinogenen Wirkung auch aufgrund unterschiedlicher wirtschaftlicher und politischer Interessen strittig (23).

Anthropogene Quellen von PCDD und PCDF

Mit Ausnahme von analytischen Standards wurden Dioxine nie gezielt hergestellt, sondern gelangten unbeabsichtigt bei einer Vielzahl von Verbrennungsprozessen und Prozessen in der chemischen Industrie in die Umwelt. Damit sind sie von den *dioxin-like* PCB zu unterscheiden: polychlorierte Biphenyle wurden in großen Mengen produziert und

beispielsweise als Kühl- und Isolierflüssigkeiten in Transformatoren und Hydraulikanlagen oder als Weichmacher und Flammschutzmittel eingesetzt. Schätzungsweise 1,5 Mrd. t PCB wurden seit 1929 weltweit hergestellt (102).

In den Jahren 1987 und 1988 wurden allein in den USA ungefähr 1700 t PCB bei Unfällen (Transformatorenbrände etc.) freigesetzt. PCB-Mischungen sind mit chlorierten Furanen verunreinigt. So würden 1700 t PCB schätzungsweise 17 kg Furane enthalten. Bei der Verbrennung der PCB könnten nach Schätzungen von Meharg und Osborn mehr als 3000 kg Dibenzofurane in die Umwelt gelangen (76).

PCDD und PCDF werden bei Verbrennungsprozessen wie z.B. von Müll, Holz, Kohle oder Öl gebildet. Weitere primäre Quellen sind das Bleichen von Papier, die Herstellung von Chlor und PVC sowie die Erzeugung von Metallen und Legierungen (38).

Die gegenwärtigen Emissionen von Dioxinen aus bekannten Quellen können nur etwa zehn Prozent des jährlichen Eintrags erklären, wie Arbeiten zur Dioxin-Bilanz in Schweden und Grossbritannien (49) gezeigt haben. Ähnliche Diskrepanzen kalkuliert die U.S. EPA für die Vereinigten Staaten (24). Eisenberg *et al*. (24, 32) gehen davon aus, dass der Eintrag in Böden die Emissionen sogar um den Faktor 20 übersteigt, möglicherweise auch um den Faktor 60. Vermutlich sind noch nicht alle primären, eventuell auch natürlichen Quellen von PCDD/F identifiziert oder die sekundäre Freisetzung und Mobilisierung aus Reservoiren, besonders aus den beiden wichtigsten *sinks*, Boden und Sediment, ist weitaus bedeutender als bisher angenommen. Das steht auch mit dem partikelgebundenen oder aerosolischen Transport volatiler und semi-volatiler Kongenere über teilweise weite Strecken in Zusammenhang (68). Der Eintrag von POPs besonders in arktische und antarktische Ökosysteme über Verdunstung und Kondensation (*global distillation = grasshopper effect*) beruht auf einer Reihe komplexer klimatischer und geographischer Mechanismen (95). Nur wenige Bilanzierungsmethoden

berücksichtigen die sekundäre Freisetzung aus Reservoiren nach Chemieunfällen oder kalkulieren ähnliche Emissionen von anderen *large-scale sites* (76). Wie neue Forschungsberichte zeigen, wurden beispielsweise während des Vietnam-Krieges mehr als 360 kg 2,3,7,8-TeCDD als Verunreinigung in den Entlaubungsmitteln *Agent Orange*, *-Purple*, *-Green* und *-Pink* über Vietnam versprüht (97). Im Vergleich dazu wurden in Seveso schätzungsweise 600 g bis 2 kg 2,3,7,8-TeCDD freigesetzt, allerdings auf einer Fläche von nur etwa 18 km^2 (83).

Kongener-spezifische Analysen von Dioxin-Belastungen erlauben es, zwischen einzelnen Verteilungsmustern zu unterscheiden und Signaturprofile den verursachenden Prozessen zuzuordnen. So entstehen bei der Chlorbleiche von Papier vor allem 2,3,7,8-TeCDD und 2,3,7,8-TeCDF (99). Hingegen ist für die Chloralkali-Elektrolyse ein hoher Anteil von polychlorierten Dibenzofuranen, vor allem tetra- bis hexachlorierter Kongenere typisch (87). Im Augenblick ist es noch ungeklärt, in welchem Maß diese spezifischen Muster abiotisch oder biotisch verändert werden. Unter den Bedingungen einer Transformation wäre eine Zuordnung zu einem bestimmten industriellen oder natürlichen Prozess nur bedingt möglich. Biotransformation wurde für komplexe Mischungen von PCB-Isomeren (Aroclor) nachgewiesen und erstmalig durch Arbeiten von Brown *et al*. (18) gezeigt. Für polychlorierte Dioxine und Dibenzofurane konnten Beurskens *et al*. (15) für das Ketelmeer, ein Sedimentationsgebiet des Rheins in den Niederlanden, Veränderungen der Kongenerprofile nachweisen. Datierte Sedimentkerne aus einer Probenahme im Jahr 1990 zeigten für einige höher chlorierte Kongenere in der Schicht von 1970 geringere Konzentrationen als in archivierten Proben, die 1972 von der Oberfläche des Sedimentes gewonnen wurden. Umgekehrt waren die Konzentrationen für 2,3,7,8-TeCDD und 1,2,3,7,8,9-HexaCDD in der Schicht von 1970 aus den frisch genommenen Sedimentkernen signifikant erhöht (15).

Ursachen der Dioxinbelastung im Sediment des Spittelwassers

Die vorliegende Arbeit basiert zum grossen Teil auf Versuchen mit Anreicherungskulturen aus dem Sediment des Flusses Spittelwasser. Die Sedimentbelastung des Spittelwassers mit polychlorierten Dibenzo-*p*-dioxinen und -Dibenzofuranen resultiert aus einer jahrzehntelangen Verschmutzung mit Abwässern aus der chemischen Industrie in Bitterfeld. Abwasser gelangte ungereinigt von den Produktionsstätten über den Schachtgraben, dessen Sediment ebenfalls mit PCDD/F kontaminiert ist (113), in das Spittelwasser. Die geringe Fliessgeschwindigkeit begünstigt an einigen Stellen die Sedimentation, was Sedimentschichten von bis zu zwei Metern Dicke hervorgebracht hat. Sedimente des Spittelwassers sind nach Angaben von Götz *et al*. (43) mit bis zu 3000 ng I-TEQ je kg Trockensubstanz belastet. Nach einem Bericht von Wilken *et al*. (113) sind Bodenproben aus tieferen Schichten (30-60 cm Tiefe) des Ufers mit extremen Werten von bis zu 203000 ng I-TEQ je kg Trockensubstanz kontaminiert. Die gleiche Probe war in einer Tiefe von 10-30 cm mit 1,3 g Hexachlorcyclohexanen und 400 ng DDX pro kg Trockensubstanz belastet. Möglicherweise können vergleichbare *hot spots* auch in tieferen Schichten des Sedimentes vorkommen. Viele Sedimente zeigen Dioxinbelastungen mit ausgeprägten Maxima (z.B. (15)) besonders in den Schichten von etwa 1960 bis 1980, dem Zeitraum der höchsten Produktion von Dioxin-kontaminierten Pestiziden. So war zum Beispiel das in Bitterfeld hergestellte *Trizilin 25* (Nitrofen) mit bis bis 3 g PCDD und PCDF je kg extrem verunreinigt (112).

Für Analysen zur Belastung mit PCDD und PCDF wurden in dieser Arbeit zwei Sedimentkerne (0 bis 50 cm) des Spittelwassers untersucht. Alle Sedimentproben waren stark mit PCDD/F kontaminiert, die Konzentrationen waren signifikant höher (maximal 120000 ng I-TEQ/kg Trockensubstanz) als bisher publizierte Daten. Die Toxizitätsäquivalente reflektieren in diesen Proben nicht die Gesamtbelastung mit PCDD/F. Polychlorierte

Dibenzofurane dominierten die Verteilung der Kongenere (>90 %) (Abb. 2). Besonders Octachlordibenzofuran (OCDF) war erhöht, welches in die Berechnung der Toxizität nach dem Modell der EPA nur mit einem Faktor von 0,001 eingeht. Es wurde eine Gesamtbelastung von ca. 6,8 mg PCDD und PCDF pro kg Trockensubstanz festgestellt.

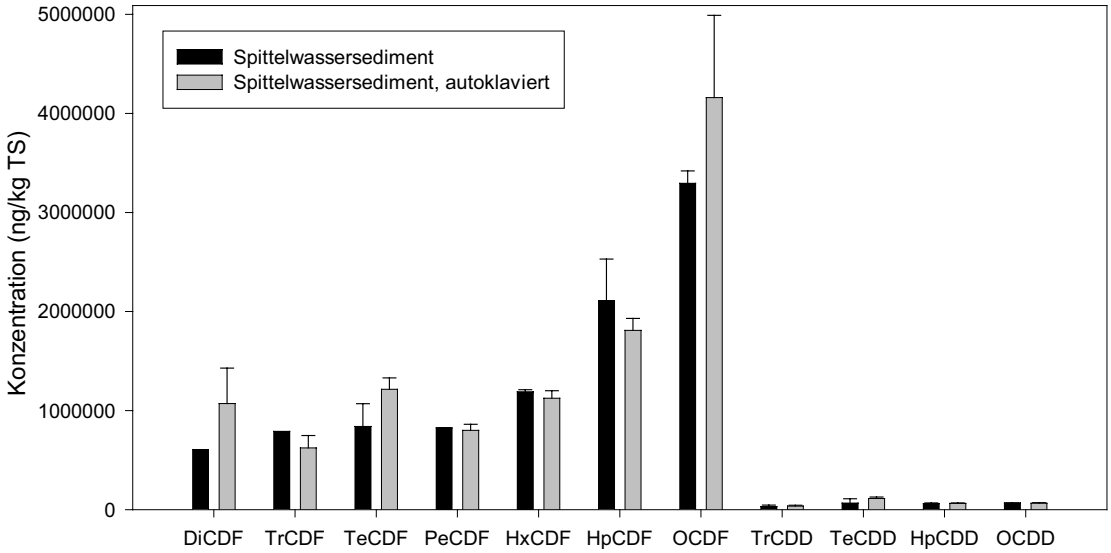

Abbildung 2 Verteilung der Homologengruppen von polychlorierten Dioxinen und Dibenzofuranen in Sedimentproben aus dem Spittelwasser (20). DiCDF, Di-; TrCDF, Tri-; TeCDF, Tetra-; PeCDF, Penta-; HxCDF, Hexa-; HpCDF, Hepta- und OCDF, Octachlordibenzofuran. TrCDD, Tri-; TeCDD, Tetra-; HpCDD, Hepta- und OCDD, Octachlordibenzo-*p*-dioxin.

Die Mobilisierung von Schadstoffen aus dem Bitterfelder Raum ist ein ernstes Problem für stromabwärts gelegene Flußbereiche. Die Belastungen der Elbe und des Hamburger Hafens mit PCDD/F stammen zu einem grossen Teil aus dem Bitterfelder Raum (43); Schwebstoffe werden über das Spittelwasser und die Mulde in die Elbe transportiert. Die Konzentrationen in der Elbe sind nach Zufluss der Mulde erhöht und zeigen das charakteristische Profil des Spittelwassers (43). Das Kongenerenmuster im Sediment des Spittelwassers ist ungewöhnlich und resultiert offenbar teilweise aus der Überlagerung verschiedener Signaturen. Allerdings ist

der Anteil von chlorierten Dibenzofuranen hoch, besonders von Octachlordibenzofuran (Abb. 2). Wie von Götz *et al*. (43) diskutiert wurde, stammt die Belastung vermutlich in starkem Maß aus Prozessen, die mit der Produktion von Magnesium und seiner Legierungen (z.B. *Elektron*) in Zusammenhang stehen. Die Region war zwischen 1920 bis 1945 das Zentrum der deutschen Magnesiumindustrie und die naheliegenden Flugzeugwerke (z.B. Junkers, Dessau) waren die Hauptabnehmer. Vermutlich sind für den größten Teil der Kontamination mit PCDD/F industrielle Prozesse vor 1945 verantwortlich zu machen. Sie datieren damit vor dem Beginn der üblicherweise als Quelle angesehenen Entwicklung der Chlorchemie in der Zeit zwischen 1950 und 1970.

Das charakteristische Spittelwasser-Muster ist der Hepta- und Octachlordibenzofuran-dominierten Isomerenverteilung in Fisch, Crustaceen, Muscheln und Sedimenten in einer durch die Magnesium-Produktion verunreinigten Fjordregion in Südnorwegen ähnlich (79). Weitere Anhaltspunkte für die Magnesiumproduktion als gemeinsamer Ursache kommen aus der Geschichte des Werkes in Herøya. Im 2. Weltkrieg wurde unter deutscher Besatzung ein Werk errichtet, welches Magnesium basierend auf dem I.G. Farben-Verfahren aus Bitterfeld herstellen sollte. Nach Wiederaufbau der zerstörten Produktionsanlagen wurde 1950, auf Grundlage der deutschen Technologie, die Produktion wieder aufgenommen. Die Hauptquelle ist der Produktionsschritt, bei dem Magnesiumchlorid durch gemeinsame Erhitzung von Koks und Magnesiumoxid in einer Chloratmosphäre bei 700-800°C gewonnen wird. Überschüssiges Chlorgas wird gewaschen und mit dem Abwasser entsorgt (71). Greenpeace kalkulierte für das Abwasser des Werkes in Herøya eine PCDD/F-Freisetzung von 9000 ng I-TEQ ***pro kg*** hergestelltes Magnesium. Bei einer jährlichen Kapazität von 55000 t entspräche das in etwa 500 g I-TEQ (http://archive.greenpeace.org/toxics/reports/dioxinsources.pdf). Das Werk wurde im Jahr 2002 geschlossen.

Die biologische Sanierung durch *biostimulation*, also der Stimulierung autochthoner Mikroorganismen durch Substratzugabe, und *bioaugmentation*, der Zugabe von angereicherten Organismen (11), könnte eine interessante und kostengünstige Variante für die Behandlung besonders von großflächig belasteten Böden und Sedimenten (*large-scale sites*) sein. Auch für das Spittelwasser wurde eine biologische *in situ*-Behandlung, teilweise in Kombination mit einer Abdeckung des Sedimentes (*capping*) vorgeschlagen (28). Diese Verfahren als mögliche Alternativen zu chemisch-physikalischen Sanierungsverfahren müssen Risiken bei einer Sanierung des Spittelwasser berücksichtigen. Es handelt sich um ein akut durch Hochwasser gefährdetes Gebiet: bei ungenügender Immobilisierung des Sedimentes durch *capping* kann es durch die Überschwemmungen zu einer Remobilisierung von Schadstoffen aus den Reservoiren und zu einer Verteilung auf den Überflutungsflächen kommen. Noch sind die Risiken einer biologischen Sanierung unkalkulierbar, obwohl das Vorhandensein von PCDD-dehalogenierenden Bakterien im Spittelwasser gezeigt wurde (20). Es fehlen im Augenblick jedoch Daten für die *in situ*-Dehalogenierung, insbesondere zum Einfluß von (halogenierten) Co-Kontaminationen oder halogenierten und nicht-halogenierten alternativen Elektronenakzeptoren auf die Dechlorierung komplexer Mischungen von PCDD und PCDF.

Natürliches Vorkommen halogenierter Dioxine

Wie man erst seit kurzer Zeit versteht, werden halogenierte organische Verbindungen in bedeutendem Maßstab natürlich gebildet (zusammengefaßt in Referenz (45)). Gribble beziffert in einem Artikel aus dem Jahr 2003 die Zahl bekannter natürlich gebildeter halogenierter organischer Verbindungen auf 3800 (46). Diese Zahl bezieht sich auf die Bildung durch abiotische Prozesse wie Waldbrände (z. B. (92)), Vulkanausbrüche und andere geothermische Prozesse aber auch auf die Bildung durch lebende Organismen in marinen und terrestrischen

Habitaten. Organische Halogenverbindungen werden unter anderem von Bakterien, Pilzen, Algen, Schwämmen und Insekten gebildet, wahrscheinlich um ökologische Aufgaben zu erfüllen, wie die Abwehr von Feinden und Konkurrenten, oder Signalfunktion auszuüben (45).

Für Dioxine und ähnliche Verbindungen galt eine derartige natürliche Bildung bis vor kurzem als ausgeschlossen. Der klare Nachweis der Entstehung von halogenierten Dioxinen und Dibenzofuranen bei natürlichen biogenen und abiotischen Prozessen gehört zu den überraschenden Befunden der letzten Jahre (36, 41, 44, 54, 75, 78, 84, 85, 90, 94, 103). Einige der natürlich gebildeten Organohalogenverbindungen, darunter Dioxine und Dioxin-ähnliche Verbindungen, reichern sich ebenfalls in Nahrungsketten an (73, 101, 107-109).

Als am Ende der 90er Jahre eine erhöhte Dioxin-Belastung in Geflügel und Fisch aus Farmhaltung festgestellt wurde (39, 51), erkannte man, dass bestimmte dem Futter zugesetzte Tone (*ball clay*) das gleiche ungewöhnliche Kongenerenmuster wie im Fettgewebe der Tiere aufwiesen (39, 86). In nachfolgenden Untersuchungen konnte eine starke Dioxin-Belastung in den verwendeten Tonen der Mississippi-Ebene (36) und in Flußsedimenten aus der gleichen Region nachgewiesen werden (85). Die Zusammensetzung ist von Octachlordibenzo-*p*-dioxin und 1,2,3,4,6,7,8-Heptachlordibenzo-*p*-dioxin dominiert und steht aufgrund der Isomerenverteilung in keinem Zusammenhang mit einem industriellen Prozess (36, 84, 85). Analysen von Kaolin aus Deutschland zeigten eine ähnliche Belastung (63, 84). Interessanterweise kommt ein ähnliches Verteilungsmuster auch in australischen Küstensedimenten (Queensland) vor (z.B. (42)). Anhand von datierten Sedimentkernen konnte gezeigt werden (41), dass seit mindestens 300 Jahren eine Ablagerung von dioxinhaltigem Material im Küstenbereich erfolgte. Eine anthropogene Quelle als Ursache für die Dioxin-Belastung konnte damit nahezu ausgeschlossen werden (41).

Die Exposition gegenüber natürlich vorkommenden Halogenverbindungen war und ist wahrscheinlich ein wichtiger Faktor in der Evolution von bakteriellen Mechanismen, diese Verbindungen zu entgiften oder sie als Energie- oder Kohlenstoffquelle zu nutzen.

Mikrobielle Transformation von halogenierten Dioxinen

Der oxidative Abbau von Dioxinen mit Sauerstoff als Elektronenakzeptor ist möglich, wobei das Substrat als Energie- und Kohlenstoffquelle fungieren kann (115). Da die meisten höherchlorierten Substrate bereits sehr oxidiert sind, ist die Mineralisierung unter aeroben Bedingungen auf die unchlorierten Grundkörper oder niedrig chlorierte Kongenere beschränkt (114, 115). Charakteristische Reaktionen sind zunächst die Ringaktivierung durch eine anguläre Dioxygenase-Reaktion (Positionen 1 und 1a, Abb. 1), bei der ein instabiles *cis*-Dihydrodiol gebildet wird. Die Etherbrücke wird spontan gespalten und es kommt zu einer *meta*-Dioxygenase-katalysierten Ringöffnung der Verbindung 2,2',3-Trihydroxydiphenylether. In der folgenden Hydrolase-katalysierten Reaktion werden 2-Hydroxy-*cis*,*cis*-muconsäure und Brenzcatechin gebildet, dass in *meta*- oder *ortho*-Position gespalten werden kann und über Acetyl-CoA in den Tricarbonsäurezyklus eingeht (88).

Beim Abbau von chlorierten Dioxinen entstehen zum Teil chlorierte Brenzcatechine als vorläufige Endprodukte, die zusammen mit dem Abbauprodukt Acylchlorid *meta*-spaltende Dioxygenasen hemmen ("Suizid-Inhibitoren") (116).

Hong *et al*. konnten mit *Sphingomonas wittichii* Stamm RW1^{T} (122) eine begrenzte Biotransformation von 2,7-Dichlor- und 1,2,3,4-Tetrachlordibenzo-*p*-dioxin feststellen (60). Es wurde gezeigt, dass der Stamm RW1^{T} auch im Boden Dibenzo-*p*-dioxin und Dibenzofuran (74) sowie 2-Monochlordibenzo-*p*-dioxin abbauen kann (47).

In den als Senken für Dioxine geltenden aquatischen Sedimenten spielt der aerobe Abbau wahrscheinlich nur eine geringe Rolle. Nur die obersten Sedimentschichten sind aerob. Unter anaeroben Bedingungen ist der bisher einzige bekannte mikrobielle Transformationsweg für Dioxine die reduktive Dechlorierung (65), formal die Substitution eines Chloratoms durch Wasserstoff.

$$R\text{-}Cl + 2e^- + 2H^+ \longrightarrow R\text{-}H + H^+ + Cl^-$$

Die Verfügbarkeit von Elektronenakzeptoren ist unter anaeroben Bedingungen oftmals limitierend für bakterielles Wachstum. Organismen, die über die Fähigkeit verfügen, halogenorganische Verbindungen als terminale Elektronenakzeptoren für eine anaerobe Atmung zu nutzen, sollten daher einen Wachstumsvorteil besitzen. Man nennt diesen Atmungsprozeß Halorespiration, Dehalorespiration (58) oder Chloridogenese (65). Dehalorespiration wurde zum ersten Mal für *Desulfomonile tiedjei* Stamm DCB-1 beschrieben (δ-*Proteobacteria*). Seither wurde für eine Reihe von Organismen ("Deltaproteobacteria", "Gammaproteobacteria", "Epsilonproteobacteria", "Clostridia" und "Chloroflexi") Wachstum durch Dehalorespiration mit unterschiedlichen halogenierten Aliphaten und Aromaten beschrieben und in einer Reihe von Übersichtsartikeln zusammengefasst (37, 58, 65).

Huang *et al*. (61) geben für die möglichen Dechlorierungswege von PCDD $\Delta G°'$-Werte von -130 bis -180 kJ/mol pro Dechlorierungsschritt an (E'_0= +300 bis +470 mV). Die Redoxpotentiale von chlorierten Dioxinen sind vergleichbar mit dem von Nitrat als Elektronenakzeptor (NO_3^-/NO_2^-, E'_0= +433 mV) und signifikant höher als das für Sulfatreduktion (SO_4^{2-}/SH^-, E'_0= -116 mV) (100). Das heißt auch, dass chlorierte Verbindungen

unter sulfatreduzierenden und methanogenen Bedingungen konkurrenzfähige Elektronenakzeptoren sein können. Dehalogenierer konkurrieren mit Acetogenen und hydrogenotrophen Methanogenen um den Elektronendonor Wasserstoff. Wie durch Untersuchungen gezeigt wurde, besitzen reduktiv dehalogenierende Bakterien eine höhere Affinität zu Wasserstoff und können bei geringeren H_2-Schwellenwert-Konzentrationen operieren (<0.3 nM) (67). Das macht sie aus thermodynamischen Gesichtspunkten auch zu bevorzugten Partnern im syntrophen Abbau von Fettsäuren, der auf *interspecies hydrogen transfer* beruht (91).

Während eine Reihe von Reinkulturen beschrieben wurde, die die Dehalogenierung von halogenierten Aliphaten und chlorierten Phenolen und Benzoaten katalysieren (65), ist weit weniger über die anaeroben Dechlorierer der hydrophoben PCB, Dioxine und Chlorbenzole bekannt. Im Fall von PCB sind verschiedene Dechlorierungsmuster gefunden worden, die speziellen physiologischen Gruppen zugeordnet werden konnten und teilweise auch unter *in situ*-Bedingungen stattfinden (13, 110). Ein bedeutender Schritt war die Möglichkeit, PCB-Dechlorierung ohne Sedimentzusatz zu erhalten (26). Häufig ist jedoch der Zusatz von teilweise undefinierten Bestandteilen zu Kulturen von dehalogenierenden Bakterien weiterhin erforderlich (z. B. steriler Kulturüberstand (72, 111), fermentierter Hefeextrakt (57), Aminosäuren (55)). Die komplexen Ansprüche an Komponenten im Medium sind vielfach ungeklärt. Es konnte bisher noch keine PCB-dechlorierende Reinkultur isoliert werden.

Ein Hinweis, dass PCB als terminale Elektronenakzeptoren für anaerobe Atmungsprozesse fungieren, wurde durch die Konzentrationsabhängigkeit von PCB-Dechlorierungsprozessen (1, 82) erhalten. Die Stimulation der PCB-Dechlorierung durch *priming* mit verschiedenen chlorierten Substraten (22) oder 2,6-Dibrombiphenyl (120) und der

Nachweis einer damit verbundenen Zunahme der Zellzahl von PCB-Dechlorierern konnte diese Hypothese bestätigen.

In einer *ortho*-PCB-dechlorierenden Kultur wurde durch molekularbiologische Methoden ein Bakterium mit entfernter Verwandtschaft zu *Dehalococcoides ethenogenes* nachgewiesen (59). Dieser Organismus (*o*-17) konnte durch Cutter *et al*. (27) als das 2,3,5,6-Tetrachlorbiphenyl-dechlorierende Bakterium in einer Mischkultur identifiziert werden. Von Wu *et al*. (121) wurde ein anderes Bakterium, Stamm DF-1 nachgewiesen, das doppelt flankierte Chloratome von PCB-Kongeneren wie etwa 2,3,4,5-Tetrachlorbiphenyl abspalten kann.

Die reduktive Dechlorierung von chlorierten Dioxinen durch Mikroorganismen wurde vor etwa zehn Jahren zum ersten Mal von Adriaens und Grbic-Galic (4) beschrieben. In der Zwischenzeit wurde reduktive Dehalogenierung von Dioxinen in einer Reihe von Mischkulturen dokumentiert (2, 3, 7, 9, 10, 16, 20, 106). Vor kurzem konnten wir zeigen, dass der *Dehalococcoides* Stamm CBDB1 in Reinkultur verschiedene chlorierte Dioxine (1,2,3,4-Tetrachlordibenzo-*p*-dioxin [1,2,3,4-TeCDD], 1,2,3- und 1,2,4-Trichlordibenzo-*p*-dioxin [1,2,3-TrCDD und 1,2,4-TrCDD], 2,3-Dichlordibenzo-*p*-dioxin [2,3-DiCDD] und 1,2,3,7,8-Pentachlordibenzo-*p*-dioxin [1,2,3,7,8-PeCDD]) reduktiv dehalogenieren kann (19). Dieser Organismus wurde ursprünglich aufgrund seiner Fähigkeit isoliert, Trichlorbenzole als Elektronendonatoren für anaerobe Dehalorespiration zu nutzen (6). Die anaerobe Transformation von Chlorbenzolen war bis zur Isolierung von Stamm CBDB1 ebenfalls nur von Mischkulturen bekannt (5, 17, 33, 56).

Eigenschaften von *Dehalococcoides*

Die in Reinkultur vorliegenden Stämme der Gattung *Dehalococcoides* gehören zusammen mit einigen unkultivierten Vertretern taxonomisch zu einer separaten Gruppe

innerhalb der *Chloroflexi* (Abb. 3) und sind durch eine Reihe ungewöhnlicher morphologischer und physiologischer Merkmale gekennzeichnet. Auf elektronenmikroskopischen Aufnahmen sind kokkenähnliche Zellen ohne eine typische bakterielle Zellwand zu sehen, teilweise mit Einkerbungen und Zellanhängen (6, 52, 72). Wahrscheinlich ist die Morphologie aber eher scheibenförmig, wie die Aufnahmen von Stamm BAV1 zeigen (52). Die Stämme 195, CBDB1 und BAV1 wachsen nur unter strikt anaeroben Bedingungen und sind offenbar auf Dehalorespiration spezialisiert. Das Spektrum der bekannten Elektronenakzeptoren ist in Tab. 1 zusammengefasst.

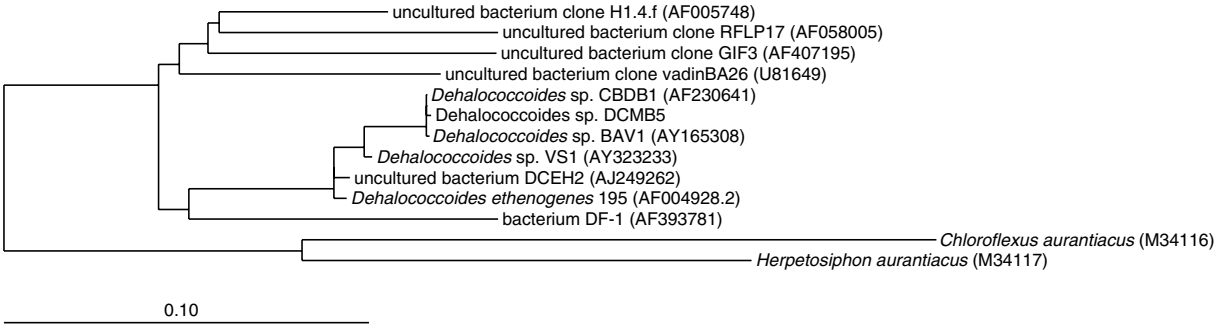

Abbildung 3 Phylogenetischer Stammbaum (*maximum parsimony*) von Bakterien der *Dehalococcoides*-Gruppe und weiterer Vertreter der *Chloroflexi*, basierend auf Sequenzen von 16S rRNA-Genen. Die GenBank Accession-Numerierung ist in Klammern angegeben. "*Dehalococcoides* sp. DCMB5" bezieht sich auf das in Kapitel 5 identifizierte Bakterium. Balken: evolutionäre Distanz in Nukleotidsubstitution pro Basenposition.

Dehalococcoides ethenogenes Stamm 195 kann mit Wasserstoff als Elektronendonor und Chlorethenen als Elektronenakzeptoren wachsen (72). Dieses Bakterium wurde durch seine ungewöhnliche Fähigkeit bekannt, Tetrachlorethen bis zu Ethen zu dechlorieren, wobei es alle Schritte bis auf den letzten (Vinylchlorid zu Ethen) zur Energiekonservierung nutzt. Demgegenüber kann *Dehalococcoides* Stamm BAV1 durch die reduktive Dechlorierung von Vinylchlorid zu Ethen wachsen (52).

Kürzlich wurde auf einem Poster beschrieben, dass *Dehalococcoides ethenogenes* Stamm 195 ebenfalls Dioxine dechlorieren kann (35). Neben einer Reihe von Chlorbenzolen kann der Stamm auch 2,3,4,5,6-Pentachlorbiphenyl, 1,2,3,4-Tetrachlordibenzofuran und 1,2,3,4-Tetrachlornaphthalen umsetzen (35). Das zeigt das hohe Potential von Vertretern dieser phylogenetischen Gruppe für die Transformation hydrophober haloaromatischer Verbindungen, z. B. von PCBs, Dioxinen und Chlorbenzolen und macht diese Organismen auch für Untersuchungen der potentiellen mikrobiellen Umsetzung von Verbindungsklassen interessant, für die es noch keine Daten zur anaeroben Transformation gibt, wie etwa polybromierte Diphenylether. Belastungen mit polybromierten Diphenylethern (PBDE) zählen zu den POPs mit den am stärksten zunehmenden Konzentrationen in der Umwelt (z. B. (70)) und wahrscheinlichen dioxin-ähnlichen Wirkungen.

Tabelle 1 Zusammenfassung der Elektronendonor-Spezifität für die kultivierten *Dehalococcoides*-Stämme.

Organismus	**Elektronenakzeptoren**	**Literatur**
Dc. ethenogenes Stamm 195	PCE, TCE, *cis*-DCE, 1,1-DCE, 1,2-Dichlorethan	72
Dc. sp. BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, Vinylchlorid, Vinylbromid, 1,2-Dichlorethan	52
Dc. sp. CBDB1	1,2,3-TrCB, 1,2,4-TrCB, 1,2,3,4-TeCB, 1,2,3,5-TeCB, 1,2,4,5-TeCB, PeCB, HCB, 1,2,3,4-TeCDD, 1,2,3-TrCDD, 1,2,4-TrCDD, 2,3-DiCDD, 1,2,3,7,8-PeCDD	6, 19, 62

Dc., *Dehalococcoides*
PCE, Tetrachlorethen; TCE, Trichlorethen; DCE, Dichlorethen; TrCB, Trichlorbenzol; TeCB, Tetrachlorbenzol; PeCB, Pentachlorbenzol; HCB, Hexachlorbenzol; TeCDD, Tetrachlordibenzo-*p*-dioxin; TrCDD, Trichlordibenzo-*p*-dioxin; DiCDD, Dichlordibenzo-*p*-dioxin; PeCDD, Pentachlordibenzo-*p*-dioxin.

Indizien für die Nutzung eines breiten Substratspektrums halogenierter Verbindungen lassen sich auch aus der Genomsequenz von *Dehalococcoides ethenogenes* 195 (http://www.tigr.org/tdb/mdb/mdbinprogress.html) ableiten. Mindestens 15 verschiedene

Dehalogenase-Operone wurden gefunden. Weder für Stamm 195 noch für Stamm CBDB1 konnten bisher andere, nicht-halogenierte Elektronenakzeptoren nachgewiesen werden. Es wird daher angenommen, dass es sich um obligate Dehalogenierer handelt. Diese Vermutung wird durch eine Reihe von Arbeiten gestützt, die *Dehalococcoides* in dechlorierenden Mischkulturen oder Chlororganika-kontaminierten Standorten nachweisen konnten (25, 30, 34, 53, 66).

Regiospezifität der reduktiven Dehalogenierung von chlorierten Dioxinen

Die Produkte der reduktiven Dechlorierung von Dioxinen sind weniger dechloriert, damit werden sie zu geeigneten Substraten des aeroben Abbaus. Außerdem sind die niedriger chlorierten Transformationsprodukte besser wasserlöslich und damit wahrscheinlich bioverfügbarer, was den mikrobiellen Zugang erleichtern könnte. Im Gegensatz zur reduktiven Dechlorierung von PCB-Kongeneren, bei denen die bevorzugte Abspaltung der Chloratome in *meta*- und *para*-Stellung die Toxizität von Mischungen mit *dioxin-like* PCB verringert (13, 81), wird für halogenierte Dioxine und Dibenzofurane auch die Möglichkeit diskutiert, dass Zwischen- oder Endprodukte der reduktiven Dechlorierung toxischer sein können (10). Legt man die Toxizitätsäquivalente der WHO von 1998 zugrunde (104), so würde die reduktive Dechlorierung von Octachlordibenzo-*p*-dioxin in *peri*-Position (Positionen 1, 4, 6 und 9) zu 2,3,7,8-TeCDD eine Zunahme der Toxizität um den Faktor 10000 bedeuten (104). Deshalb ist die Kenntnis von Dechlorierungsmechanismen auch für die Beurteilung der potentiellen Entwicklung der Toxizität in der Umwelt von Relevanz.

Barkovskii und Adriaens (10) haben zwei Dechlorierungswege in Mischkulturen aus Passaic River Sediment beobachtet, den *peri*-Weg von 2,3,7,8-substituierten hepta- bis pentachlorierten Dioxinen, der zu einer vorübergehenden Bildung von 2,3,7,8-TeCDD führte,

und einen gemischten *peri*-lateralen Dechlorierungsweg von nicht-2,3,7,8-substituierten Kongeneren. Für den gemischten *peri*-lateralen Dechlorierungweg waren offenbar kombinierte abiotische und mikrobielle Dechlorierungsaktivitäten verantwortlich, wohingegen die Transformation über den *peri*-Weg indirekt nicht-methanogenen, nicht-sporenbildenden Populationen zugeordnet werden konnte (10).

Anaerobe Mischkulturen aus stark PCDD/F-kontaminiertem Spittelwassersediment (21) dechlorierten frisch zugegebenes 1,2,3,4-Tetrachlordibenzo-*p*-dioxin nach einem ähnlichen Muster wie Hexachlorbenzol-adaptierte Bakterien aus dem Rhein-Sedimentations-gebiet des Ketelmeeres (16). Die Modellverbindung 1,2,3,4-Tetrachlordibenzo-*p*-dioxin wurde zu 1,3- und 2,3-Dichlordibenzo-*p*-dioxin (DiCDD) umgesetzt. Die Intermediate 1,2,3- und 1,2,4-Trichlordibenzo-*p*-dioxin waren im Gegensatz zu einer Kultur aus Saale-Sediment (9) nachweisbar. In nachfolgenden Transfers mit den trichlorierten Intermediaten konnte ein gemischter *peri*-lateraler Dechlorierungsweg identifiziert werden, der mit Prozess M bezeichnet wurde (20).

Demgegenüber setzten zwei Kulturen, interessanterweise aus den am höchsten mit halogenorganischen Verbindungen belasteten Sedimentschichten des Spittelwassers, 1,2,3-TrCDD nur zu 1,3-DiCDD um (20). 1,2,4 TrCDD wurde nicht dehalogeniert. Dieser Weg deutet darauf hin, dass nur laterale Chloratome abgespalten werden, die von weiteren Chlor-Substituenten flankiert sind (Prozess SP) (20).

In einer anderen Kultur aus Leine-Sediment wurde eine bevorzugte Eliminierung in *peri*-Position beobachtet, die 1,2,4-TrCDD zu 1,3-DiCDD umsetzt und ausgehend von 1,2,3-TrCDD fast ausschliesslich zur Bildung von 2,3-DiCDD führte (Kapitel 3). *Dehalocoocoides* sp. Stamm CBDB1 entspricht dieser Spezifität und dehalogeniert trichlorierte Dioxine ebenfalls über einen bevorzugten *peri*-Dechlorierungsweg. 1,2,4-TrCDD

wird durch Abspaltung der Chloratome 1 und 4 strikt in *peri*-Position über 1,3-DiCDD zu 2-MCDD umgesetzt (19). Aus 1,2,3-TrCDD werden die beiden Intermediate 2,3- und 1,3-DiCDD im Verhältnis von >2:1 gebildet, dieser Weg geht also ebenfalls mit einer bevorzugten *peri*-Dehalogenierung einher. Interessanterweise sollte dieser Weg aus thermodynamischen Gesichtspunkten nicht bevorzugt sein (61). In diesen Fällen wird die Möglichkeit diskutiert, dass Co-Kontaminationen aus dem Sediment die entsprechenden Enzymsysteme induziert haben könnten (31), was im Fall von *Dehalococcoides* sp. Stamm CBDB1 jedoch ausgeschlossen werden kann. Stamm CBDB1 behielt in vier aufeinanderfolgenden Transfers mit 1,2,3-TrCDD oder 1,2,4-TrCDD die Dechlorierungsaktivität und die Regiospezifität (19). Hier sollte in Betracht gezogen werden, dass durch das gleiche Bakterium auch 2,3-DiCDD in einem weiteren Schritt in lateraler Position zu 2-MCDD umgesetzt werden kann. Die Bildung von 2-MCDD aus 2,3-DiCDD ist gegenüber der Dechlorierung von 1,3-DiCDD zu 2-MCDD thermodynamisch klar bevorzugt (61).

1,2,3,4-TeCDD wird von *Dehalococcoides* Stamm CBDB1 über das vermutliche Zwischenprodukt 1,2,3-TrCDD zu 2,3-DiCDD und 2-Monochlordibenzo-*p*-dioxin umgesetzt. Bestimmte Mischkulturen aus Mulde und Spittelwasser bildeten aus 1,2,3-TrCDD bevorzugt 1,3-DiCDD (Kapitel 3 und Referenz (20)). Unter der Annahme, dass bestimmte Dechlorierungswege spezifischen Organismen zugeordnet werden können, lässt sich ableiten, dass in diesen Mischkulturen zunächst andere Bakterien als *Dehalococcoides* dominant waren. Aus der Verbindung 1,2,4-TrCDD können die beobachteten Dechlorierungsprodukte 1,3-DiCDD und 2-MCDD ausschließlich durch eine *peri*-Dechlorierung gebildet werden. Nach mehreren Passagen dieser Kulturen mit 1,2,4-TrCDD, konnten wir in den meisten späteren Passagen der Kulturen eine veränderte Regiospezifität beobachten, bei der aus

1,2,3-TrCDD bevorzugt 2,3-DiCDD gebildet wurde. Dieser Befund spricht für eine Anreicherung von bevorzugt *peri*-dechlorierenden Bakterien (Stamm CBDB1-ähnliche Regiospezifität) und eine Zurückdrängung von Organismen mit anderer Spezifität der Chlorabspaltung. Ein weiterer Befund, der den Einfluß von Kultivierungsbedingungen auf die Anreicherung von Bakterien mit bestimmter Regiospezifität der Dechlorierung zeigt, wurde aus folgendem Anreicherungsexperiment mit der Spittelwasserkultur "Spit B (0-10 cm)" erhalten. In dieser Kultur war der laterale Dechlorierungsweg von 1,2,3-TrCDD zu 1,3-DiCDD klar bevorzugt (20). Die Bildung von 1,3-DiCDD war ebenfalls bei der Dehalogenierung von 1,2,3,4-TeCDD und 1,2,4-TrCDD begünstigtes Produkt. In späteren Kulturen (nach fortlaufender Kultivierung mit 1,2,4-TrCDD) änderte sich die Selektivität: die Bildung von 2,3-DiCDD aus 1,2,3-TrCDD, also ein CBDB1-ähnlicher Dehalogenierungsweg war bevorzugt. *Dehalococcoides* wurde in dieser Kultur tatsächlich durch molekularbiologische Methoden nachgewiesen (Kapitel 3 und 5). Nach Kultivierung mit 1,2,3-Trichlorbenzol in einem Zweiflüssigphasensystem (Kapitel 5) und Weichagar-Verdünnungsreihen wurden Einzelkolonien isoliert und mit einem Gemisch aus 1,2,3- und 1,2,4-TrCDD inkubiert. Dass in den dehalogenierenden Kulturen zusätzlich zu 1,2,3-TrCDD-*peri*-dechlorierenden Bakterien auch 1,2,3-TrCDD-lateral-dechlorierende Mikroorganismen anwesend waren, wurde in der der Einzelkolonieisolierung folgenden Flüssigkultur gezeigt. Diese Kultur bestand aus mehreren Populationen, aber *Dehalococcoides* konnte in 16S rDNA-Klonbibliotheken nicht nachgewiesen werden. Das Gemisch aus 1,2,3-TrCDD und 1,2,4-TrCDD wurde durch diese Kultur in einer für *Dehalococcoides* untypischen Dechlorierung ausschliesslich zu 1,3-DiCDD dehalogeniert. 2-Monochlor-dibenzo-*p*-dioxin konnte nicht nachgewiesen werden. Diese Regiospezifität entspricht der

Transformation in einem frühen Transfer der Kultur, der gemischten *peri*-lateralen Dechlorierung mit 1,3-DiCDD als bevorzugtem Produkt.

Interessant sind die vorläufigen Befunde für *Dehalococcoides ethenogenes* Stamm 195 (35), die einerseits das Potential von Vertretern der Gattung *Dehalococcoides* für reduktive Dechlorierung von Arylverbindungen, andererseits aber auch eine vollkommen andere Regiospezifität für Dioxine und Chlorbenzole als Stamm CBDB1 zeigen.

Stamm 195 dechloriert die Modellverbindung 1,2,3,4-TeCDD zu 1,2,4-TrCDD und 1,3-DiCDD. 2,3-Dichlordibenzo-*p*-dioxin wurde nicht als Dechlorierungsprodukt nachgewiesen. Weder einzeln eingesetztes 2,3-DiCDD noch 2,3,7,8-TeCDD wurden dechloriert. Das steht im Gegensatz zu den Fähigkeiten von Stamm CBDB1, der 1,2,3,4-TeCDD zu 2,3-DiCDD und 2-MCDD dechloriert und eingesetztes 2,3-DiCDD ebenfalls zu 2-MCDD transformieren kann. Der Umsatz von 2,3,7,8-TeCDD durch CBDB1 wurde nicht getestet, die Dechlorierung von 1,2,3,7,8-Pentachlordibenzo-*p*-dioxin zu 2,3,7,8-TeCDD, 1,3,7,8-TeCDD, 2,3,7-TrCDD und 2,7- oder 2,8-DiCDD legt aber eine solche Aktivität nahe. Beim Vergleich von Stamm 195 und Stamm CBDB1 zeigt sich, dass diese relativ nahe verwandten Stämme durchaus ein unterschiedliches Substratspektrum aufweisen oder regiospezifisch unterschiedliche Dehalogenierungsreaktionen durchführen können, wie bereits im vorangegangenen Abschnitt erwähnt wurde.

In einem kürzlich erschienenen Artikel wird diskutiert, dass eine strikt laterale mikrobielle Dechlorierung von natürlich gebildetem Octachlordibenzo-*p*-dioxin in den bereits genannten australischen Küstensedimenten die Zunahme des Anteils von heptachlorierten Kongeneren in den Sedimentkernen mit grösserer Tiefe erkären könnte (40).

Bioverfügbarkeit, *Aging* und *Priming* - die Grenzen mikrobieller Transformationen in der Umwelt

Wenn aerober Abbau und reduktive Dechlorierung möglich sind, warum sind PCDD/F in den wichtigsten *sinks*, aquatischen Sedimenten und in Böden, so extrem persistent? Für Hexachlordibenzo-*p*-dioxine (HxCDD) in Ketelmeer-Sediment wurden Halbwertszeiten von 13 Jahren angegeben (15). Sinkkonen und Paasivirta (96) gehen für OCDD in Sedimenten und Böden von einer Halbwertszeit von etwa 150 Jahren aus, für 1,2,3,4,7,8-HxCDD wird eine Halbwertszeit von etwa 270 Jahren angenommen.

Der biologische Umsatz in den wichtigsten Reservoirs ist durch eine Reihe von Umweltfaktoren bedingt. Oftmals werden Unterschiede in der Biotransformation unter Umweltbedingungen beobachtet. Eine Substanz die in Laborstudien abbaubar ist, wird in der Umwelt nicht oder nur teilweise umgesetzt. So bestimmen Temperatur, pH-Wert, Redoxpotential, die Verfügbarkeit von Elektronendonatoren, Spurenelementen und Vitaminen den Rahmen, in dem mikrobielle Umsätze durch bestimmte physiologische Gruppen möglich sind.

Andere kritische Faktoren, die besonders mit der erhöhten Halogenierung und der chemischen Struktur von Dioxinen im Zusammenhang stehen, sind eine verminderte Wasserlöslichkeit und Bioverfügbarkeit. "Bioverfügbarkeit" beschreibt im umweltmikrobiologischen Zusammenhang die Zugänglichkeit einer Verbindung für intrinsische Mikroorganismen unter bestimmten Umweltbedingungen.

Die Verfügbarkeit der halogenierten Substrate ist durch Sorption an Partikel begrenzt, nur ein kleiner Teil ist in der Wasserphase verfügbar. Der Sorptionsprozeß ist von physikochemischen Eigenschaften der Verbindungen abhängig. Mit dem Octanol/ Wasser-Verteilungskoeffizient (K_{OW}) kann die Tendenz einer Verbindung, sich in der organischen Matrix zu verteilen, abgeschätzt werden. Zwei Prozesse, die die Verteilung der

Verbindungen beeinflussen und wahrscheinlich simultan vorkommen, sind Sorption an hydrophobe Oberflächen und die Verteilung innerhalb der Struktur der organischen Matrix (Migration). Die Prozesse sind zeitabhängig und im Laufe der Zeit scheint ein immer größerer Anteil unverfügbar zu werden. Daraus resultiert auch eine verminderte Aufnahme durch mehrzellige Organismen (69) und eine geringere Verfügbarkeit für Mikroorganismen. Man spricht in diesem Zusammenhang von *aging* oder *weathering* (50). Einerseits hat dieser Prozess toxikologische Konsequenzen, potentiell toxische Stoffe werden in ihrer akuten Toxizität überschätzt (8). Auf der anderen Seite bedeutet die geringe Bioverfügbarkeit keine Entgiftung und hat keinen Einfluss auf die Transportfähigkeit; die halogenierten Verbindungen können weiterhin mit den Partikeln in der Umwelt verteilt werden. Sorptionsprozesse haben Konsequenzen für die Biotransformation durch aerobe oder anaerobe Mikroorganismen und stellen entsprechende Anforderungen an künftige biologische Sanierungsverfahren. Hohe Konzentrationen von halogenorganischen Verbindungen oder deren Abbauprodukte (z. B. Chlorcatechole aus der oxidativen Umsetzung von Dioxinen) können toxisch für Mikroorganismen sein. Möglicherweise ist dann eine reduzierte Bioverfügbarkeit für Mikroorganismen von Vorteil, d.h. wenn die Matrix vor toxischen Effekten mancher Verbindungen schützen kann.

Mit zunehmender Halogenierung nimmt die Löslichkeit von Dioxinen in Wasser ab. Bakterien verfügen jedoch offenbar über Mechanismen nicht nur in der Wasserphase gelöste Verbindungen zu nutzen. *Biosurfactants* (89) oder auch ein direkter physikalischer Kontakt ohne Desorption wurden diskutiert (21). Außerdem ist die Möglichkeit, sorbierte Verbindungen zugänglich zu machen, offenbar stark vom metabolischen Zustand der Mikroorganismen abhängig (48). In der Literatur werden für Sediment und Boden *minute pores* mit Durchmessern von <100 nm und *nanopores* mit Durchmessern von 0.3-1.0 nm

diskutiert (8), die, besonders wenn sie hydrophobe Oberflächen besitzen, eine Desorption von hydrophoben Substanzen erschweren oder unmöglich machen (77).

Die Beobachtung, dass frisch zugesetzte alternative halogenierte Verbindungen die Umsetzung historischer Belastungen z. B. von PCB stimulieren können, sogenanntes *priming*, sollte auch im Zusammenhang mit der Bioverfügbarkeit diskutiert werden (29, 105, 120). So können die frisch zugesetzten Verbindungen einerseits aufgrund ihrer günstigeren physikochemischen Eigenschaften besser verfügbar sein, auf der anderen Seite sind sie noch nicht in die organische Matrix diffundiert, was einen bakteriellen Angriff erleichtern sollte. Der stimulierende Effekt von bromierten Verbindungen auf die reduktive Dechlorierung von PCDD (7) und PCB (14, 120) könnte außerdem auf die höheren $\Delta G°'$-Werte im Vergleich zu den chlorierten Verbindungen zurückzuführen sein (31).

Eine Stimulation der *para*-Dechlorierung von Aroclor 1260 durch *priming* mit 2,5,3',4'-Tetrachlorbiphenyl wurde durch Bedard *et al*. gezeigt (12). Im gleichen Sediment konnte eine *meta*-Dechlorierung durch priming mit 2,3,4,5,6-Pentachlorbiphenyl nachgewiesen werden (105). Das ist, zusammen mit der Temperaturabhängigkeit bestimmter regiospezifischer Dechlorierungswege (117-119), ein weiterer Hinweis darauf, dass spezifische Dehalogenierungswege durch bestimmte Bakterien oder physiologische Gruppen vermittelt werden.

Ausblick auf die Arbeit

Im Rahmen der vorliegenden Arbeit wurde die reduktive Dechlorierung von chlorierten Dioxinen durch anaerobe Bakterien anhand von Modellverbindungen untersucht. Eine Einführung in die Eigenschaften der Stoffklasse, in die Verbreitung von Dioxinen in der Umwelt und bereits bekannte, durch Mikroorganismen vermittelte Transformationen, wird in Kapitel 1 gegeben. In Kapitel 2 wird die reduktive Dechlorierung der Modellverbindung

1,2,3,4-TeCDD in Mikrokosmen und die Transformation trichlorierter Intermediate der 1,2,3,4-TeCDD-Abbausequenz in Subkulturen aus Sedimenten des stark mit Dibenzo-dioxinen und -furanen kontaminierten Flusses Spittelwassers beschrieben. Regiospezifische Unterschiede bei der reduktiven Dechlorierung in Anreicherungskulturen aus unterschiedlichen Sedimentschichten zweier Sedimentkerne werden untersucht. Kapitel 3 geht der Frage nach, ob reduktive Dechlorierung auch in Anreicherungskulturen aus weniger belasteten Sedimenten möglich ist. Welche Transformationswege sind hier bevorzugt? Können spezielle, für ihre Fähigkeit zur Dechlorierung bekannte Organismen in den Kulturen durch molekularbiologische Techniken nachgewiesen werden und korrelieren bestimmte Dechlorierungsmuster mit dem Vorhandensein dieser anaeroben Bakterien? Kapitel 4 beschreibt die Dechlorierung ausgewählter Dioxine durch *Dehalococcoides* sp. Stamm CBDB1. Im 5. Kapitel wird eine Möglichkeit gezeigt, Dioxin-dechlorierende Bakterien mit einer alternativen chloraromatischen Verbindung (1,2,3-Trichlorbenzol) in einem Zweiflüssigphasensystem anzureichern. Die Entwicklung der bakteriellen Gemeinschaft während des Anreicherungsschrittes wird mit Restriktionsfragmentlängen-Analyse beschrieben. In Kapitel 6 wird eine dieser Kulturen mit einer weiteren *fingerprinting*-Methode charakterisiert.

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Regiospecific Dechlorination of Spiked Tetra- and Trichlorodibenzo-*p*-dioxins by Anaerobic Bacteria from PCDD/F-Contaminated Spittelwasser Sediments

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Abstract

Samples were taken from sediment of the creek Spittelwasser (district Bitterfeld, Germany), which is highly polluted with PCDD/Fs and other chloroorganic compounds. The sediment cores were separated into 10- to 20-cm thick layers, spiked with 50 µM of 1,2,3,4-tetrachlorodibenzo-p-dioxin and incubated for 8 months under anaerobic conditions in the presence of cosubstrates. Reductive dechlorination of the tetrachlorinated congener and formation of tri- and dichlorinated products was observed in all biologically active incubations. Analysis of subcultures spiked with 1,2,3- and 1,2,4-trichlorodibenzo-p-dioxin, respectively, revealed two different dechlorination pathways within the sediment cores. Pathway M was characterized by the simultaneous dechlorination of peri- and lateral chlorine atoms, whereas sequence SP was restricted to the dechlorination at positions flanked by chlorine atoms on both sides.

INTRODUCTION

Sediments are important sinks of polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/F). Therefore, the study of transformation processes in these environments is of great concern. Reductive dechlorination of dioxin congeners might be an environmentally important reaction and has been investigated in different anaerobic microcosms derived from historically contaminated sediments. Previous studies have demonstrated the presence of biotic (Adriaens and Grbic-Galic, 1994, Beurskens *et al*., 1995, Ballerstedt *et al*., 1997) as well as abiotic processes (Adriaens *et al*., 1996, Fu *et al*., 1999) or combined reactions (Barkovskii and Adriaens, 1998). Barkovskii and Adriaens (1996) proposed different pathways leading to fewer chlorinated dioxin congeners. The *peri*-dechlorination of 2,3,7,8-substituted hepta- to penta-CDDs catalyzed by non-spore-forming bacteria was characterized by the (transient) formation of 2,3,7,8-tetraCDD, whereas the *peri*-lateral dechlorination pathway of pasteurized cells resulted in fewer chlorinated dioxins with non-2,3,7,8-substituted congeners as intermediate products (Barkovskii and Adriaens, 1996). In no case the bacteria or defined consortia involved in these dechlorination processes have been identified.

The region close to Bitterfeld belongs to the most organochlorine-polluted areas in Germany. Due to the former presence of extensive chlorine industry and the lack of effective waste water and exhaust gas purification, the surrounding environment was contaminated with chloroorganic and many other compounds. Sediments and soils of flooding areas of the rivers Elbe and Mulde and of its tributary Spittelwasser are highly contaminated with PCDD/F. In the latter case, PCDD/F concentrations of up to 3000 pg I-TEQ/g d.w. for sediments and 180,000 pg I-TEQ/g d.w. for soils were reported (Götz *et al*., 1998). Our own study revealed a dioxin contamination level of 120,000 pg I-TEQ/g d.w. in non-top layers of the Spittelwasser sediment (unpublished data). Waste water of industry and households have been discharged

into the river Spittelwasser for decades. The low streaming speed favored sedimentation resulting in sediment layers of up to two meters.

The number of bacteria in sediments is known to decrease with increasing depth, while microbial activity as carbon mineralization by methanogens may have subsurface maxima (Wellsbury *et al*., 1996). However, it is not known, if dechlorinating bacteria are present in deeper layers of freshwater sediments, where the availability of potential cosubstrates, i.e., electron donors for microbial reductive dechlorination might be low. Therefore, the objective of the present study was to investigate the capability of anaerobic bacteria obtained from different depths of the contaminated sediment of Spittelwasser to dehalogenate spiked dioxin congeners. Microcosms, inoculated with sludge of different sediment layers, were incubated with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and subcultivated with 1,2,4- and 1,2,3-trichlorodibenzo-*p*-dioxin (TrCDD), respectively. Dechlorination pathways were elucidated from the analysis of the disappearence of the parent congener and the formation of specific lower chlorinated products.

MATERIALS AND METHODS

Sediment characteristics. Adsorbable organic halogen (AOX) was analyzed using the Metrohm (Switzerland) AOX analyzer 686 according to the DIN standard 38 409 (H14) after an overnight shaking of the sediment sample with acid nitrate solution and activated carbon. The chemical oxygen demand (COD) was determined using the chromate method.

Sampling and primary incubations. Sediment samples (A and B; distance 5 m) were collected from different sediment layers of the Spittelwasser site (coordinates (*x,y*): 4520150; 5729500). Sediment cores (0-40 cm depth, 3.5 cm diameter) were obtained by pushing a glass tube vertically through the sediment. Individual sections of approximately 10 cm thickness

were collected from the cores and stored under N_2/CO_2 (80:20) at 4°C prior to the experiments. Anaerobic incubations of the sediment were carried out in 125-ml serum bottles capped with butyl rubber stoppers and sealed with aluminium crimps. The sediment slurries were prepared by inoculation with sediment (50 % [w/v]) into 30 ml of anaerobic mineral medium (Holliger *et al*., 1992) and supplemented with formate (9 mM), fumarate, pyruvate, acetate and benzoate (5 mM each), yeast extract (0.005 % [w/v]) and 1,2,3,4-TeCDD (50 μM). In addition, controls containing the same amendments were prepared by autoclaving the sediments on three consecutive days at 121°C (25 min). Dioxins were purchased from AccuStandard, Inc. (New Haven, CT) and were added from stock solutions in acetone as described by Ballerstedt *et al*. (1997). At time zero and after 8 months of incubation, duplicate 2-ml subsamples were removed for the analysis using sterile techniques. The slurries were mixed to secure a uniform suspension. All cultures were incubated with agitation (130 rpm) in the dark at 20°C.

Preparation of subcultures. The dechlorination pathway of 1,2,3,4-TeCDD was investigated using subcultures (10 % transfers of the primary culture into fresh medium, see above) which were spiked with 25 μM of the possible intermediates 1,2,3- or 1,2,4-TrCDD, respectively, and were incubated in several aliquots of 3-ml volumes in Hungate tubes containing N_2/CO_2 (80:20) in the gas phase. At regular time intervals (0, 2, 4, 6, 8 weeks), subsamples were removed in duplicate and stored at -20°C until analysis.

Analysis. Extraction, clean-up procedure and analysis of selected congeners by capillary gas chromatography (GC) equipped with a DB-5 column (J&W Scientific, Folsom, CA) and ^{63}Ni-electron capture detector (ECD) followed the procedures described previously (Ballerstedt *et al*., 1997). We used a nine point calibration curve using a quadratic fit of the data ranging from 0.78 μM to 200 μM for quantification. Dioxin congeners were identified by

matching retention times with those of authentic standards. Recovery efficiencies for PCDDs after the clean-up procedure were 65-100 % based on the internal standard used (2,4,8-trichlorodibenzofuran). The identifications were later confirmed by mass spectrometric detection in the selected ion monitoring mode (GC-MS-SIM) as described elsewhere (Ballerstedt *et al*., 1997).

RESULTS

The contents of organic carbon (OC) and adsorbable organic halogen were determined in the individual layers of both sediment cores (Table 1). Sample A possessed in general higher amounts of organic carbon and a slightly higher content of AOX. Whereas sample A exhibited the highest AOX values in a depth of 20 to 40 cm, the AOX load of sample B was comparable low at this depth.

Table 1 Characteristics of the sediment layers and molar distribution of spiked 1,2,3,4-TeCDD and its dechlorination products in primary enrichment cultures from Spittelwasser sediment after 8 months of incubation

Sediment core (layer)	Dry weight (%)	Organic carbon (%)[b]	AOX (mg/kg d.w.)	Relative molar distribution of congeners after 8 months of incubation (mol%)[c]				
				1,3-DCDD	2,3-DCDD	1,2,3-TrCDD	1,2,4-TrCDD	1,2,3,4-TeCDD
A (30-40 cm)[a]	12.8	14.1	3672	0.5	0.7	n.d.[d]	10.4	88.4
A (20-30 cm)[a]	16.4	13.7	4019					
A (10-20 cm)	14.2	13.1	2810	83.4	3.5	n.d.[d]	2.8	10.3
A (0- 10 cm)	5.2	11.9	1950	33.1	10.1	n.d.[d]	3.4	53.5
B (30-40 cm)[a]	25.9	0.5	307	13.5	1.4	n.d.[d]	39.2	45.9
B (20-30 cm)[a]	26.6	3.2	1696					
B (10-20 cm)	18.0	8.9	1936	1.4	1.0	n.d.[d]	11.9	85.7
B (0- 10 cm)	18.5	5.8	1486	43.8	11.3	n.d.[d]	23.7	21.2

[a] The layers from a depth of 20-30 and 30-40 cm were mixed before use as inoculum, [b] As calculated from COD-measurement, [c] Mean values of duplicate samples representing the molar fraction of the total concentration of all congeners measured, [d] Not detectable.

To study the capability of anaerobic bacteria to dechlorinate dioxins, cultures were inoculated with material of the different layers and were spiked with 1,2,3,4-TeCDD. The deepest layers (30-40 and 20-30 cm) of core A and B, respectively, were combined before inoculation. After 8 months of incubation reductive dechlorination of 1,2,3,4-TeCDD was observed in all sediment layers investigated (Table 1). Recovery efficiency obviously decreased during the incubation time and differed between samples, resulting in 5-30 % of the total PCDD measured at time zero. Therefore, the concentration of each congener was reported as mole percent of the sum of all congeners measured. 1,2,3,4-TeCDD was predominantly dechlorinated to 1,2,4-TrCDD, 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) and 2,3-dichloro-dibenzo-*p*-dioxin (2,3-DCDD) by 243 days. 1,2,3-TrCDD was not detected as an intermediate transformation product. Dechlorination was negligible in sterile controls (below 1 mol% of 1,3-, 2,3-DCDD and 1,2,4-TrCDD) during the incubation period with the exception of autoclaved sample B (10-20 cm), where 4 mol% of 2,3-DCDD was formed. However, dechlorination activity in the live sediment slurries differed in the extent and position of chlorine removal as indicated by the formation of different less chlorinated products.

The most extensive dechlorination of 1,2,3,4-TeCDD to mainly 1,3-DCDD (> 80 mol%) occurred in enrichment cultures from core A at a depth of 10-20 cm. The highest dechlorination activity of core B was found in the surface layer (0-10 cm), but besides 1,3-DCDD noticeable amounts of 2,3-DCDD were formed, and 1,2,4-TrCDD accumulated to a large extent in these enrichment. Formation of appreciable amounts of 2,3-DCDD (ratio of 2,3-DCDD:1,3-DCDD greater than 1:4) was only found in enrichment cultures from the top layers (0-10 cm) of both sediment cores. Accumulation of 1,2,4-TrCDD as the final dechlorination product from 1,2,3,4-TeCDD (indicating that only one lateral chlorine was removed from 1,2,3,4-TeCDD) was detected in primary incubations from layers of both cores

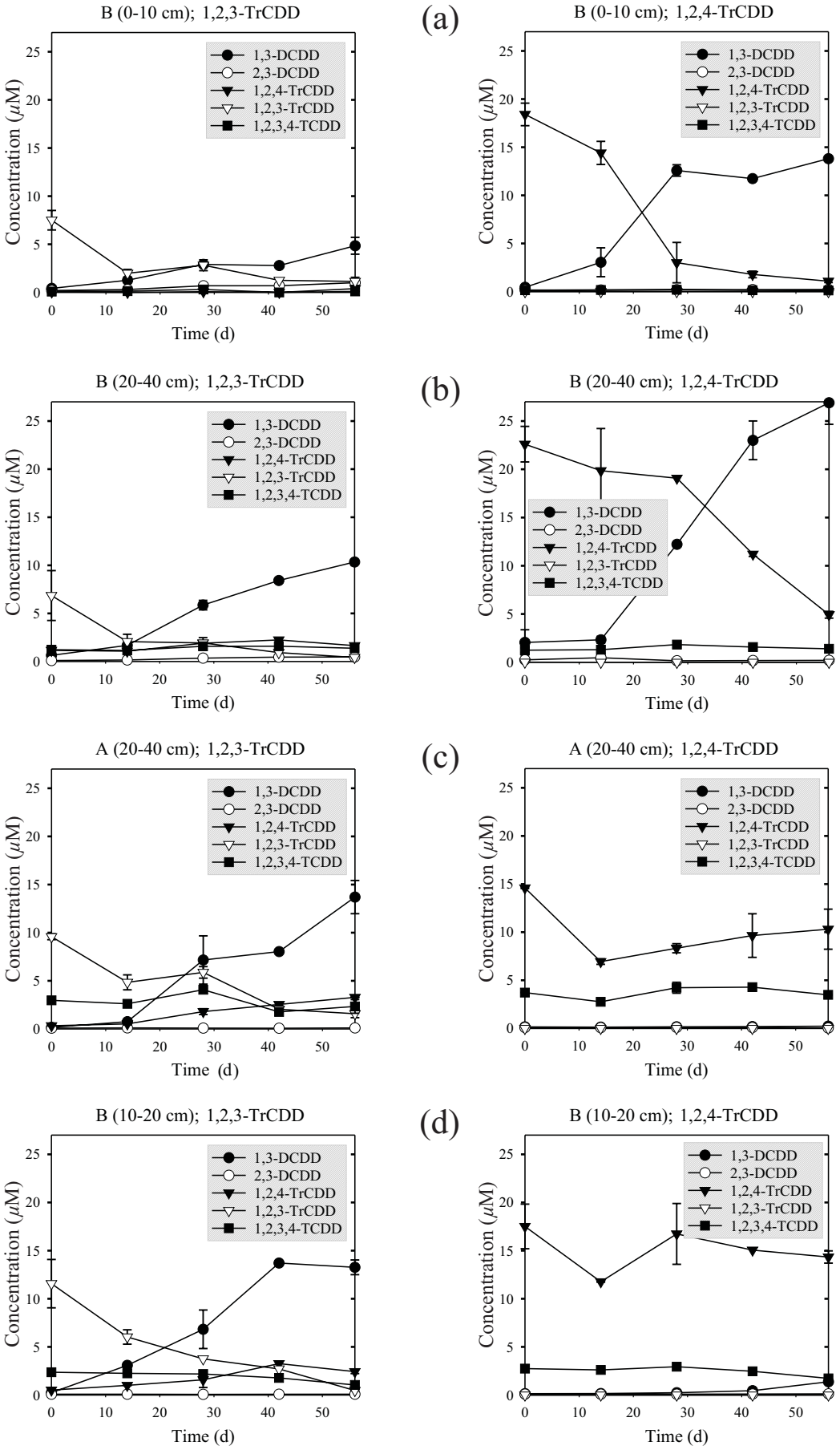
(A: 20-40 cm; B: 10-20 cm).

Subcultures were inoculated from the primary sediment incubations, spiked with the possible intermediary trichlorinated congeners 1,2,3-TrCDD and 1,2,4-TrCDD, respectively, and analyzed at intervals of two weeks for the occurrence of reductive dechlorination. Subcultures from the two upper layers (0-10 cm; 10-20 cm) of core A exhibited no dechlorination within a period of 8 weeks and were not further studied. Subsamples from sediment layers of core B (0-10 cm and 20-40 cm) reductively dechlorinated both congeners (Fig. 1 a, b). 1,2,4-TrCDD was exclusively dechlorinated to 1,3-DCDD. A fairly rapid transformation within 56 days (dechlorination rate of up to 0.8 μM d^{-1} on the basis of the appearance of the lesser chlorinated compound, Fig. 1 b) was observed. Dehalogenation of 1,2,3-TrCDD resulted in the formation of 1,3- and to a very low extent of 2,3-DCDD.

In contrast, two enrichment cultures from core A (20-40 cm) and core B (10-20 cm) transformed 1,2,3-TrCDD to 1,3-DCDD as the only product. 1,2,4-TrCDD was not degraded by these cultures (Fig. 1 c, d). These results were in good agreement with the identification of 1,2,4-TrCDD as the only lesser chlorinated congener during the long-term primary incubation with 1,2,3,4-TeCDD (Table 1).

Next page:

Figure 1 Reductive dechlorination of 1,2,3- and 1,2,4-TrCDD in subcultures inoculated with the primary incubations of the individual layers of sediment cores A (c) and B (a, b, d). The 1,2,3,4-TeCDD determined originated from the inoculum. Mean values of duplicate samples are shown. The error bars indicate the standard deviation.


B (0-10 cm); 1,2,3-TrCDD
(a)
B (0-10 cm); 1,2,4-TrCDD
B (20-40 cm); 1,2,3-TrCDD
(b)
B (20-40 cm); 1,2,4-TrCDD
A (20-40 cm); 1,2,3-TrCDD
(c)
A (20-40 cm); 1,2,4-TrCDD
B (10-20 cm); 1,2,3-TrCDD
(d)
B (10-20 cm); 1,2,4-TrCDD
1,3-DCDD
2,3-DCDD
1,2,4-TrCDD
1,2,3-TrCDD
1,2,3,4-TCDD
Concentration (μM)
Time (d)

DISCUSSION

Anaerobic enrichment cultures inoculated with material of two highly PCDD/F-contaminated sediment cores were all able to reductively dechlorinate spiked 1,2,3,4-TeCDD. The products formed differed in the number and position of chlorines removed. Only one of the two possible intermediary trichlorodibenzo-*p*-dioxins (1,2,4-TrCDD) was detected. Studying the fate of 1,2,3- and 1,2,4-TrCDD in subcultures revealed the existence of two different dechlorination pathways. One dechlorination sequence was designated as Process **M** (referring to enrichments from river Mulde sediment, where this process was also found, Bunge *et al.*, 1999). It was characterized by the formation of 1,3-DCDD ***and*** 2,3-DCDD as the final dechlorination products of 1,2,3,4-TeCDD. Almost the same 2,3-/1,3-DCDD ratios (1:4 and 1:10, respectively) were formed from spiked 1,2,3-TrCDD by the respective subcultures (B [0-10 cm] and B [20-40] cm), suggesting that the main dechlorination route proceeded via 1,2,3-TrCDD rather than 1,2,4-TrCDD (which was exclusively dechlorinated to 1,3-DCDD). Fig. 2 shows the proposed dechlorination pathway. This process resembles the previously published dechlorination of 1,2,3,4-TeCDD by a methanogenic enrichment culture from Lake Ketelmeer (Beurskens *et al.*, 1995), indicating a combination of simultaneous lateral and *peri*-dechlorination activities (Fig. 2). These observations were in contrast to the dechlorination of 1,2,3,4-TeCDD in primary enrichment cultures obtained from Saale river (Ballerstedt *et al*., 1997), where 1,3-DCDD was exclusively formed via 1,2,4-TrCDD. This sequence required a successive dechlorination activity in lateral and *peri*-positions and was designated as Process **S** (Bunge *et al*., 1999). A change in the selectivity of chlorine removal from *peri*- to lateral positions was also described for the dechlorination of OCDD via 2,3,7,8-TCDD to 2-monochlorodibenzo-*p*-dioxin (Albrecht *et al*., 1999, Barkovskii and Adriaens, 1996) .

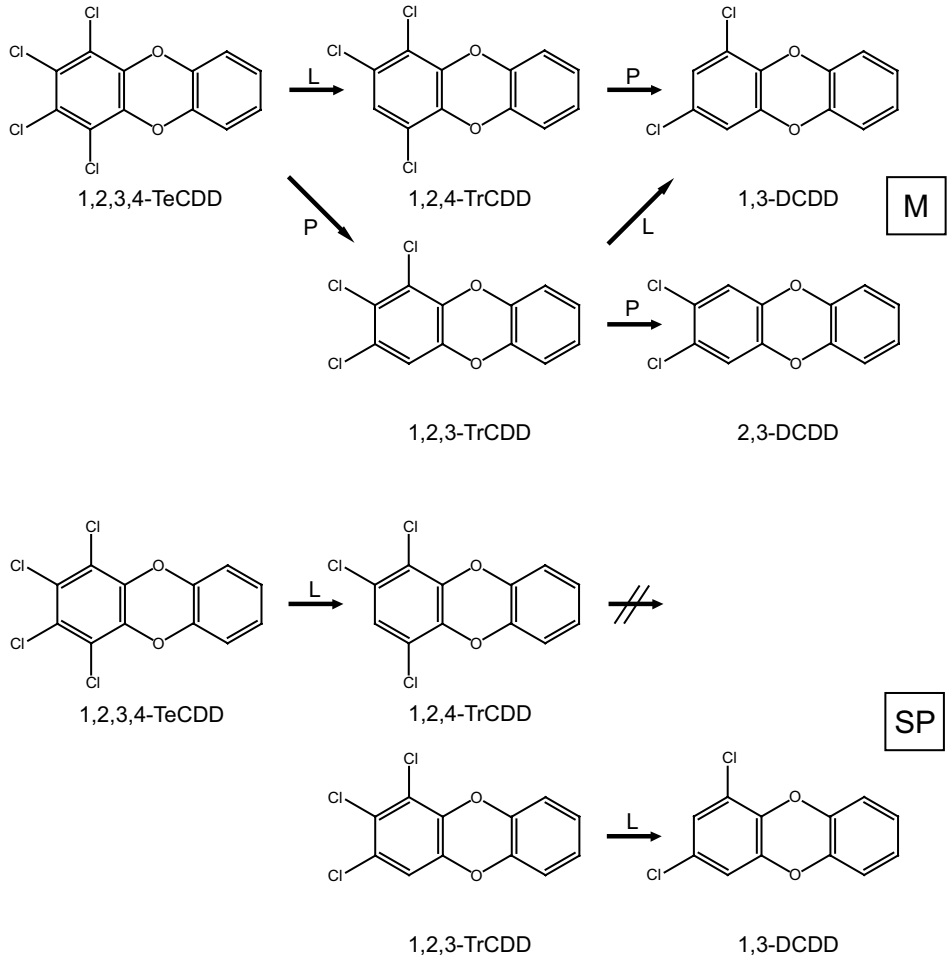


Figure 2 Comparison of two 1,2,3,4-TeCDD-dechlorination processes (M, SP) observed in slurries of Spittelwasser sediment. Removal of chlorine atoms in *peri*- (P) and lateral (L) positions.

Another dechlorination activity designated as Process **SP** (detected for the first time in Spittelwasser sediment) was restricted to positions flanked by chlorines on both sides. Therefore, 1,2,4-TrCDD accumulated from 1,2,3,4-TeCDD and resisted further dehalogenation. 1,2,3-TrCDD added to subcultures was transformed to 1,3-DCDD, and no 2,3-DCDD was detectable. The regiospecificity of these dehalogenation reactions is in accordance with the thermodynamically most favorable reductions ($\Delta G^{\circ\prime}$ values were calculated to be -174.8 and -162.7 kJ/reaction [redox pairs 1,2,3,4-TeCDD/1,2,4-TrCDD and

1,2,3,4-TeCDD/1,2,3-TrCDD] and -165.2 and -152.1 kJ/reaction [redox pairs 1,2,3-TrCDD/ 1,3-DCDD and 1,2,3-TrCDD/2,3-DCDD, respectively]; Huang *et al*., 1996). It should be noted, however, that we observed dechlorination pathway **SP** exclusively in those layers of the Spittelwasser sediment, which exhibited the highest AOX contents within cores A and B (4019 mg/kg d.w. and 1936 mg/kg d.w., respectively). One could speculate, that the high level of sediment contamination with chloroorganic compounds (e.g., up to 6 mg/kg d.w. of hexachlorocyclohexane, 3 mg/kg d.w. of chlorobenzenes and others) affected the dechlorination process. It seems, that halogenated contaminants may select for a specific dehalogenation activity or for the bacteria involved in this process. Priming of distinct dechlorination processes by other halogenated compounds has been recently described for PCBs (Van Dort *et al*., 1997, DeWeerd and Bedard, 1999). Wu *et al*. (1999) showed, that priming with 2,6-dibromobiphenyl promoted the growth of PCB dechlorinating microbes and thus accelerated the dechlorination process. The Process **SP** exhibits a potential for detoxification of dioxin contaminations, assuming that chlorines in lateral (flanked) positions can be also removed from higher chlorinated 2,3,7,8-substituted congeners.

Our data clearly show the occurrence of different dechlorination pathways within a single sediment core, suggesting that different dechlorinating populations are involved in these processes. Position and congener specificity of microbial PCDD reductive dechlorination might depend on specific bacteria. The different regiospecificities of microbial PCDD and PCB dehalogenation present in one sediment sample could be separated by various treatments, e.g. pasteurization (Barkovskii and Adriaens, 1996, Ye *et al*., 1992) or incubation at different temperatures (Wu *et al*., 1997). More research is needed to better understand the role of microorganisms that mediate this process in order to predict the behavior and fate of chloroorganic contaminants in the environment. Further work will be carried out to investigate the

significance of the microbial PCDD/F degradation *in situ* to evaluate the suitability of this attractive alternative to conventional treatment strategies for bioremediation of large-scale contaminated sites.

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Anaerobic Transformation of Dioxins by Bacteria from River Sediments: Diversity of the Dehalogenating Community

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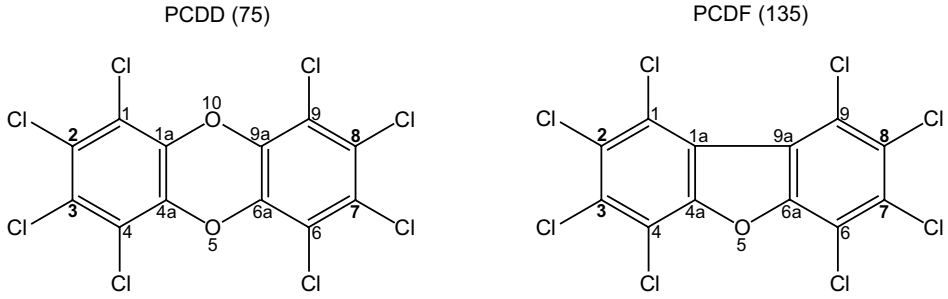
Abstract

Mixed cultures of anaerobic bacteria capable of reductively dechlorinating freshly spiked 1,2,3,4-tetrachlorodibenzo-p-dioxin (1,2,3,4-TeCDD) were obtained from freshwater sediments of streams near Bitterfeld, Germany. All of these sediments have been impacted by industrial wastewater for decades, but have comparably low levels of polychlorinated dibenzo-p-dioxins and -furans (PCDD and PCDF), not exceeding 500 ng toxicity equivalents (I-TEQ) per kg dry weight. Reductive dechlorination of 1,2,3,4-TeCDD in the primary enrichments as well as dechlorination of trichlorinated dioxin congeners in subsequent transfers showed that dioxin-dechlorinating bacteria could be enriched from these low-level contaminated sites. The amount of two transformation products, 1,3- and 2,3-di-chlorodibenzo-p-dioxin was different in the studied cultures, demonstrating preferential substitution of chlorine in either peri- or lateral positions of the molecule. Ten different dioxin-dechlorinating cultures were analyzed with a 16S rDNA-based polymerase chain reaction (PCR) approach to detect phylotypes of bacteria known to be able to reductively dechlorinate several halogenated aliphatic and aryl compounds. Using this qualitative approach, we could not assign a specific dechlorination pathway to the presence or absence of specific dehalogenating organisms. However, it was shown that members of Desulfito-bacterium and Dehalococcoides were present in all communities studied, including cultures from Spittelwasser sediments (Bunge et al., 2001), and might be involved in reductive dehalogenation of chlorinated dioxins.

INTRODUCTION

For more than 25 years, since the devastating accident in a 2,4,5-trichloro-phenol-producing plant near Seveso, Italy, polychlorinated dibenzo-*p*-dioxins and -furans (PCDD and PCDF), especially the 2,3,7,8-substituted congeners, have been in the focus of attention. The different congeners of these persistent organohalogens vary in physical-chemical data (33), toxicity (see reference (35) for a comprehensive review), and bioaccumulation- and biomagnification properties, which result from differences in the degree and position of chlorine substitution (Fig. 1).

Figure 1 Chemical structure of polychlorinated dibenzo-*p*-dioxins (PCDD) and -furans (PCDF) and numbering of the carbon atoms. The fully (octa)chlorinated compounds are shown. The number of possible different congeners is shown in parentheses. Seventeen individual 2,3,7,8-substituted congeners of PCDD/F are thought to have "dioxin-like" toxicity.



Dioxins, including the most hazardous 2,3,7,8-chlorinated compounds, are formed as inadvertant by-products of manufacturing and incineration processes (18, 35). Because of their high hydrophobicity, dioxins are strongly sorbed to organic matter; aquatic sediments and soils therefore constitute important sinks of these pollutants in the environment. In the Bitterfeld area of Germany, sediments and soils (particularly sediments and soils of flooding areas of Spittelwasser creek) are contaminated with high levels of PCDD, PCDF and other chlorinated

compounds such as hexachlorocyclohexane (HCH), DDT and degradation products (DDX), chlorobenzenes, chlorophenols and polychlorinated biphenyls (PCB) (19). There is no consensus regarding the sources of these compounds since many potential dioxin-releasing facilities (chlorine production, chlorinated pesticides, PVC and magnesium production) were settled in this old industrial region and their effluents have been discharged into the environment for decades.

In order to eliminate these environmental contaminants, microbiological processes using the capabilities of naturally occurring microbial communities to carry out intrinsic bioremediation offer significant promise. Treatment strategies for the most affected areas (Spittelwasser) proposed by different European teams include capping the sediment by clay, which would prevent the transport of PCDD/F-contaminated particles downstream. In addition, this process might create stronger reducing conditions, thus enhancing natural attenuation processes by reductive dehalogenation of the organochlorine compounds (14). We have recently described the dioxin-dechlorinating capabilities of anaerobic bacteria derived from Spittelwasser sediments (13). Despite these promising laboratory results, only a few tests evaluating their effectiveness and relevance to PCDD contamination in the field have been reported. Whether reductive dechlorination of dioxins can provide a powerful tool for natural attenuation strategies remains to be seen.

One of the few reports about *in situ* dioxin transformation reactions is a study in sediment cores of Lake Ketelmeer (a sedimentation area of the Rhine River, The Netherlands), which indicated that PCDD/F were transformed (9). This observation has been used to support the hypothesis that reductive dechlorination of highly halogenated dioxins occurs as a natural process of intrinsic microorganisms and makes a significant impact on the dioxin contamination. Beurskens *et al*. (10) demonstrated the reductive dechlorination of 1,2,3,4-

tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by hexachlorobenzene-adapted anaerobic mixed cultures from Lake Ketelmeer sediment. Other reports of microbial dehalogenation of dioxins include articles by Adriaens and Grbic-Galic (1), Barkovskii and Adriaens (8) and Ballerstedt *et al*. (7).

It has been argued that the (temporary) products of PCDD/F dechlorination can be, to some extent, more toxic (4, 8). Thus, knowledge of the transformation processes is of major concern for evaluation of the long-term fate of PCDD/Fs. One objective of the present study was to demonstrate the microbial dioxin reduction potential in anaerobic mixed cultures from contaminated sediments. So far, no pure culture of a reductively dioxin-dechlorinating bacterium has been described and nothing is known about the microorganisms involved in the reductive dechlorination of dioxins, but it is generally assumed that they might occur in very low numbers within dechlorinating communities. Therefore, we conducted a *nested* PCR approach using specific 16S rDNA primers to detect bacteria with a known dehalogenating potential for a variety of halogenated compounds in dioxin-dechlorinating enrichment cultures obtained from river sediments in the region near Bitterfeld.

MATERIALS AND METHODS

Enrichment cultures. Sediment samples were collected from River Saale, River Mulde, and Leine creek (near Bitterfeld, Germany), and were analyzed by routine dioxin analyses according to the DIN EN 1948-3 standard method (15) . The samples contained 12 (Saale), 44 (Mulde3), 464 (Mulde1) and 70 (Leine) ng toxicity equivalents (I-TEQ) per kg dry weight and were thus far less contaminated than the Spittelwasser sediment samples from the same area (13). The samples were transferred into 1-l serum bottles flushed with N_2/CO_2 (80%/20%). Sediment was used to inoculate primary enrichment cultures (50 % [wt/vol]) in mineral

medium (21) supplemented with 25 μM 1,2,3,4-TeCDD and a mixture of organic acids as described elsewhere (7). Samples were withdrawn anaerobically after 2, 4, and 8 months of incubation at 20°C and extracted with hexane according to Ballerstedt *et al*. (7). After a long-term incubation of 240 days at 20°C in the dark, the primary enrichments were transferred (10 % [vol/vol]) into fresh medium supplemented with 25 μM 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD) or 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and organic acids as previously described (13). Duplicate samples were withdrawn at specific times and stored at -20°C prior to analysis. Controls containing the same amendments were prepared without inoculum. Another set of controls received material autoclaved on three consecutive days at 121°C (25 min). The secondary enrichment cultures from Saale, Mulde and Leine as well as secondary enrichment cultures from Spittelwasser described elsewhere (13) were further transferred up to five times into fresh medium for studies of the community composition.

Analysis. Extraction, clean-up, and analysis of selected congeners using gas chromatography (GC) equipped with a DB-5 capillary column (J&W, Folsom, CA) and a ^{63}Ni-electron capture detector (ECD) followed the methods as described (7). The compounds were identified by matching retention times with those of authentic standards (AccuStandard, New Haven, CT) and quantified using a nine-level calibration curve for each congener. Recovery efficiencies for dioxin compounds after the clean-up procedure were usually between 65 and 100 % based on the internal standard used (2,4,8-trichlorodibenzofuran). Identified congeners were confirmed by mass spectrometry as described previously (7).

Isolation of DNA. The sources of genomic DNA were ten different actively 1,2,4- or 1,2,3-TrCDD-dechlorinating cultures (see Table 3). Enrichment cultures from Mulde, Leine, and Saale had undergone seven serial transfers before DNA extraction was initiated, while

DNA was extracted from creek Spittelwasser cultures after five consecutive transfers. Cells were harvested from 1 ml of culture by centrifugation at 9,500 x g for 20 min. Bead-based cell disruption was essentially performed as described by Kuske *et al*. (26). The resulting community DNA was precipitated with ethanol and sodium acetate (31), purified according to standard procedures (31), and dissolved in sterile deionized water.

PCR detection. The first PCR amplification was performed using either the *Bacteria*-specific oligonucleotide primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3', *E. coli* positions 8-27) and rP2 (5'-ACGGCTACCTTGTTACGACTT-3', *E. coli* positions 1512-1492) or the primers fD2 (5'-AGAGTTTGATCATGGCTCAG-3', *E. coli* positions 8-27) and rP1 (5'-ACGGTTACC TTGTTACGACTT-3', *E. coli* positions 1512-1492) (37). The reaction mixtures contained 1 x *Taq* PCR buffer (Promega), 1.8 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 210 nM of each forward and reverse primer, and 0.020 U of *Taq* DNA polymerase (Promega) per μl reaction volume. Reaction mixtures were incubated in a "PCR Sprint" thermocycler (Thermo Hybaid, Germany) according to the following conditions: denaturation at 94°C for 2 min, followed by 30 cycles of 15 s denaturation at 94°C, 30 s annealing at 54°C, and extension at 72°C for 1 min. The last 20 extension steps were performed using a 20 s time increment per cycle. Amplicons of the two PCR reactions were combined, and 0.75 μl of the purified amplicons (QIAquick PCR purification Kit, Qiagen, Hilden, Germany) was used as a template for the second (*nested*) PCR amplifications, which were performed with the same reaction mixtures except for the species-specific or genus-specific primers and their annealing temperatures, as follows: DCH205/DCH1033, 58°C; DRE445/DRE1248, 54°C; DTI178/ DTI1001, 52°C; DMU175/DMU623, 52°C; DET730/DET1350, 52°C; DES436/DES1027, 55°C; LPA208/LPA1247, 54°C. These *nested* oligonucleotide primers were directed against variable regions of the 16S rDNA of the respective organisms as listed in Table 2. The primers

selected for this study were designed using the ARB program package (http://www.arb-home.de/) and were subsequently checked by using the CHECK_PROBE (2.1) function provided by the Ribosomal Database Project (RDP-II, http://rdp.cme.msu.edu/html/).

Sequencing. Cycle sequencing was performed using the automated laser fluorescence DNA sequencer ABI Prism 377, version 4.0 (PE Applied Biosystems, Langen, Germany), using the *nested* primers as described above. Relatives were identified using the Fasta33 program at EMBL-EBI and the SEQUENCE_MATCH (2.7) tool of RDP-II. Obtained sequences were screened for chimeras using RDP's CHIMERA_CHECK (2.7) program.

RESULTS AND DISCUSSION

Dehalogenation of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin. Reductive dechlorination of 1,2,3,4-TeCDD was observed in all sediment cultures collected from different low PCDD/F-contaminated sites, although comparatively long incubation periods (four months) were required to detect significant amounts of dechlorination products. Table 1 shows the results of dioxin analyses for sediment slurries from the rivers Mulde, Leine and Saale after eight months of incubation. It is generally agreed that PCDD-dechlorinating bacteria grow and reduce substrates slowly, which is probably due to the low bioavailability of the substrates. Furthermore, alternative halogenated and non-halogenated electron acceptors also present in the sediment samples, and possible toxic effects of co-contaminants, might explain the low dechlorination rate in the primary enrichments.

Dichlorodibenzo-*p*-dioxins (DiCDD) constituted the final products of dechlorination in initial enrichment cultures. The comparison of data obtained from live and abiotic treatments (autoclaved and uninoculated controls) strongly indicated that the dechlorination was

microbially mediated and that PCDD-dechlorinating bacteria should have been present in the sediments investigated.

The highest dechlorination rate was observed in the cultures inoculated with sediments from the sites "Saale" and "Mulde1". 1,3-DiCDD was the major dehalogenation product (about 50-60 mol%), whereas 2,3-DiCDD was only detected in trace amounts.

Table 1 Reductive dechlorination of spiked 1,2,3,4-TeCDD in primary enrichment cultures from stream sediments after eight months of incubation. For comparison, the data obtained for sediment slurries of the Spittelwasser site are included (13).

Origin[a] of the sediment sample (sediment layer)	Relative molar distribution of congeners after 8 months of incubation (mol%)[b]				
	1,3-DiCDD	2,3-DiCDD	1,2,3-TrCDD	1,2,4-TrCDD	1,2,3,4-TeCDD
Mulde 1	49.0	0.4	0.4	0.8	49.4
Leine	6.1	3.5	0.4	0.9	89.1
Mulde 3	37.0	16.4	1.4	4.0	41.2
Saale	62.5	2.0	1.2	3.0	31.3
Spittelwasser A (20-40 cm)	0.5	0.7	n.d.[c]	10.4	88.4
Spittelwasser A (10-20 cm)	83.4	3.5	n.d.[c]	2.8	10.3
Spittelwasser A (0- 10 cm)	33.1	10.1	n.d.[c]	3.4	53.5
Spittelwasser B (20-40 cm)	13.5	1.4	n.d.[c]	39.2	45.9
Spittelwasser B (10-20 cm)	1.4	1.0	n.d.[c]	11.9	85.7
Spittelwasser B (0- 10 cm)	43.8	11.3	n.d.[c]	23.7	21.2

[a] Mulde1 and Mulde3 refer to two different sampling sites. Spittelwasser A/B represent two sediment cores collected from neighboring sites (13). [b] mean values of duplicate samples representing the molar fraction of the total concentration of all congeners measured. [c] not detectable.

Two main products of reductive dechlorination of 1,2,3,4-TeCDD were formed in the mixed cultures “Mulde 3” und “Leine”: 1,3-DiCDD und 2,3-DiCDD in a ratio of approximately 2:1. The two possible trichlorinated congeners 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD) and 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) were only found in low concentrations.

These results are contrary to a previous study using primary cultures inoculated with sediment from the Spittelwasser site, where 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) was detected in considerably high concentrations (13). Additionally, in two of these cultures from sediment cores of creek Spittelwasser (A (20-40cm) and B (10-20cm), see Table 1), 1,2,4-TrCDD was the only dechlorination product formed, indicating that only positions flanked by two chlorine substituents were accessible to dechlorination. These results were in good agreement with the identification of 1,3-DiCDD as the final product during incubations with 1,2,3-TrCDD and the inability to transform 1,2,4-TrCDD by these cultures (13).

Dehalogenation of trichlorinated dioxin congeners. As shown recently, dehalogenation of the trichlorinated congeners 1,2,3-TrCDD and 1,2,4-TrCDD was an order of magnitude faster than that observed for the tetrachlorinated congener (7). To test this possibility, the investigated cultures were sequentially subcultured and spiked with 1,2,3-TrCDD and 1,2,4-TrCDD, respectively. Concentrations of the parent compounds and the lesser clorinated products were monitored over time (Fig. 2) and used to elucidate the transformation pathways and to calculate the transformation rates.

The position of chlorine removal from 1,2,3- and 1,2,4-TrCDD and the rates of appearance of lesser chlorinated products were in most cultures similar to those shown in Figure 2a for the culture "Mulde3". 1,2,4-TrCDD was exclusively dechlorinated to 1,3-DiCDD. A rather fast transformation within 41 days was observed. The maximum dechlorination rate of 1.4 μM d^{-1} was calculated based on the known amount of 1,2,4-TrCDD

added and the increase in the molar ratio of 1,3-DiCDD formed. Reductive dehalogenation of 1,2,3-TrCDD yielded two dechlorination products: 1,3-DiCDD and a low amount of 2,3-DiCDD, indicating a combination of simultaneous *peri*- and preferential lateral dechlorination activities.

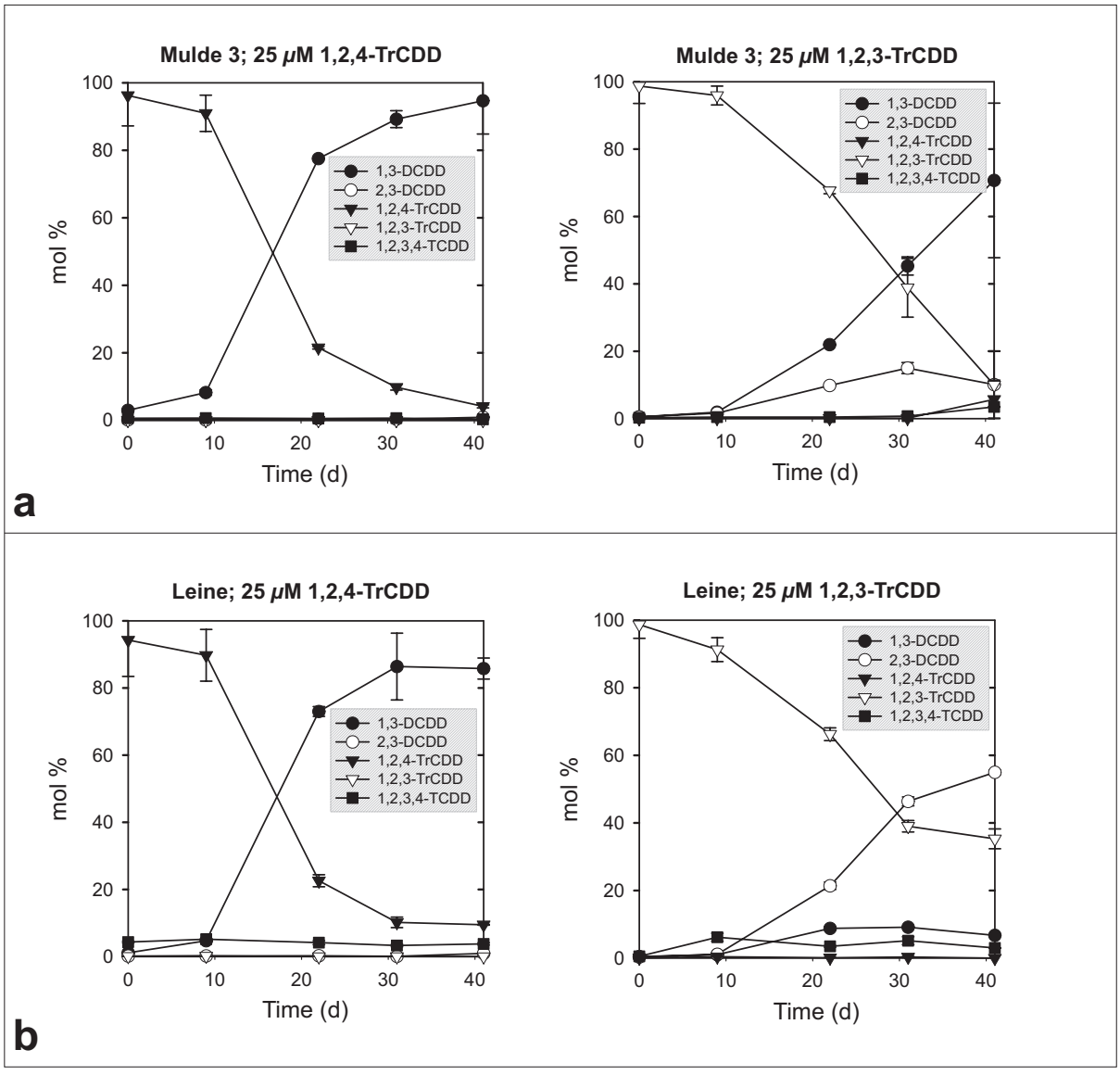


Figure 2 Reductive dechlorination of 1,2,3- and 1,2,4-TrCDD by subcultures from the sampling sites "Mulde3" (a) and "Leine" (b). Opposite ratios of the transformation products 1,3-DiCDD/2,3-DiCDD were observed in the two cultures during dechlorination of 1,2,3-TrCDD. All subcultures were inoculated with the primary enrichments spiked with 1,2,3,4-TeCDD, which was still detectable in the following culture from Leine sediment. Mean values of duplicate samples are shown. The error bars indicate the standard deviation.

Between different 1,2,3-TrCDD-dechlorinating cultures, the ratio of these two processes was not the same (Figure 2b). In fact, the different ratio of 1,3-DiCCD to 2,3-DiCDD formation from 1,2,3-TrCDD was the most significant difference between the investigated cultures. One culture (Leine) formed more 2,3-DiCDD than 1,3-DiCDD from 1,2,3-TrCDD, demonstrating that substitution of chlorines in *peri* position was favored.

Based on the appearance of the lower chlorinated products from 1,2,3,4-TeCDD, 1,2,3-TrCDD and 1,2,4-TrCDD, some if not all of the observed dechlorination pathways can be assigned to process M as previously described and characterized by the occurrence of coexisting *peri*- and lateral dechlorination pathways (13).

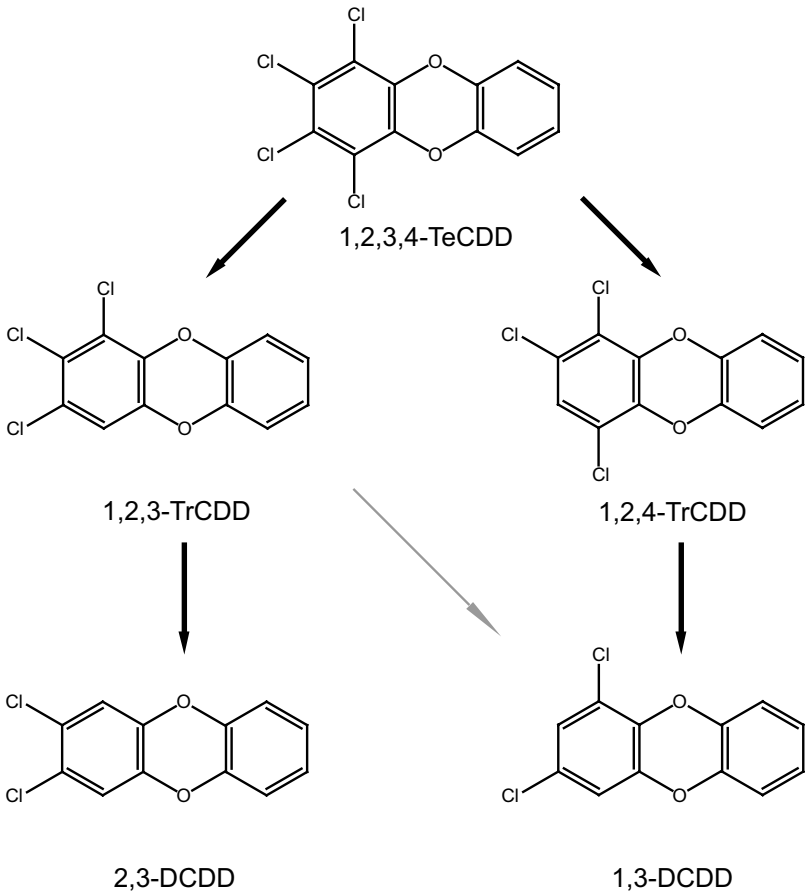


Figure 3 Dechlorination pathway of spiked dioxin congeners in enrichment cultures from Leine sediment. A grey arrow indicates that the formation of 1,3-DiCDD from 1,2,3-TrCDD (lateral dechlorination) is less favored than the regioselective dechlorination in *peri* position.

Nonetheless, such a strong preference for the formation of 2,3-DiCDD from 1,2,3-TrCDD as observed in the Leine sediment cultures has not been reported yet. The regiospecificity of this special case of reductive dechlorination is summarized in Fig. 3.

If these results could be extrapolated to reductive dechlorination of dioxins chlorinated on both rings, favored removal of peripheral chlorine atoms implies the risk of formation of 2,3,7,8-TeCDD, a compound that is more toxic than the parent congeners. Indeed, a preferred *peri*-dechlorination of 2,3,7,8-substituted hepta- to pentachlorinated dibenzo-*p*-dioxins and the (transient) formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was observed by Barkovskii and Adriaens (8). The authors assigned the *peri*-regioselective removal of chlorines to non-spore-forming bacteria. A more detailed view regarding the bacteria involved in dioxin dechlorination is of great importance.

A molecular method to identify dehalogenating bacteria in the mixed cultures. Attempts to isolate the dechlorinating bacteria were unsuccessful. The number of 1,2,4-TrCDD dechlorinating bacteria in mixed cultures from River Saale sediments was estimated at 2.5 x 10^3 cells per ml vs. a total number in the range of 10^8 bacteria per ml (5). Therefore, isolating a pure culture using conventional isolation strategies is unlikely. We therefore performed a molecular approach using PCR and specific primers directed against variable regions of the 16S rDNA to detect the presence of various microorganisms with a known dehalogenating potential (22). The particular bacteria are phylogenetically very diverse and belong to physiologically distinct microbial groups. Different sets of oligonucleotide primers were designed for the detection of dechlorinators and for strain Coc4, previously isolated from a dioxin dehalogenating community (Table 2).

After five (Spittelwasser) and seven transfers (Mulde, Leine, Saale) of the dehalogenating communities, genomic DNA was isolated from 1,2,3- or 1,2,4-TrCDD dechlorinating

cultures and used as template for the amplification of rRNA genes with primers binding to evolutionarily conserved regions of the molecule (37). Aliquots of the 16S rDNA amplicons were used for the reamplification in a PCR performed with the set of *nested* primers. *Nested* PCR has been shown to be a sensitive and specific method for the detection of bacteria both in culture and the environment and was used recently also for the detection of dechlorinating bacteria (17, 27). Löffler *et al*. demonstrated that 1 to 10 cells of the *Desulfuromonas* strain BB1 and of *Dehalococcoides* sp. FL2 were sufficient to yield a visible band (27).

Table 2 16S rDNA-directed primers for *nested* PCR − sequences, binding sites and target organisms. 16S rDNA of "target organisms" exactly matches the sequences of both primers.

Primer	Sequence (5'-3')	Target site[a]	Target organisms and GenBank accession numbers
DCH 205[f]	AACCTTCGGGTCCTGCCGTC	204-222	*Desulfuromonas chloroethenica* (U49748)
DCH 1033[r]	GCCGAACTGACCCCTATGTT[b]	1034-1015	
DRE 445[f]	GGAAGAACGGCATCTGTG	446-464	*Dehalobacter restrictus* (U84497)
DRE 1248[r]	GGCTTCCGTTCCGTCTG	1265-1249	
DTI 178[f]	ATGAGACCACATGAGCTC	179-193	*Desulfomonile tiedjei* (M26635)
DTI 1001[r]	GTTTCCACGACTGTCCG	1017-1001	
DMU 175[f]	CCCATACTCCTTCTTGTC	176-193	*Dehalospirillum multivorans* (X82931)
DMU 623[r]	TTCGAGAGCAGTTCAACG	641-624	
DET 730[f]	GCGGTTTTCTAGGTTGTC	731-748	*Dehalococcoides ethenogenes* (AF004928)
DET1350[r]	CACCTTGCTGATATGCGG	1368-1351	
DES 436[f]	TGTCTTCAGGGACGAACG	437-454	*D. frappieri* strains PCP-1 (U40078), TCE1 (X95742), TCP-A (AJ404686); *D. hafniense* (X94975), *D. dehalogenans* (L28946), *D. chlororespirans* (U68528), *D.* sp. PCE1 (X81032)
DES 1027[r]	CTCATAGCTCCCCGAAGG	1043-1027	
LPA 208[f]	TTGTGCTGTCGCTTATGG	209-227	Bacterium Coc4[c], *Trichococcus pasteurii* KoTa2 (X87150), *Trichococcus flocculiformis* (Y17301)
LPA 1247[r]	GGTCTTGCTGCTCGTTGT	1265-1248	

[a] corresponding to *E. coli*-positions (11), [b] according to reference (27), [c] This strain was isolated from a previous dioxin-dechlorinating mixed culture (7), *D. Desulfitobacterium.*

The results of the *nested* PCR approach and sequence analysis of 16S rDNA partial sequences are summarized in Table 3. Most surprisingly, with the primer sets designed for the genus *Desulfitobacterium* and for *Dehalococcoides ethenogenes* we identified target organisms in all cultures from different sampling sites. In both cases, the partial 16S rDNA sequences derived from ten different mixed cultures were absolutely identical. The sequence obtained with the *Dehalococcoides*-specific primers was identical to the 16S rDNA of strain CBDB1, a chlorobenzene dehalogenating bacterium from Saale River sediment (2, 3). The sequences of the two *Dehalococcoides* strains FL2 (AF357918.2) and BAV1 (20) corresponding to the 16S rDNA fragments of our study were also the same. In addition to a number of uncultured members of the genus which exhibited identical or very similar sequences, *Dehalococcoides ethenogenes* 195 shared 98.8 % identity with the amplicons of the specific PCR reactions (AF004928.2) (30).

The presence of *Desulfitobacterium* in some of the cultures has already been shown by fluorescence *in situ* hybridization (12). The ten obtained products from the *Desulfito-bacterium*-specific PCR were in all cases identical to *Desulfitobacterium frappieri* PCP-1 (U40078) and *Desulfitobacterium* sp. strain TCE1 (X95742), and also very similar (97.4 to 100 %) to the 16S rDNA of other members of the genus.

Our results showed a positive signal in all cultures using a primer pair designed for strain Coc4, previously isolated from a dioxin-dehalogenating community (6). According to its 16S rDNA and several physiological data, strain Coc4 is closely related to *Trichococcus pasteurii* KoTa2, formerly described as *Lactosphaera pasteurii* (24). Strain Coc4 is probably a predominant member of the communities but no dechlorination of 1,2,4-TrCDD was observed by the isolated strain. Therefore, it is not assumed that the strain is directly involved in the dehalogenation process. Using the Coc4-specific primer set, we also amplified a 16S rDNA

fragment (culture "Mulde3" incubated with 1,2,3-TrCDD) similar to molecular clones WCHB1-71 (98.5 %) and WCHB1-82 (97.2 %), both retrieved from a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation (16).

Table 3 Detection of organochlorine-transforming organisms by a *nested* PCR approach and sequencing of 16S rDNA fragments [a].

Target organisms / Culture	*Dm. chloro-ethenica*	*Db. restrictus*	*Ds. multi-vorans*	*Dc. etheno-genes*	*Df. tiedjei*	*Desulfito-bacterium*
Leine; 123-TrCDD	–	+	–	+ S (571 bp)	–	+ S (539 bp)
Mulde 3; 123-TrCDD	–	+ S (774 bp)	–	+ S (579 bp)	–	+ S (541 bp)
Spittelwasser A (20-40 cm); 123-TrCDD	+ S (782 bp)	–	+ S (435 bp)	+ S (566 bp)	–	+ S (541 bp)
Spittelwasser B (10-20 cm); 123-TrCDD	–	–	+ S (432 bp)	+ S (589 bp)	–	+ S (516 bp)
Mulde 1; 124-TrCDD	+	–	+	+ S (605 bp)	–	+ S (547 bp)
Leine; 124-TrCDD	–	–	–	+ S (605 bp)	–	+ S (537 bp)
Mulde 3; 124-TrCDD	–	–	–	+ S (600 bp)	–	+ S (565 bp)
Saale; 124-TrCDD	+ S (478 bp)	–	–	+ S (615 bp)	–	+ S (547bp)
Spittelwasser B (20-40 cm); 124-TrCDD	+ S (753 bp)	–	+	+ S (605 bp)	–	+ S (518 bp)
Spittelwasser B (0- 10 cm); 124-TrCDD	+ S (760 bp)	–	+	+ S (605 bp)	–	+ S (567 bp)

[a] PCR product of the expected size formed (+), not formed (-), bidirectionally sequenced (S), sequence length in brackets; *Dm. Desulfuromonas, Db. Dehalobacter*, *Ds. Dehalospirillum, Dc. Dehalococcoides*, *Df. Desulfomonile*.

Sequenced amplification products of the *Desulfuromonas chloroethenica*-specific PCR reactions shared a maximum similarity of only 97.8 % with the sequence of *Desulfuromonas chloroethenica* (25) but 100 % sequence similarity with clone RFLP 108 derived from a 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community (23). The next closest cultivated relatives were *Desulfuromonas acetexigens* (up to 99.6 %) (28), and the two tetrachloroethene-dechlorinating *Desulfuromonas michiganensis* strains BB1 (AF357915.2) and BRS1 (AF357914.2) with a maximum sequence identity of 98 % (34).

In the PCR approach using the *Dehalobacter*-targeted primers, only two cultures from sediments of Mulde and Leine incubated with 1,2,3-TrCDD resulted in an amplified product. Most interestingly, the sister cultures of the same origin (initial 1,2,3,4-TeCDD-dehalogenating community) enriched with 1,2,4-TrCDD did not yield an amplicon. The sequence from "Mulde3" was 96.6 % similar to *Dehalobacter restrictus* strain TAE (38), and similar to the clones SJA-19 (95.5 %) derived from a trichlorobenzene-transforming microbial consortium (36) and SHA-67 (95.4 %) from an anaerobic 1,2-dichloropropane-dechlorinating mixed culture (32). Sequences about 99 % identical to the tetrachloroethene-dehalorespiring bacteria *Dehalospirillum multivorans* (=*Sulfurospirillum multivorans*, (29)) and *Sulfurospirillum halorespirans* strain PCE-M2 (29) were detected in all TrCDD-dechlorinating microbial communities from Spittelwasser sediment and in one 1,2,4-TrCDD-dehalogenating culture initially inoculated with Mulde sediment ("Mulde1").

Concluding remarks. Different dechlorination pathways might be attributed to different populations of dechlorinating bacteria. However, by our qualitative 16S rDNA detection approach we could not assign a specific dioxin transformation pathway based on the presence of certain phylogenetic groups and physiological properties (*i.e.*, reductive dechlorination) of some of their members. For several reasons, data based on 16S rDNA studies need to be

interpreted with considerable caution. These data alone cannot be used as a predictive indicator of metabolic function and it remains unclear whether or not the bacteria identified by molecular methods are involved in the biologically mediated transformation of dioxins. Thus, the isolation of the concerning bacteria is crucial for the successful study of dehalogenation. The cultures can serve as a source for the isolation of dioxin-dechlorinating bacteria and the results of phylogenetic analyses can be a guide for the isolation procedure. Additionally, it might be useful to evaluate the dehalogenation properties of bacteria clearly detected in this study with respect to reductive dechlorination of dioxins.

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Reductive Dehalogenation of Chlorinated Dioxins by an Anaerobic Bacterium

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Abstract

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs) are among the most notorious environmental pollutants. Some congeners, particularly those with lateral chlorine substitutions at positions 2, 3, 7 and 8, are extremely toxic and carcinogenic to humans[1]. One particularly promising mechanism for detoxification of PCDDs and PCDFs is microbial reductive dechlorination. So far only a limited number of phylogenetically diverse anaerobic bacteria have been found that couple the reductive dehalogenation of chlorinated compounds – the substitution of a chlorine for a hydrogen atom – to energy conservation and growth in a process called dehalorespiration[2]. Microbial dechlorination of PCDDs occurs in sediments and anaerobic mixed cultures from sediments, but the responsible organisms have not yet been identified or isolated. Here we show the presence of a Dehalococcoides species in four dioxin-dechlorinating enrichment cultures from a freshwater sediment highly contaminated with PCDDs and PCDFs. We also show that the previously described chlorobenzene-dehalorespiring bacterium Dehalococcoides sp. strain CBDB1 (ref. 3) is able to reductively dechlorinate selected dioxin congeners. Reductive dechlorination of 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD) demonstrates that environmentally significant dioxins are attacked by this bacterium.

INTRODUCTION

PCDDs and PCDFs are ubiquitous and recalcitrant environmental pollutants[4,5]. Continuing anthropogenic contamination with PCDD/Fs, formed as unwanted by-products of manufacturing and incineration processes, is of great public concern due to the compounds' toxicity and tendency to bioaccumulate and biomagnify in wildlife and humans. Natural sources of dioxins include volcanic activities, forest fires, production by biological systems[6,7] and as yet unknown formation processes[8,9]. Because of their high hydrophobicity, dioxins are strongly adsorbed on organic matter and they therefore accumulate in aquatic sediments and soils, where conditions might be anaerobic. The only known biological process leading to a transformation of the highly chlorinated congeners under anaerobic conditions is the microbially mediated reductive dechlorination observed in microcosms or mixed cultures[10-15]. Different sources of PCDDs and PCDFs introduce different complex mixtures of PCDD and PCDF congeners into the environment. The extent to which intrinsic microbes change these source-specific profiles *in situ* is largely unknown although studies of sediment cores of Lake Ketelmeer, a sedimentation area of the river Rhine in The Netherlands, have shown a change of congener distribution over time[16]. This observation can be taken as an indication that highly chlorinated dioxins are subject to anaerobic dehalogenation processes *in situ*. Our knowledge of the organisms involved in PCDD dechlorination is currently very limited. Until now, no pure culture with the ability to reductively dechlorinate dioxins has been described.

We have previously examined the reductive dehalogenation of selected dioxin congeners by anaerobic mixed cultures[17]. These enrichment cultures were established with various sediment samples from the stream Spittelwasser (Bitterfeld district, Germany), which contains dioxin at concentrations of up to 120,000 pg toxicity equivalents (I-TEQ) per g dry

weight. Spiked 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD) (50 μM) was converted to a mixture of 1,3- and 2,3-dichlorodibenzo-*p*-dioxin (DiCDD). In previous experiments, the transformation pathways were elucidated with subcultures spiked with the possible intermediate trichlorodibenzo-*p*-dioxins (TrCDD) 1,2,4- and 1,2,3-TrCDD. A comparison of data obtained from the cultures with abiotic treatments (autoclaved and uninoculated controls) clearly demonstrated that the dechlorination was mediated by microorganisms[17].

RESULTS

In this study, the cultures from Spittelwasser sediment were transferred six times (10% v/v each time) into synthetic medium, to obtain sediment-free cultures reproducibly dechlorinating TrCDDs. 1,2,4-TrCDD was dechlorinated to 1,3-DiCDD, and 1,2,3-TrCDD was dechlorinated to 1,3-DiCDD and 2,3-DiCDD as observed in the initial cultures. However, in contrast to the initial cultures, most of the later transfers formed more 2,3- than 1,3-DiCDD from 1,2,3-TrCDD, demonstrating that dechlorination in the peripheral (*peri*)-position was now preferred. In addition, 2-monochlorodibenzo-*p*-dioxin (2-MCDD) was detected as the final dechlorination product of both TrCDDs. Most-probable-number analysis detected only about 10^4 dechlorinating bacteria ml^{-1} in the mixed cultures compared with a total cell number of more than 10^7 cells ml^{-1}. We therefore did not attempt isolation of the dioxin-dechlorinating bacterium by conventional methods, *e.g.*, agar-shake dilutions. Instead, a polymerase chain reaction (PCR)-based approach was used to study the presence of several bacteria with known dechlorination potential[2], among them *Dehalococcoides*. This genus comprises two strains with unusual dehalogenation properties: *Dehalococcoides* sp. strain CBDB1 (ref. 3) is the only known bacterium able to dechlorinate chlorinated benzenes, and *D. ethenogenes* strain 195 completely dechlorinates tetrachloroethene to ethene[18]. Using the oligonucleotide primers

DET730 and DET1350 targeting the 16S ribosomal DNA (rDNA) of *Dehalococcoides*, PCR products were obtained from the sixth transfers of two 1,2,3- and two 1,2,4-TrCDD-dechlorinating enrichment cultures. Each of the four sequences had a length of about 600 base pairs (bp). They were identical with each other and also with the sequence of *Dehalococcoides* sp. strain CBDB1 (GenBank accession number AF230641), and shared 98.5% identity with the sequence of strain 195 (AF004928.2).

To substantiate an involvement of *Dehalococcoides* in dioxin dechlorination, the capability of strain CBDB1 to transform TrCDDs was studied. Liquid cultures pre-grown anaerobically on trichlorobenzenes were transferred (5% v/v inoculum) into a completely synthetic anaerobic medium[3] containing 5 mM acetate as a carbon source, hydrogen as an electron donor as described[3], and 25–60 μM 1,2,3- or 1,2,4-TrCDD. 1,2,3-TrCDD was dechlorinated to 1,3-DiCDD, 2,3-DiCDD and 2-MCDD, whereas 1,2,4-TrCDD was dechlorinated to 1,3-DiCDD and 2-MCDD (Fig. 1a, b). The time course of the dehalogenation of 1,2,3-TrCDD (Fig. 1a) showed 2,3-DiCDD as the initial and transient dechlorination product first detected after 14 days of incubation. Small amounts of 1,3-DiCDD and 2-MCDD were detectable after 21 days. Whereas concentrations of DiCDD remained at a low level, 2-MCDD concentrations steadily increased with decreasing levels of 1,2,3-TrCDD, leading to the transformation of about 60 mol% TrCDD to 2-MCDD within 57 days. In cultures to which 1,2,4-TrCDD had been added, the extent of reductive dechlorination was still low at day 21 (Fig. 1b). Although the fraction of 1,3-DiCDD increased rapidly thereafter, production of 2-MCDD was low during the first 46 days. At the end of the experiment (57 days), 37 mol% of TrCDD had been converted to 2-MCDD. Neither 1-MCDD nor non-substituted dibenzo-*p*-dioxin was detected throughout the study.

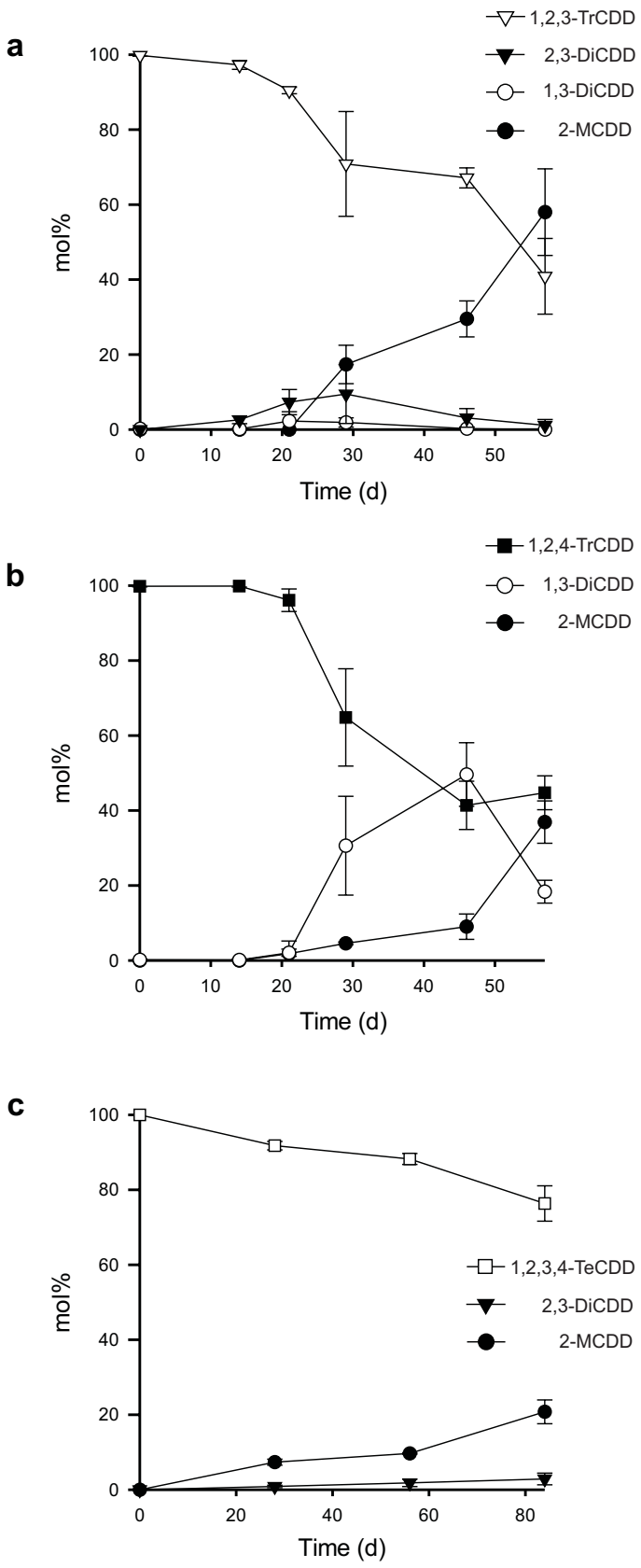

Figure 1 Time course of reductive dechlorination of 25 μM 1,2,3-TrCDD (**a**), 60 μM 1,2,4-TrCDD (**b**) and 46 μM 1,2,3,4-TeCDD (**c**) by *Dehalococcoides* sp. strain CBDB1. Molar distributions of the parent compounds and their dechlorination products are shown. Values are means and s.d. for triplicate samples. No dechlorination products were detected in sterile controls after 75 (**a**, **b**) and 84 days (**c**).

Thus, 2-MCDD constituted the final dechlorination product of both TrCDDs. Autoclaved or uninoculated controls did not show any formation of dechlorinated products.

Three further dioxin congeners available to us were studied for reductive dechlorination by strain CBDB1. 2,3-DiCDD (10 μM) was transformed to 53 mol% 2-MCDD after 28 days of incubation. 1,2,3,4-TeCDD (46 μM) was dechlorinated within 84 days to 3 mol% 2,3-DiCDD and 21 mol% 2-MCDD (Fig. 1c). Traces of 1,3-DiCDD were detected only once (after 56 days). The concentrations of 1,2,3- and 1,2,4-TrCDD were below the detection limit throughout the experiment. 1,2,3,7,8-PeCDD (3 μM) was used as a model compound for dioxins chlorinated on both rings and as a representative of the 17 most toxic PCDD and PCDF congeners substituted at positions 2, 3, 7 and 8. The applied concentration (3 μM) was similar to the total PCDD and PCDF concentration (about 6 μM) determined in Spittelwasser sediment[17]. Pure cultures of strain CBDB1 transformed this compound, albeit slowly (2.8 mol% within 104 days), to 2,3,7,8-TeCDD, 2,7-DiCDD or 2,8-DiCDD (see Methods) and small amounts of 1,3,7,8-TeCDD and 2,3,7-TrCDD (Fig. 2). A control with autoclaved inoculum did not show any product formation from PeCDD.

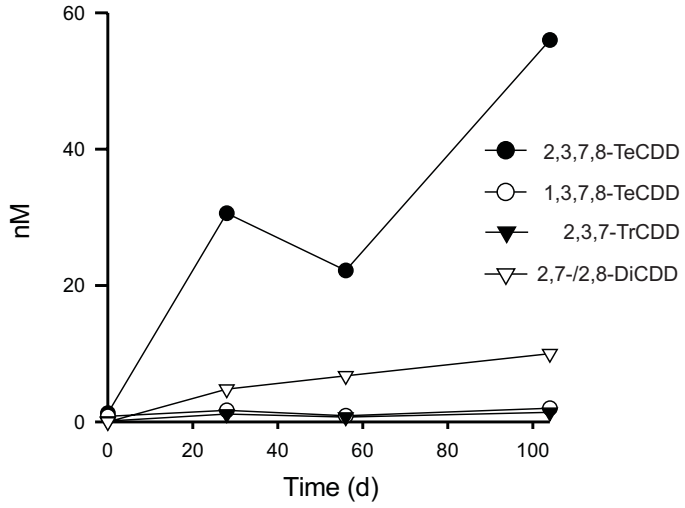

Figure 2 Formation of dechlorination products from 1,2,3,7,8-PeCDD. 1,2,3,7,8-PeCDD was added at a starting concentration of 3 μM. Within 104 days, 2.8 mol% was converted (decrease of PeCDD not shown) to products, which were formed in nanomolar concentrations. Values shown are means for two parallel samples.

To ensure that the reductive dechlorination of dioxins by strain CBDB1 was independent of the presence of chlorinated benzenes originating from the preculture, the cultures were subcultured sequentially (10% v/v each time) with either 1,2,3-TrCDD or with 1,2,4-TrCDD. The initial inoculum for this experiment originated from a culture spiked with 15 μM 1,2,3-trichlorobenzene and 15 μM 1,2,4-trichlorobenzene as the terminal electron acceptors. The cultures could be successfully transferred in synthetic medium with 1,2,3-TrCDD or 1,2,4-TrCDD at least four times (dilution factor 10,000). The dechlorination pattern remained the same through the four consecutive transfers. Adrian *et al*.[3] previously showed that *Dehalococcoides* sp. strain CBDB1 does not grow in the synthetic medium without an added chlorinated electron acceptor. Because maintenance of the cultures was not dependent on the addition of chlorobenzenes, the data support the hypothesis that PCDD congeners are used as respiratory electron acceptors.

DISCUSSION

The dioxin dehalogenation reactions observed with strain CBDB1 are summarized in Fig. 3. 1,2,3,4-TeCDD was predominantely transformed by way of 1,2,3-TrCDD, as suggested by the formation of the subsequent intermediate 2,3-DiCDD. This indicates an initial dechlorination at *peri* positions. Dechlorination of spiked 1,2,3-TrCDD by CBDB1 also proceeded preferentially at the *peri* position. However, both DiCDDs are further dechlorinated either at a lateral or peripheral carbon to 2-MCDD as the final dechlorination product. Spiked 1,2,4-TrCDD was strictly *peri*-dechlorinated by the successive removal of chlorines in positions 1 and 4. Such a dechlorination pattern has also been observed in enrichment cultures from sediment layers from the Spittelwasser[17] and the river Saale[14]. The shift from lateral to *peri* dechlorination of 1,2,3-TrCDD observed in the sixth transfers of the mixed cultures from

Spittelwasser sediment suggests that *Dehalococcoides* sp. strain CBDB1 represented one subpopulation that was enriched by our culture conditions from a greater diversity of dioxin-dechlorinating bacteria in the sediment community.

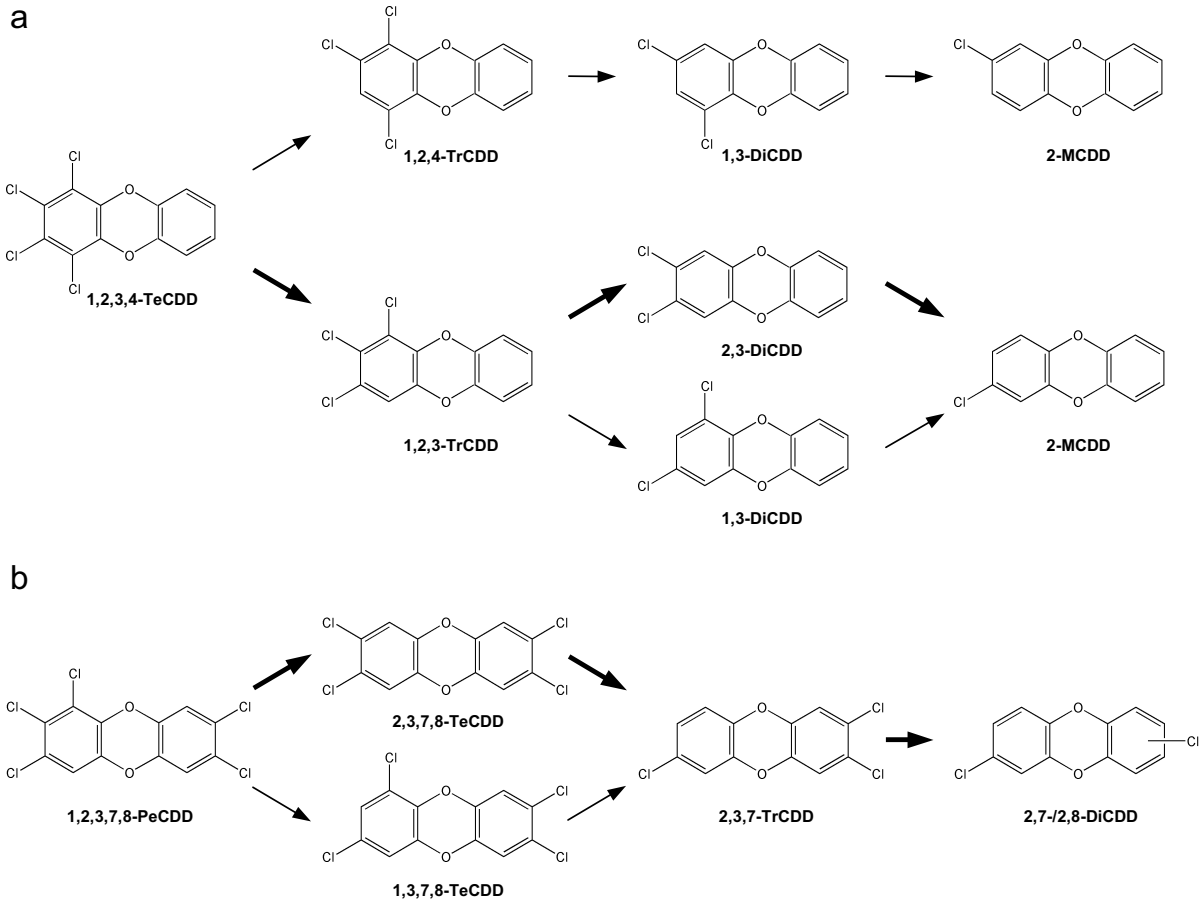

Figure 3 Proposed pathways of reductive dechlorination of spiked 1,2,3,4-TeCDD (**a**) and 1,2,3,7,8-PeCDD (**b**) by a pure culture of *Dehalococcoides* sp. strain CBDB1. The major routes are marked with bold arrows. The results of reductive dechlorination of spiked 1,2,4-, 1,2,3-TrCDD and 2,3-DiCDD are included in pathway **a**. For visualization purposes, identical structures of 1,3-DiCDD and 2-MCDD are shown inverted.

The removal of *peri*-chlorines from higher chlorinated 2,3,7,8-substituted dioxins involves the risk of forming 2,3,7,8-TeCDD, the most toxic dioxin congener (dioxin activation, upper pathway), as indicated earlier by other authors for sediment-derived mixed cultures[13]

and sediment microcosms[15]. However, these same cultures also exhibited dioxin detoxification (lower pathway) by the formation of less harmful TrCDDs and MCDD. This combined *peri*-lateral dechlorination pathway was assigned to non-methanogenic, non-spore-forming bacteria[13].

We used 1,2,3,7,8-PeCDD, the immediate precursor of 2,3,7,8-TeCDD in the upper pathway, to study the dechlorination behaviour of strain CBDB1. It also produced 2,3,7,8-TeCDD but simultaneously the less toxic 1,3,7,8-TeCDD, 2,3,7-TrCDD and 2,7-/2,8-DiCDD. The removal of chlorine atoms from the individual rings of 1,2,3,7,8-PeCDD (as suggested by the products identified) strongly resembles the dechlorination patterns observed with 1,2,3-TrCDD and 2,3-DiCDD. This suggests a transient formation of 2,3,7,8-TeCDD and its further dechlorination through 2,3,7-TrCDD (Fig. 3). With strain CBDB1 it is therefore now possible to analyse and understand the dynamics of microbial transformation of environmentally significant dioxin congeners both in culture and natural environments. This might help to predict a potential transient hazard involved in the overall detoxification process.

On the basis of 16S rDNA sequence, *Dehalococcoides* sp. strain CBDB1 is affiliated to a major subphylum of the phylum *Chloroflexi* (green non-sulphur bacteria). The tetrachloroethene-dehalorespiring bacterium *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1 are so far the only isolated and cultivated representatives of the "*Dehalococcoides*" subphylum[3,18]. For both strains, growth on fermentable substrates or the use of non-halogenated electron acceptors was not observed. Available 16S rDNA sequence information indicates the frequent presence of *Dehalococcoides*-related bacteria in mixed cultures that dehalogenate a variety of chlorinated substances[19-22]. Additionally, two bacteria with distant relatedness to *Dehalococcoides* were described recently as being

responsible for the dechlorination of polychlorinated biphenyls[23,24]. The high genetic potential of this genus for dechlorination reactions is evident from the genome sequence of *Dehalococcoides ethenogenes* (see the TIGR website at http://www.tigr.org/tdb/mdb/mdbinprogress.html). At least 15 reductive dehalogenase-homologue genes were detected in the genome, suggesting that different enzymes are involved in the reductive dehalogenation of different substrates. *Dehalococcoides* is thought to be well adapted for anaerobic reductive dehalogenation, which might be an ancient process for the turnover of naturally produced organohalogens. Owing to their dehalogenation potential, indigenous and introduced organisms of the *Dehalococcoides* cluster are an important addition to the arsenal of organochlorine-transforming microorganisms[2] that are potentially applicable to the bioremediation of contaminated sites containing anthropogenic PCDD.

MATERIALS AND METHODS

Analytical techniques. MCDD, DiCDD and non-chlorinated dibenzo-*p*-dioxin were analysed from the headspace of the cultures by solid phase microextraction (SPME, 100 μm polydimethylsiloxane coated fibers, Supelco). The conventional method, including freeze drying and concentration by evaporation[14] yielded from the same samples only up to 21 % of the 2-MCDD concentration compared with SPME, whereas the DiCDDs were found at similar concentrations. Samples were preconditioned for 2 h at 54 °C; fibres were equilibrated 35 min at 54 °C and desorbed for 195 s (injection port: 260 °C) followed by splitless injection (0.7 min). The Shimadzu GC14A/FID gas chromatograph was equipped with a DB-608 capillary column (30 m x 0.331 mm internal diameter, 0.5 μm film thickness). A six-level external calibration curve from the headspace over minimal medium (amended with the respective dioxin concentrations ranging from 0.78 μM to 25 μM) was generated. On the basis of the

similar molar response within a given homologue group[25], dichlorinated dioxins were quantified with 2,7-DiCDD as a calibration standard. After SPME analysis, the liquid cultures were extracted and analysed for the respective tetrachlorinated and trichlorinated congeners as described[14]. Identification of monochlorodioxins and dichlorodioxins was confirmed on a gas chromatograph with mass-selective detector (GC/MSD; HP6890/HP5973) from mass spectra and the relative retention of authentic standards[14].

For every data point, two or three individual 3-ml cultures were harvested. Heterogeneities of absolute dioxin concentrations, which might have been due to slight differences in starting concentrations, recovery efficiencies and sorption onto differing cell numbers, were normalized by the expression of each congener as mol% of the sum of all congeners detected. 1,2,3,7,8-PeCDD and its dechlorination products were analysed on a GC/MSD after extraction of the 3-ml cultures with toluene, gentle concentration and separation on a SP-2331 column (60 m x 0.25 mm internal diameter, 0.2 μm film thickness) in accordance with standard conditions for dioxin analyses[26]. The relative retention times and response factors were determined by analysing calibration mixtures containing five native congeners (1,2,3,7,8-PeCDD, 2,3,7,8-TeCDD, 1,2,4-TrCDD, 2,7-DiCDD, and 1-MCDD) and five $^{13}C_{12}$-labelled internal standards (1,2,3,7,8-PeCDD, 2,3,7,8-TeCDD, 1,3,6,8-TeCDD, 2,3,7-TrCDD, and 2,8-DiCDF). Before extraction, the five $^{13}C_{12}$-labelled congeners were added and the recovery efficiency compared with that of $^{13}C_6$-1,2,3,4-TeCDD was determined. The recovery efficiency ranged between 75% and 100%. 2,3-DiCDD and 2,7-DiCDD could be identified by relative retention compared with $^{13}C_{12}$-2,3,7-TrCDD, but 2,7- and 2,8-DiCDD were not expected to be separated under these conditions.

Cultivation. Chlorinated dibenzo-*p*-dioxins (amchro, Hattersheim, Germany) were dissolved in acetone and added to each cultivation tube; the solvent was evaporated by using 20% CO_2/80% N_2. The synthetic culture medium[3] was prepared with strict anaerobic techniques. *Dehalococcoides* sp. strain CBDB1 was cultivated under anaerobic conditions with a gas phase of 20% CO_2/80% N_2 in several parallel cultures in 15-ml Hungate tubes (Bellco Glass, Inc., Vineland, New Jersey, USA) containing 3 ml of culture volume sealed with thick butyl rubber stoppers (Ochs Glasgerätebau, Bovenden, Germany), which were best suited to the maintenance of highly reduced conditions over long cultivation times, essential for growth of strain CBDB1. For some experiments Teflon disks were placed below the septa to reduce potential sorption on the stoppers. Recovery of total dioxins decreased over time. After 4 weeks of incubation, recovery ranged between 54% and 92% without Teflon coats, and between 38% and 76% with Teflon coats. The cultures were supplied with 5 mM acetate and hydrogen (2.5 ml injected into the headspace of the Hungate tubes, corresponding to an approximate dissolved hydrogen concentration of 0.1 mM). The consecutive transfers into fresh medium with acetate, hydrogen and TrCDDs were performed every 24 days. The cultures were incubated at 30 °C in the dark and agitated at 115 rpm. A comparison with non-agitated cultures demonstrated that agitation promoted dechlorination. For example, after 56 days, 60 mol% of the 1,2,3-TrCDD was converted in an agitated culture and only 40 mol% in a static culture. Controls were established with autoclaved inocula, and without inoculum.

PCR detection and sequence analysis. Community DNA, extracted from dioxin-dechlorinating enrichment cultures with the use of standard methods, was used as a template for almost complete 16S rDNA amplification with the universal primer pair fD1 and rP2. PCR amplification and DNA sequencing methods have been described previously[27]. Amplified products from the initial PCR were then used as templates in the second

amplification with *Dehalococcoides*-targeted primers DET730 (5′-GCGGTTTT CTAGGTTGTC-3′) and DET1350 (5′-CACCTTGCTGATATGCGG-3′), which were specifically designed with ARB software[28]. Obtained amplicons were sequenced entirely in both directions and analysed as described[27].

ACKNOWLEDGEMENTS

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Enrichment of Dioxin-Dehalogenating Bacteria by a Two-Liquid-Phase System with 1,2,3-Trichlorobenzene

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Abstract

Enrichment cultures capable of 1,2,4-trichlorodibenzo-p-dioxin (1,2,4-TrCDD) dechlorination were derived from dioxin-contaminated Spittelwasser sediments by successive transfers to fresh defined mineral salts medium. The number of dioxin-dehalogenating bacteria was low and represented about 0.007 % of the total cell number. Addition of 1,2,3-trichlorobenzene (1,2,3-TrCB) dissolved in hexadecane to cultures supplied with a mixture of organic acids as electron donors resulted in a dechlorinating culture that transformed 1,2,3-TrCB to 1,3-dichlorobenzene (1,3-DiCB). Amendments with hydrogen instead of organic acids resulted in significantly lower dechlorination activity. The most-probable-number technique demonstrated that during 1,2,3-trichlorobenzene dechlorination, the number of 1,2,4-trichlorodibenzo-p-dioxin dechlorinating bacteria increased tremendously from 2.5 x 10^3 cells ml^{-1} to 1.1 x 10^7 cells ml^{-1}. The latter number accounted for 11 % of the total cell number indicating that 1,2,3-TrCB can serve as an alternative chlorinated electron acceptor for dioxin dechlorinators. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA clone libraries from the initial dioxin-dechlorinating culture and from the two enrichments with trichlorobenzene revealed changes in community composition. Compared to the initial culture, the community structure of the culture containing TrCB plus "organic acids" showed a proportional increase of ten different restriction patterns that might reflect the enrichment of microorganisms with dioxin-dechlorinating capabilities. Two of these patterns (5 and 6) increased remarkably - each accounting for 6 % of the investigated clones - and represented a Dehalococcoides strain

and an organism of the Bacteroidetes phylum, respectively. Inhibition of methanogens enhanced the rate of chlorobenzene dehalogenation. 1,2,3,4-Tetrachlorodibenzo-p-dioxin (1,2,3,4-TeCDD) applied in hexadecane was also rapidly dechlorinated in the absence of methanogens.

INTRODUCTION

Environmental contamination with polychlorinated dibenzo-*p*-dioxins and -furans (PCDDs and PCDFs) has raised significant concern due to their toxicity and persistence in the environment. PCDD/Fs are generally considered as environmental contaminants of anthropogenic origin. Although this is true for many large scale industrial sites all over the world (see http://www.epa.gov/superfund/sites/npl/npl.htm for the U.S. National Priorities List [NPL]), it became evident that pre-industrial (35) and unidentified natural sources exist, e.g. in lake sediments (39), in clay from the U.S. and Germany (38), and in coastal sediments of Queensland, Australia (21). The formation of dioxins and other haloorganic compounds occurs in nature through biogenic (22, 45) or geogenic processes (22) and bacteria may have evolved mechanisms to cope with these substances or even gain energy by metabolizing halogenated compounds (26).

Microbial growth linked to the mineralization of chlorinated dioxins and furans has been demonstrated under aerobic conditions (53). However, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) commonly accumulate under anaerobic conditions; thus, anaerobic subsurface environments constitute the ultimate sinks for PCDD/Fs released into the environment. Within the last decade, research has been conducted to study the ability of anaerobic microorganisms to dehalogenate chlorinated dioxins. Microbially mediated anaerobic biotransformation of dioxins can undergo sequential reductive dechlorination, in

which chlorine removal and substitution with hydrogen produces a reduced organic compound with fewer chlorines. The reductive dechlorination of spiked and historically present dioxins has been documented in a variety of microcosms and cultures from anaerobic freshwater and estuarine sediments (1, 3, 7, 8, 10, 13, 47).

To derive energy from reductive dechlorination during anaerobic respiration, dechlorinating microorganism have been proposed to use PCDD/Fs as terminal electron acceptors by coupling the reaction to the oxidation of organic compounds or molecular hydrogen. Thermodynamic calculations have shown that energy available from reductive dechlorination of PCDD/Fs is sufficient to support the growth of dechlorinating bacteria (28).

A recent study identified several members of a dioxin-dechlorinating community from river Saale sediment and detected different bacteria known for dehalogenation activity (6). Nevertheless, little is known about the overall composition of PCDD-dechlorinating communities and their population dynamics during the transformation of dioxins and other chlorinated compounds. Despite efforts to isolate the responsible organisms, up to now only one pure culture, which was originally isolated as a chlorobenzene dehalorespiring bacterium (2), has been described that grows by the reductive dechlorination of dioxins (12). Such isolation failures might be explained by difficulties in culturing the respective bacteria. Many of the dehalogenating organisms tend to grow slowly or seem to require additional growth factors, such as amino acids, vitamins, or filtered supernatant of preceding cultures (25, 33). However, the main problem in cultivating and isolating dioxin-dechlorinating bacteria are low cell numbers that are primarily due to limitations in the amount of available dioxin in these cultures (43). For example, Ballerstedt obtained a cell number of just 2.5 x 10^3 ml^{-1} for 1,2,4-TrCDD dechlorinating bacteria within a community containing a total cell number of $>10^8$ ml^{-1} (5).

16S rRNA-based molecular approaches are often considered the cultivation-independent method of choice to describe bacterial communities. However, since dechlorinating bacteria are not predominant in most dioxin-dechlorinating communities, often representing less than 1 of 10,000 cells, they may not be easily detected by molecular approaches that are based on conserved primers for initial PCR amplification (e.g., DGGE/TGGE, RFLP/T-RFLP).

One way to overcome these limitations is to increase the cell numbers of dechlorinators by adding alternative halogenated compounds. "Priming" has been used to stimulate PCB dechlorination in general, as well as to selectively enrich distinct PCB dechlorinating populations. Addition of specific PCB congeners such as brominated biphenyls or halogenated benzoates has been shown to stimulate certain types of PCB dechlorination (17, 46, 54). It is presumed that the priming effect can be attributed to enrichment of dechlorinating populations, which metabolize these alternative halogenated electron acceptors. The ability of organohalogens to enrich PCB dechlorinators has been clearly shown by Cho *et al*. (14) and Wu *et al*. (54). Such enrichment is probably due to the fact that many dehalogenating organisms and enzymes are not specific for a single compound and that the added substrates might be more bioavailable due to more favorable physical-chemical properties. In addition, these effects might be caused by the increased bioavailability of freshly added compounds rather than "aged" contaminants from the site.

Reductive dechlorination of highly hydrophobic polychlorinated dioxins is probably a slow process, especially under field conditions, and appears to occur only to a limited extent (9). Therefore, stimulating bacterial dioxin dechlorination is desirable, but an effect of priming on the biotransformation rates of dioxins has only been indicated by a few authors. The ability to reduce chlorinated dioxins can be stimulated by the addition of 2-monobromo-

dibenzo-*p*-dioxin (3) and brominated phenols (47). However, potential effects on the dehalogenating microbial community have not been studied in detail.

Here we show reductive dechlorination of 1,2,3-trichlorobenzene by a dioxin dechlorinating mixed culture. Additionally, we demonstrate that the number of dioxin-dechlorinating bacteria can be increased during cultivation with the more water-soluble substrate 1,2,3-trichlorobenzene, supplied via a hexadecane phase. Using molecular approaches that target the 16S rRNA genes, we report the community structure of the initial 1,2,4-trichlorodibenzo-*p*-dioxin dechlorinating culture prior to exposure to 1,2,3-trichlorobenzene. Furthermore, comparing two derived 1,2,3-trichlorobenzene enrichments with the initial culture, we describe changes in the composition of the dechlorinating communities according to the enrichment conditions. We also study the influence of specific inhibitors of methanogens and Gram-positive bacteria on the dechlorination of 1,2,3-trichlorobenzene, and demonstrate enhanced dechlorination of 1,2,3,4-TeCDD in the two-liquid-phase system.

MATERIALS AND METHODS

Origin of the enrichment cultures. Anoxic PCDD/F-contaminated sediment (0 cm to 10 cm core) was obtained from the creek Spittelwasser site, Germany. The 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD)-dechlorinating enrichment culture was derived from primary slurry microcosms spiked with 50 μM 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (13). The following subcultures were enriched for the ability to dechlorinate 1,2,4-TrCDD and transformed 1,2,4-TrCDD to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DiCDD) and 2-monochlorodibenzo-*p*-dioxin (2-MCDD). Consecutive transfers (10% [vol/vol]) in bicarbonate-buffered defined mineral salts medium amended with acetate, a mixture of organic acids (formate, benzoate, pyruvate, fumarate) as electron donors, and 1,2,4-TrCDD as the

putative electron acceptor yielded stable dioxin-dechlorinating cultures (12). The dehalogenating activity was maintained without the addition of sterilized sediment.

Culture media and growth conditions. Defined, reduced anaerobic mineral salts "medium 204" was prepared as follows: mineral salts were added under a continuous stream of N_2/CO_2 (80%/20%) to deionized water after it had been boiled and cooled to room temperature under N_2/CO_2 (80%/20%) (in grams per liter, unless indicated otherwise): $MgCl_2$ x $6H_2O$, 0.054; $MgSO_4$ x $7H_2O$, 0.068; $CaCl_2$ x $2H_2O$, 0.12; NH_4HCO_3, 0.41; yeast extract (Difco), 0.05; resazurin, 0.001; 1 ml of selenite- and tungstate solution (0.5 g NaOH, 3 mg Na_2SeO_3 x 5 H_2O, 4 mg Na_2WO_4 x 2 H_2O per liter of deionized water); 1 ml of trace element solution SL10 (51). The pH was adjusted to 7.2 using $NaHCO_3$. After autoclaving, 1 ml of filter-sterilized vitamin solution described by Holliger *et al*. (25), modified by the addition of 63 μM of 1,4-naphthoquinone, sodium/potassium phosphate buffer (final concentration 3.33 mM, pH 7.2) and titanium(III)-nitrilotriacetic acid (final concentration, 0.1 mM) was added by syringe. Amorphous ferrous sulfide (11) (0.15 mM, according to McCue *et al*. (34)) together with sodium sulfide (1 mM) promoted dechlorination and was used as a reducing agent for maintaining highly reduced conditions over long time periods. Transformation of trichlorobenzenes (TrCB) was tested using 60 μM of 1,2,3-TrCB and 1,2,4-TrCB, respectively, added directly to the medium from 1 M stock solutions in acetone. These cultures received a 10 % (vol/vol) inoculum from the fourth transfer of the Spittelwasser culture "B (0-10cm)" (13) which had transformed 1,2,4-TrCDD to 2-MCDD. Inoculum autoclaved on three consecutive days was used for preparation of sterile controls, and incubations without the addition of culture material served as chemical controls.

Two-liquid phase cultures were prepared and incubated in autoclaved 60-ml serum bottles containing 30 ml of sterile culture volume and sealed with Viton stoppers and aluminium crimps. The bottles were purged with N_2/CO_2 (80 %/20 %) to remove any residual air prior to the addition of anaerobic medium. Material from the fifth transfer from the Spittelwasser culture described above served as the inoculum (10% (vol/vol)). Finally, 1.5 ml of 200 mM 1,2,3-TrCB dissolved in hexadecane was added, resulting in a nominal concentration of 10 mM 1,2,3-TrCB. Cultures were grown with 5 mM acetate as the carbon source. Electron donors (formate, benzoate, fumarate, and pyruvate) were added from sterile anoxic stock solutions to give initial concentrations of 1.25 mM each in "organic acid enrichment cultures". After 68 days of incubation, another 1.25 mM was added for each of these compounds. For "hydrogen enrichment cultures", 2.5 ml (dissolved hydrogen concentration: 65 μM) of H_2 was added as the electron donor at the beginning and after 68 days. Dissolved hydrogen concentrations were calculated as previously described by Löffler *et al*. (32). All serum bottles were incubated stationary at room temperature in the dark for 35 weeks, during which they were periodically sampled in duplicates (500 μl) and analyzed to determine the extent of dechlorination. For the inhibitor studies, bromoethanesulfonic acid (BES) and vancomycin were added from filter sterilized, anoxic stock solutions in deionized water to final concentrations of 5 mM and 5 mg l^{-1}, respectively. The two-phase cultures were amended with BES, BES plus vancomycin, or no inhibitors and were grown with medium 204, the mixture of organic acids (1.25 mM each), and 5 mM acetate. Preparation of the cultures followed procedures described above; the only exceptions were (i) the two-phase enrichment culture with 1,2,3,4-TeCDD in which a nominal concentration of 3.3 mM of dioxin was supplied, (ii) a control with hexadecane but without chlorinated substrates and (iii) the inoculum (5 % v/v)

which was obtained from the preceding trichlorobenzene plus organic acids enrichment culture.

MPN determination. MPN tubes were spiked with 1,2,4-TrCDD as an electron acceptor with 50-μl Hamilton syringes from stock solutions in acetone to final concentrations of 25 μM (7). The test tubes were supplemented with medium 204 containing 2.5 mM each of formate, benzoate, fumarate, and pyruvate. All MPN tubes contained 5 mM acetate as carbon source. The cultures were incubated in 15-ml Hungate tubes with a total volume of 3 ml for 2 months in the dark at 20°C with shaking (130 rpm), and the headspace was analyzed for dechlorination products of 1,2,4-TrCDD by solid-phase microextraction and gas chromatography as described previously (12). A three-tube MPN procedure was used to estimate the number of dioxin dechlorinators. A dilution series (10^{-1} to 10^{-9} in reduced mineral medium) of each sample was prepared by serially transferring 0.5 ml-portions of the cultures at time point zero and at the end of the experiment (244 days). The MPN test vials were inoculated with 300 μl of each dilution in the 10^{0} to 10^{-9} range. Total cell numbers were determined using fluorescence microscopy (Axioplan, Carl Zeiss, Germany) by counting 4',6-diamidino-2-phenylindole (DAPI)-stained cells. The number of dioxin dechlorinators was estimated by assaying the dechlorination of 1,2,4-TrCDD in the MPN test vials. MPN vials were counted as positive when dechlorination occurred within 8 weeks of incubation regardless of the concentrations of 1,3-DiCDD and 2-MCDD. The instrument detection limit for 1,3-DiCDD and 2-MCDD analysis was below 0.78 μM.

1,2,4-TrCDD, 2,3-DiCDD, 2-MCDD and 1-MCDD were obtained from Amchro (Hattersheim, Germany) at the highest available purities. 1,3-DiCDD was provided by John R. Parsons, Amsterdam, The Netherlands; and authentic standards for 1,2-DiCDD, 1,3-DiCDD and

1,4-DiCDD were provided by Roland Weber, Tübingen, Germany. Chlorobenzenes (99 % purity) were purchased from Sigma-Aldrich (Steinheim, Germany).

Isolation. For isolation of colonies from the trichlorobenzene enrichment cultures, mineral medium 204 was completed by 0.88 % (wt/vol) high-purity Noble agar (Difco, Detroit, MI). The medium contained 5 mM acetate and 5 mM formate. After autoclaving, the temperature of the medium was held at 55°C. Hungate tubes used for the agar shakes were spiked with 7.5 μM 1,2,4-TrCDD (final concentration) and acetone was evaporated under a sterile stream of N_2/CO_2 (80%/20%). Dilution series of the enrichment cultures were prepared after 244 days (see above) and used to inoculate the agar shakes (10 ml total volume). After solidification, 3 ml of hydrogen was added to the headspace of the vials. Single colonies from 10^{-5} to 10^{-8} dilutions were picked after 21 weeks and transferred into 3-ml liquid cultures supplemented with 5 mM acetate, 2.5 ml H_2 and 25 μM 1,2,4-TrCDD. The colonies were picked under a gentle stream of sterile N_2/CO_2 using 1-ml single-use syringes and needles (0.6 x 80 mm, Braun, Melsungen, Germany) that had been flushed with N_2/CO_2. The cultures were analyzed for dechlorination products (12) after 150 days and positive cultures were subcultured with 5 mM acetate, a mixture of 1,2,3-TrCDD and 1,2,4-TrCDD (25 μM each), and hydrogen (2.5 ml) or organic acids (2.5 mM each for formate, benzoate, fumarate and pyruvate) as electron donors.

Chlorobenzene analysis. Hexane extracts were analyzed for 1,2,3-TrCB and its dechlorination products using a Shimadzu GC 14A equipped with a flame ionization detector (FID) connected to a DB608 column (30 m length, 0.331 mm i.d., 0.5 μm film thickness; J&W Scientific; Folsom, CA, USA). The make-up gas was nitrogen, and helium was used as the carrier gas at a flow of 2.2 ml min^{-1}. 2,4-Dichlorotoluene was the recovery standard and

1,3,5-tribromobenzene was used as the internal standard. Chlorinated benzenes were separated using the following temperature program which allowed baseline separation of all dichlorinated benzenes: initial hold at 50°C for 1.1 min, 40°C per min to 70°C (1.9 min), increase to 140°C at a rate of 20°C per min (1.2 min), 40°C per min to 160°C (1.2 min), increase to 220°C at a rate of 25°C per min (0.5 min), 40°C per min to 280°C, final hold at 280°C for 5 min. Injector and detector temperatures were 250 and 300°C, respectively. The chlorobenzenes were identified by matching the retention times with those of authentic standards. Identification of chlorobenzenes was confirmed with a Hewlett-Packard model 6890 gas chromatograph equipped with a HP5973 mass selective detector and a HP-5ms capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies).

Chloride release measurement. Chloride release was measured to monitor dechlorination in the two-liquid phase cultures. Samples (500 μl in duplicates) were periodically withdrawn from the aqueous phase of the cultures by syringe and centrifuged at 9,500 x g for 5 min to remove particles. The supernatants were analyzed for chloride concentration using a Chlor-o-counter MKII (Flohr Instrumenten, Nieuwegein, The Netherlands). The variability among duplicate samples was generally less than 8 %.

Extraction of DNA. Cells were harvested from 1 ml of culture by centrifugation at 9,500 x g for 20 min. The procedure for bead-based cell disruption was essentially performed as described by Kuske *et al*. (31). The resulting community DNA was precipitated with ethanol and sodium acetate (40), purified according to standard procedures (40), and dissolved in sterile deionized water.

Restriction Fragment Length Polymorphism (RFLP). PCR was performed with bacterial domain specific 16S rDNA primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (50). The 16S rRNA genes were amplified using

approximately 50 ng of genomic DNA in reaction mixtures containing 1 x *Taq* PCR buffer (Promega), 1.8 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 210 nM of each forward and reverse primer, and 0.025 U of *Taq* DNA polymerase (Promega) per μl reaction volume. Reaction mixtures were incubated in a "PCR Sprint" thermocycler (Thermo Hybaid, Germany). Conditions for PCR were as follows: an initial denaturation step at 94°C for 2 min was followed by 30 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The last 10 elongation steps were performed with a 20 s time increment per cycle. The resulting PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany), then ligated into the pGEM T-Easy-vector (Promega, Madison, WI, USA), and transformed into *E. coli* XL1-Blue (Stratagene, La Jolla, CA). The cloned 16S rDNA fragments were reamplified from recombinant clones with reaction mixtures similar to those described above, but a primer set specific to the polylinker region of the pGEM T-Easy-vector (PG1f: 5'-TGGCGGCCGCGGGAATTC-3', PG2r: 5'-GGCCGCGAATTCACTAGTG-3'). The PCR reaction was performed using an initial denaturation step at 94°C for 3 min, 30 cycles consisting of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. Fifteen μl of reamplified 16S rDNA (DNA concentration approximately 200 ng/μl) were digested overnight at 37°C with 1.7 units per μl of *Msp* I and *Rsa* I (MBI Fermentas, St. Leon-Rot, Germany). Digested fragments were separated on a 4 % agarose gel (Agarose NEEO, Carl Roth, Karlsruhe, Germany) in 1 x TBE (40).

Sequence analysis of 16S rDNA clone libraries. Cycle sequencing was performed using the automated laser fluorescence DNA sequencer ABI Prism 377, version 4.0 (PE Applied Biosystems, Langen, Germany), universal bacterial primers that recognized conserved regions of the 16S rDNA (41, 50), and the vector-specific primers PG1f and PG2r. Closest relatives were identified using the Fasta33 program at EMBL-EBI and RDP's Sequence Aligner.

Obtained sequences were checked for chimeras using RDP's Chimera Check and the *Bellerophon* server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl). Sequences were imported and aligned in the ARB software package (http://www.arb-home.de/) and added to an alignment of about 8600 bacterial sequences (ARB alignment by Philip Hugenholtz, January 2002, http://rdp.cme.msu.edu/html/alignments.html) for phylogenetic tree reconstruction. For addition to the maximum parsimony tree, filters excluding the most variable positions and terminal sequences were used. Phylogenetic analyses were performed using maximum parsimony and maximum-likelihood methods.

Nucleotide sequence accession numbers. GenBank accession numbers for the sequences used to construct phylogenetic trees are as follows: *Clostridium lituseburense* ATCC 25759^T, M59107; *Clostridium putrefaciens* DSM 1291^T, Y18177; *Cytophaga fermentans* ATCC 19072^T, M58766; *Dehalococcoides ethenogenes* 195, AF004928.2; *Dehalococcoides* sp. CBDB1, AF230641; *Desulfonema limicola* DSM 2076^T, U45990; *Sedimentibacter saalensis* ZF2^T, AJ404680; *Synergistes jonesii* ATCC 49833^T, L08066; *Syntrophus gentianae* HQgö1^T, X85132; *Trichococcus pasteurii* KoTa2^T, X87150; strain S2551, AF177428; clone IIIB-28, AJ488099; clone BA053, AF323776; clone DCE29, AJ249260; clone DCEH2, AJ249262; clone SHA-300, AJ249112; clone SJA-58, AJ009468; and clone WCHB1-69, AF050545.

The determined 16S rRNA gene sequences representing RFLP types 1, 2, 3, 4, 5, 6, 7, 10, 14, and 26 were submitted to GenBank under the accession numbers ... .

RESULTS

Chlorobenzene dechlorination. Enrichment cultures established with material from the fourth serial transfer of an actively 1,2,4-TrCDD-transforming culture from Spittelwasser sediment showed complete reductive dechlorination of 60 μM 1,2,3-trichlorobenzene

(1,2,3-TrCB) to 1,3-dichlorobenzene (1,3-DiCB) within 16 weeks; however, dechlorination of 1,2,4-trichlorobenzene did not occur over this same time period. The dechlorination pathways for 1,2,4-TrCDD and for 1,2,3-TrCB are shown in Fig. 1. Controls without inoculum or with autoclaved inoculum did not show any significant dechlorination of trichlorobenzenes.

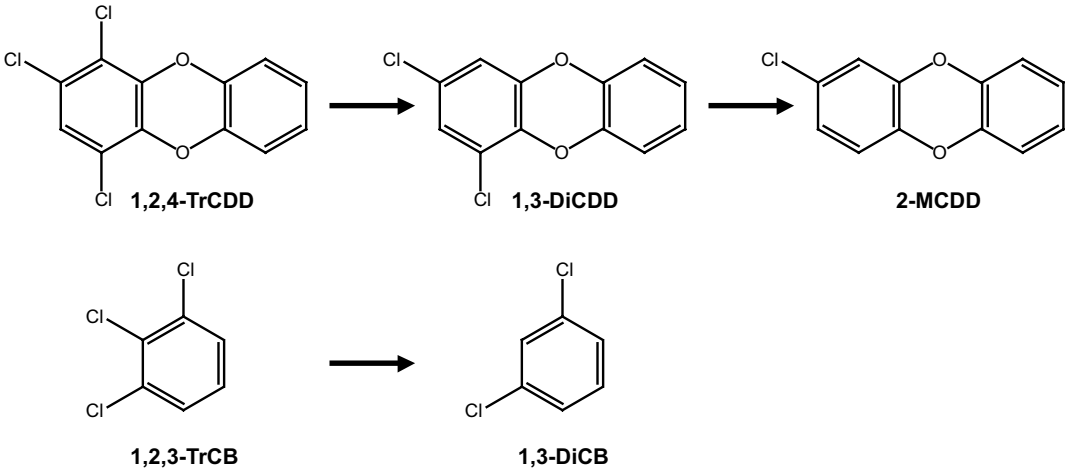

Figure 1 Pathways for reductive dechlorination of 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) in cultures from Spittelwasser sediment and of 1,2,3-trichlorobenzene (1,2,3-TrCB) observed in the following enrichment cultures. DiCDD, dichlorodibenzo-*p*-dioxin; MCDD, monochlorodibenzo-*p*-dioxin; DiCB, dichlorobenzene.

A two-phase-system originally described by Holliger *et al*. (25) was used to study the effect of 1,2,3-TrCB dechlorination on dioxin dechlorinating bacteria from the fifth transfer of a 1,2,4-TrCDD-dechlorinating Spittelwasser culture. 1,2,3-TrCB (200 mM) was supplied in hexadecane, providing a constant flow of chlorinated substrate to the dechlorinating bacteria and avoiding inhibitory effects caused by high concentrations of chlorinated benzenes. The nominal 1,2,3-TrCB concentration corresponded to 10 mM.

Two enrichment cultures were established. One culture received organic acids, while the other received hydrogen as electron donor. Dechlorination was qualitatively checked for the presence of dechlorination products in the water phase and was quantitatively monitored by

measuring the chloride concentration. After 168 days, 4.2 mM chloride was released into the aqueous phase of the culture containing organic acids corresponding to the conversion of 42 mol % of 1,2,3-TrCB to 1,3-DiCB (Fig. 2). 1,3-DiCB was also found as the dechlorination end product in cultures that received H_2 as the electron donor. The lag time before dechlorination started was equal to that of the organic acids-amended enrichment culture (Fig. 2), but 1,2,3-TrCB dehalogenation was slower and only approximately half the amount of chloride was released after 168 days. At the final sampling point after 244 days, 5.5 and 2.6 mM of chloride were released into the aqueous phase of the organic acids culture and hydrogen culture, respectively.

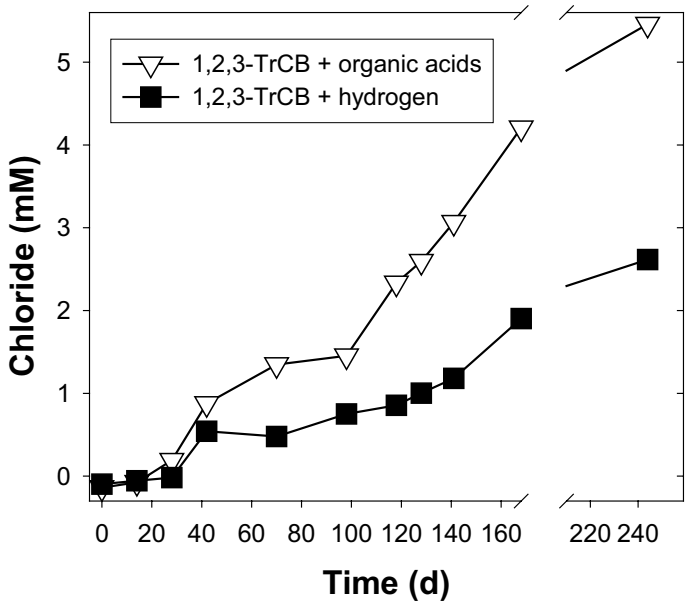

Figure 2 Time course of reductive dechlorination of 1,2,3-trichlorobenzene to 1,3-dichlorobenzene by the two-phase enrichment cultures. Each data point represents the mean of duplicate samples. Samples for MPN analysis and RFLP analyses were taken at day 0 and after 244 days.

MPN estimation. The number of 1,2,4-TrCDD dechlorinating bacteria was estimated in the chlorobenzene-amended two-phase culture containing organic acids by the MPN technique in the beginning and after 244 days. Dechlorination of the spiked compound 1,2,4-TrCDD

followed the previously observed sequential removal of chlorine, and 1,3-DiCDD and 2-MCDD were the dechlorination end products after 2 months of incubation. The initial level of 1,2,4-TrCDD dechlorinators increased by 3 orders of magnitude, from 2.5 x 10^3 to 1.1 x 10^7 cells ml^{-1}. The total number of cells increased from initial 3.7 x 10^7 to 1.0 x 10^8 cells ml^{-1} indicating that the abundance of dioxin dechlorinators in the community had increased from 0.007 % to 11 %.

Community structure. Restriction fragment length polymorphism (RFLP) analysis (Fig. 3) and sequencing of amplified 16S rDNA was used to study the community composition of the two trichlorobenzene enrichment cultures and of the 1,2,4-TrCDD-spiked culture which served as the inoculum. A total of 295 clones containing 16S rDNA inserts were analyzed and 39 different patterns were identified. Selected cloned fragments were sequenced. Seventeen of these patterns appeared only once in one of the libraries and suggested that the respective organisms may not be essential for the dechlorination process.

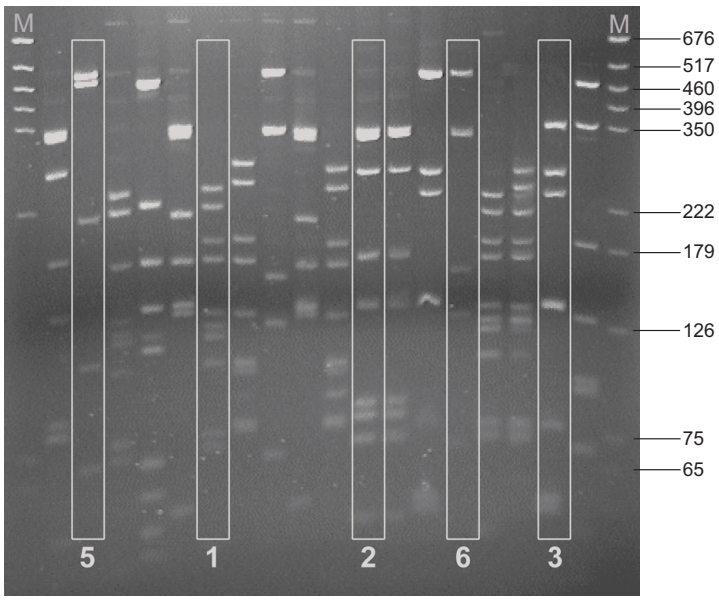

Figure 3 RFLP analysis of 16S rDNA inserts of 18 clones selected from the clone library obtained from the trichlorobenzene-dechlorinating culture grown with organic acids. The predominant restriction patterns 1, 2 and 3 as well as pattern 5 and 6 are highlighted (for phylogenetic assignment see Fig. 5). The fragments (≤ 676 base pairs) of the size standard pGEM-DNA marker/*Hinf* I, *Rsa* I, *Sin* I (Promega) are shown in lanes "M".

The clone libraries were dominated by three sequence types, represented by the restriction patterns 1, 2 and 3 (Fig. 4). The numerically dominant restriction pattern was pattern 2 (up to 49 % of the clone library from the organic acids enrichment). The 16S rDNA sequence of the pattern 2 representative shared 99.9 % identity with *Trichococcus collinsii* strain 37AN3 (GenBank accession no. AJ306612) and 99.8 % with *Trichococcus pasteurii* strain KoTa2^T, formerly described as *Lactosphaera pasteurii* (30). A similar *Trichococcus* strain, designated Coc4, was isolated previously from organic acids amended dioxin-dechlorinating subcultures from the Saale River but showed no dioxin-transforming activity (6). However, these bacteria are known to ferment pyruvate to formate and acetate (30).

RFLP type 1, the second most predominant sequence type, accounted for about 16 % in the clone library of the initial culture and 15 % in the library of the trichlorobenzene plus organic acids enrichment. Interestingly, this sequence type was not observed in the clone library from the hydrogen enrichment. The 16S rDNA sequence was 98 % identical to the sequence of *Syntrophus gentianae* strain HQgö1 (49). Physiologically, organisms of the genus *Syntrophus* are known to obtain energy from the anaerobic oxidation of benzoate (e.g. (49)) and other organic acids (29) to acetate and hydrogen in syntrophic association with hydrogen-consuming partners. Pattern 3, the last of the most abundant RFLP types, represented a bacterium most closely related to *Clostridium lituseburense* ATCC 25759 (M59107). The 16S rRNA genes shared 97 % identity.

RFLP and sequence analysis, performed using double-stranded 16S rRNA gene sequencing, revealed shifts in the community composition of the trichlorobenzene-spiked culture as compared to the initial culture grown with 1,2,4-trichlorodibenzo-*p*-dioxin. Despite the dominance of patterns 2 and 3, the three libraries were markedly different from each other (Fig. 4). Enriched sequence types might represent organisms potentially involved in

chlorobenzene dechlorination, which could also explain the increased cell number of 1,2,4-TrCDD dechlorinators during chlorobenzene cultivation found by MPN analysis. Comparing the clone libraries of the initial dioxin-dechlorinating culture and the organic acids plus 1,2,3-TrCB enrichment, the relative abundance of ten patterns (2, 4, 5, 6, 7, 10, 11, 14, 16, and 26) increased during cultivation with trichlorobenzene.

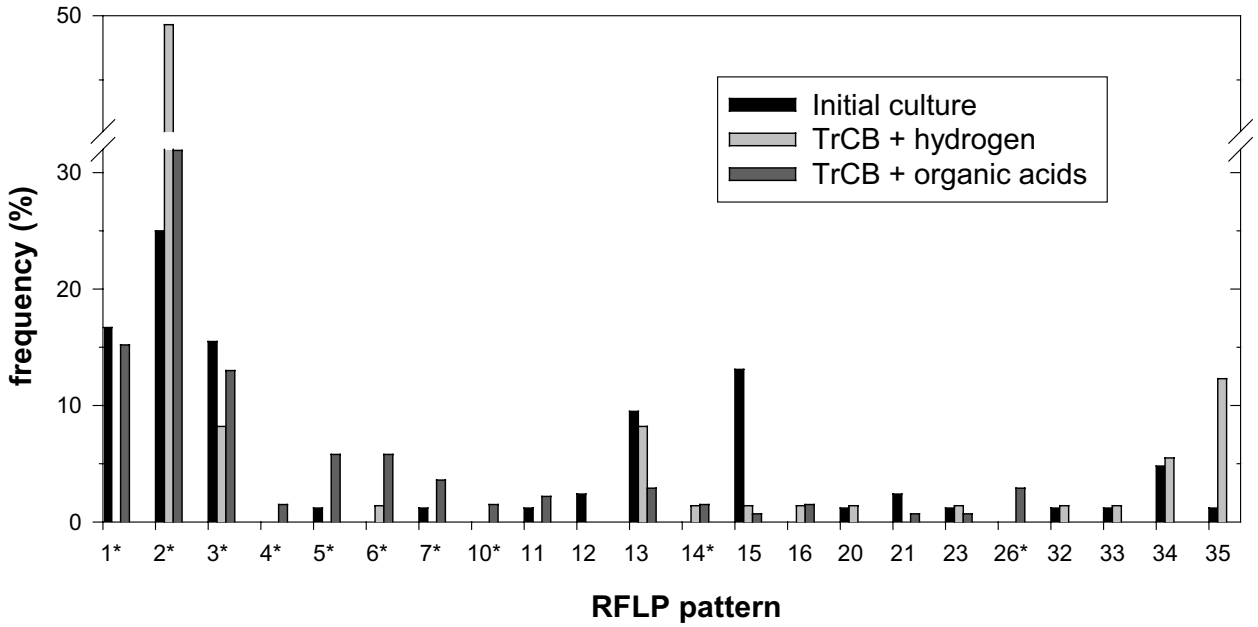

Figure 4 Community structure of the trichlorobenzene-dehalogenating two-phase enrichment cultures and of the 1,2,4-TrCDD-dechlorinating initial culture. Each column represents the relative abundance of each RFLP type in the respective clone library. A total of 295 randomly chosen clones (organic acids enrichment: 138 clones, hydrogen enrichment: 73 clones, initial culture: 84 clones) was analyzed by restriction fragment length polymorphism (RFLP). *The respective 16S rDNA inserts were entirely sequenced. For phylogenetic affiliation see text and Fig. 5.

The phylogenetic affiliation based on full length sequences of the three predominant and seven enriched 16S rDNA sequence types is shown in Fig. 5. The enriched patterns 11 and 16 were not included in Fig. 5 because only partial 16S rDNA sequences were available. Both 16S rDNA sequence types were similar to members of the genus *Clostridium*; the sequence of pattern 11 was related to *Clostridium putrefaciens* DSM 1291 (92 %, AF127024) while pattern

16 showed similarity to *Clostridium hastiforme* DSM 5675 (95.5 %, X80841). Sequences representing patterns 4 and 26 were also found to be most closely related to bacteria of the *Clostridiaceae*. The 16S rDNA sequence from pattern 14 organisms aligned closely with clone sequences of uncultured bacteria of the *Deferribacteres* phylum including bacterium DCE 29 (98 %) obtained from a chloroethene-dehalogenating mixed culture. *Synergistes jonesii* was the closest cultivated relative (86 % identity, L08066). Sequences of clones representing pattern 7 grouped with members of the δ-*Proteobacteria*, whereas the 16S rDNA from pattern 10 showed the highest sequence similarity to unidentified green non-sulfur bacteria found in cultures capable of dehalogenating chlorinated compounds such as 1,2-dichloropropane (42) and trichlorobenzene (48).

Four of the ten restriction patterns that were enriched in the organic acids culture increased also in the hydrogen-fed culture (Pattern 2, 6, 14, and 16; Fig. 4). One pattern, designated pattern 35, was exclusively enriched in the hydrogen culture.

The abundance of pattern 5 inreased remarkably accounting for nearly 6 % of all sequence types found in the organic acid culture. The insert of one clone was completely sequenced on both strands. It showed a 16S rRNA gene sequence identical to that of *Dehalococcoides* sp. strain CBDB1 and strain FL2, except for one mismatched base. Sequences of eight additional clones could not confirm the sequence difference at this position suggesting that the mismatch was most likely caused by PCR artifacts. Therefore, we conclude that the 16S rRNA genes represent a single *Dehalococcoides* population. The novel *Dehalococcoides* strain, designated DCMB5, belongs to the Pinellas group, a phylogenetic branch within the *Dehalococcoides* group (green non-sulfur bacteria) (23).

Similarly to pattern 5, pattern 6 became one of the most numerous sequence types in the trichlorobenzene plus organic acids culture. Its abundance increased to 6 % of all clones

examined in this culture, whereas the RFLP type was not encountered in the library from the initial culture. The 16S rDNA showed highest similarity (99 %) to an uncultured bacterium of the *Bacteroidetes* phylum. Clone IIIB-28 (AJ488099) was derived from a bacterial consortium removing predominantly singly flanked chlorine substituents from chlorobenzenes. The 16S rDNA sequence grouped also with other sequences of uncultured "Flexibacteraceae" obtained from contaminated sites including clone WCHB1-69 (97.0 %) (18) and from dehalogenating mixed cultures including clone SHA-5 from a 1,2-dichloropropane-dechlorinating culture (91 % identity, AJ306736). The next cultivated relatives were members of the *Sphingo-bacteriaceae* (*Pedobacter saltans*, 85 %, AJ438173.2) and "Flexibacteraceae" (*Cytophaga fermentans*, 84.5 %, M58766).

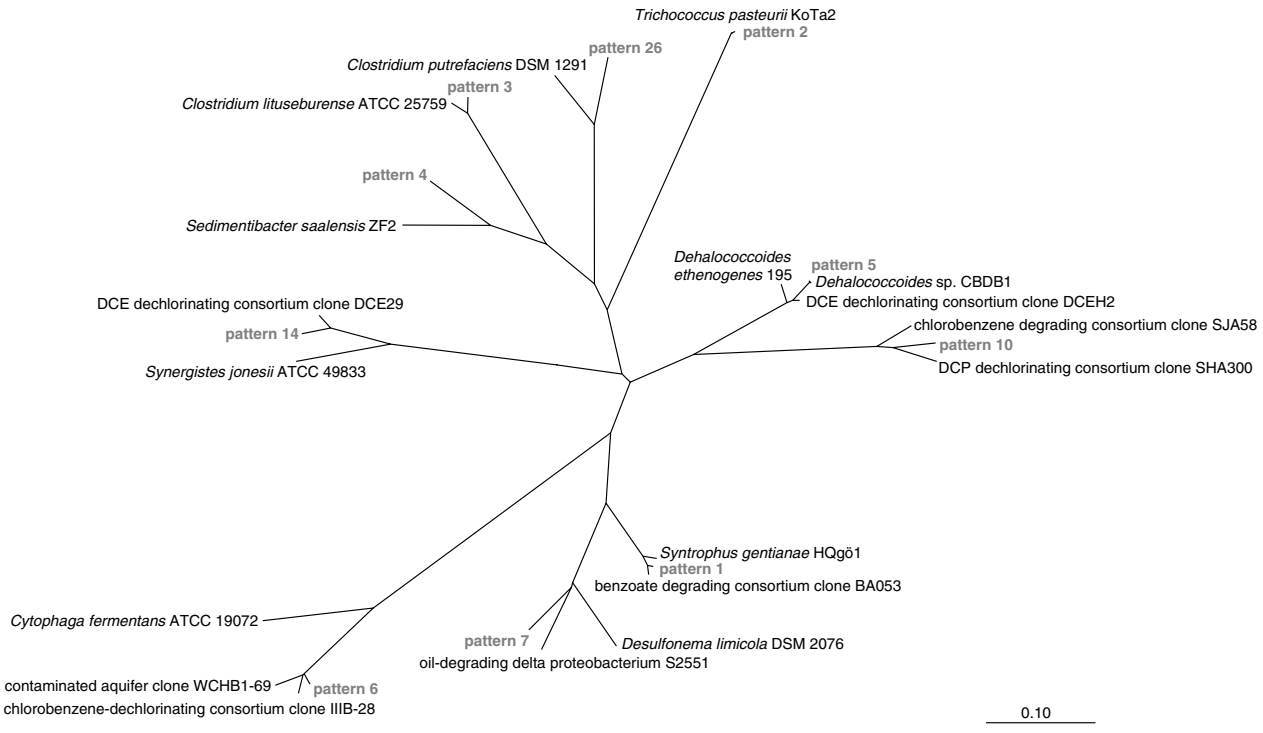

Figure 5 16S rRNA phylogenetic tree of predominant sequence types (pattern 1, 2, and 3) and enriched RFLP types in the clone library from the 1,2,3-trichlorobenzene plus organic acids enrichment, expanded with reference sequences of representative bacteria. The tree is based on the results of a maximum-parsimony analysis, as implemented in the ARB software package. A similar tree topology was generated for a phylogenetic tree constructed using maximum-likelihood methods. Bar = 0.1 base changes per position.

Isolation Efforts. Attempts to isolate the dioxin-dechlorinating population(s) by cultivating in solid medium containing 0.88 % (w/v) high-purity agar were performed with inoculum from the two 1,2,3-trichlorobenzene enrichments after 244 days of cultivation. One hundred twenty-five single colonies were picked from 10^{-5} to 10^{-8} dilutions of the original enrichment cultures and were transferred back into liquid medium spiked with 25 μM 1,2,4-TrCDD. Dechlorination activity of 1,2,4-TrCDD in defined basal salts medium amended with hydrogen and 5 mM acetate was recovered from 4 colonies derived from the organic acids culture at dilutions of 10^{-5}, 10^{-6} and 10^{-7}. Only one of them could be successfully transferred into a second liquid subculture with hydrogen and acetate. It transformed a mixture of 1,2,3- and 1,2,4-TrCDD exclusively to 1,3-DiCDD. 2-Monochlorodibenzo-*p*-dioxin was not detected as a dechlorination product. To describe the microbial constituents of the actively dechlorinating culture, we conducted an RFLP approach. The community consisted of at least six different populations. The previously detected RFLP pattern 6 was represented by 11 % of 37 clones examined. Patterns 2, 3, 16, 26 and 32 were also identified. Attempts to detect *Dehalococcoides*-like microorganisms by RFLP in the dechlorinating cultures from agar shakes have failed so far, probably due to the fact that this bacterium cannot grow in semisolid medium with agar. In this regard, it should be noted that Adrian *et al*. (2) reported dichlorobenzene formation for strain CBDB1 only in soft media containing 0.3 % of a low melting agarose, but not in media with standard agarose or with agar.

Contribution of methanogens and Gram-positive bacteria to the dechlorination process. Strong F_{420} fluorescence was observed (data not shown) in the two-liquid phase cultures indicating the presence of methanogens. The lower dechlorination rate in the hydrogen-amended enrichment culture (Fig. 2) suggested competition for hydrogen between dechlorinating bacteria and methanogens or other hydrogen-consuming bacteria.

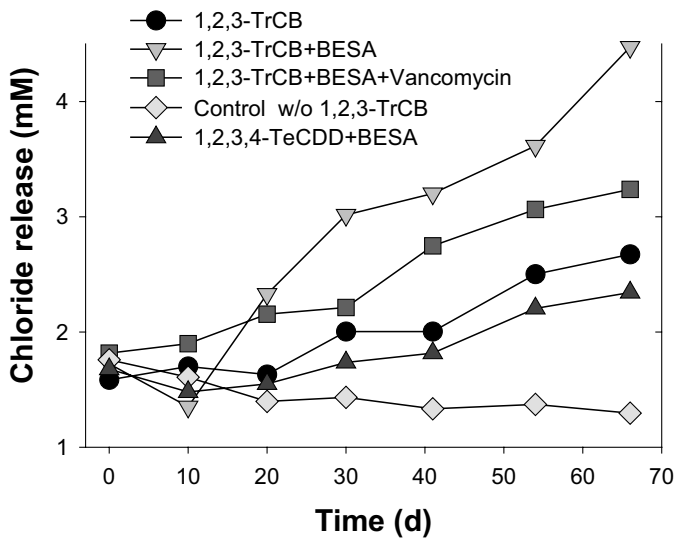

Figure 6 Reductive dechlorination of 1,2,3-TrCB (10 mM) and 1,2,3,4-TeCDD (3.3 mM) in two-liquid phase cultures amended with BES and vancomycin as indicated, and organic acids as electron donors. The inoculum (10 % vol/vol) originated from the "organic acids" plus 1,2,3-TrCB enrichment culture.

To specifically inhibit hydrogenotrophic populations, bromoethanesulfonic acid (BES) was added either separately or in combination with vancomycin to a set of two-liquid phase cultures grown with the organic acid mixture and 1,2,3-trichlorobenzene. The preceding organic acids enrichment was used as inoculum. Suppression of methanogenesis by BES resulted in an enhanced dechlorination rate as compared with that of the control (Fig. 6). However, the addition of vancomycin partly reversed the stimulating effect. Another two-liquid phase culture was amended with BES and received a nominal concentration of 3.3 mM 1,2,3,4-tetrachlorodibenzo-*p*-dioxin instead of 10 mM trichlorobenzene (Fig. 6). The release of chloride at a rate of about 16 μmol chloride l^{-1} d^{-1} between day 10 and 66 from the dioxin molecule was apparent and demonstrated that the ability to dechlorinate dioxins was not lost during the two-liquid-phase cultivation with trichlorobenzene.

DISCUSSION

Reductive dechlorination of different chlorinated substrates including tetrachloroethene, chlorinated benzenes (6) and chlorinated biphenyls (data not shown) by dioxin-dechlorinating mixed cultures has been tested in our laboratory. 1,2,3-Trichorobenzene was found to be rapidly transformed by dioxin-dechlorinating mixed cultures from Spittelwasser sediment. 1,2,3-TrCB is by three orders of magnitude more soluble in water than the trichlorinated dioxin model compound 1,2,4-TrCDD (25, 43). Due to its partition coefficient between hexadecane and water ($\log K_{HW}$ 4.04; (25)), about 20 μM of 1,2,3-TrCB were expected to have partitioned into the water phase. The application of dioxins via a decane phase was described by Barkovskii and Adriaens (8). The authors demonstrated that dioxins - despite their higher logs of partition coefficients (e.g., $\log K_{Octanol/Water}$ of 1,2,4-TrCDD is 6.35; (43)) are available for reductively dechlorinating bacteria in a biphasic system. However, high costs and the toxicity risk of dioxins might have so far precluded application of dioxins in the millimolar range via a solvent phase to enrich for dioxin-dehalogenating bacteria. Here, we exemplarily demonstrated that 1,2,3,4-TeCDD dissolved in hexadecane served as a substrate for dechlorination with a very high rate, which exceeded 300fold the rates reported for dechlorination of 1,2,3,4-TeCDD supplied as undissolved crystals (7, 10). Nevertheless, dechlorination of 1,2,3-TrCB was four times faster, accounting for about 50 μmol chloride l^{-1} d^{-1}.

Our results show that the number of dioxin-dechlorinating microorganisms increased significantly during 1,2,3-TrCB cultivation. Thus, it appears that at least some populations utilizing chlorobenzenes are also involved in dioxin dechlorination. This observation indicates that trichlorobenzene dechlorination is directly coupled to the growth of dechlorinating microorganisms and is consistent with the hypothesis that these dehalogenating organisms also consume dioxins as part of their energy metabolism by using dioxins as terminal electron

acceptors. Comparable results were obtained for PCB dechlorination where the addition of structurally similar (54) as well as structurally non-related halogenated compounds (14) promoted growth of PCB dechlorinators.

The increased number of dioxin dechlorinators should be represented by changes in the community composition. The parent dioxin-dechlorinating and the derived chlorobenzene-dechlorinating mixed cultures were compared by RFLP of 16S rDNA clone libraries. The organic acids enrichment had received the same organic acids, but at a somewhat reduced total amount (15 vs. 25 mM) than the parent culture. Despite comparable growth conditions employed in both cultures, a striking difference existed between the concentration of the halogenated electron acceptors (nominal concentration 10 mM of 1,2,3-TrCB vs. 25 μM of 1,2,4-TrCDD). Since anaerobic oxidation of hexadecane is only known under sulfate-reducing or denitrifying conditions (15, 19, 44), its contribution as an electron donor might be negligible in our cultures. Therefore, we conclude that the enriched sequences represent organisms that might be involved in chlorobenzene transformation and might be associated with dioxin-dechlorinating activity as well.

Two populations were enriched remarkably in the presence of 1,2,3-TrCB in the more active organic acids culture. Based on the 16S rDNA, the new *Dehalococcoides* strain is closely related to the strains FL2 and CBDB1. *Dehalococcoides* sp. strain CBDB1 is the first described bacterium capable of dehalogenating dioxins and chlorobenzenes in pure culture, but preliminary results obtained with *Dehalococcoides ethenogenes* strain 195 indicate that this capability is probably more distributed throughout the *Dehalococcoides* cluster (20). Whereas strains DCMB5 and CBDB1 belong to the Pinellas group, strain 195 is a member of the Cornell group of *Dehalococcoides* (23), indicating that specific dehalogenation capabilities cannot be used for classification of *Dehalococcoides* species. The second bacterium belongs to

a group poorly represented by cultivated organisms within the *Sphingobacteria* lineage of the *Bacteroidetes* phylum. It shares 16S rDNA similarity with an unidentified bacterium (AJ488099) detected in another chlorobenzene dechlorinating culture and with other unidentified bacteria including sequences that have been found in several dechlorinating communities. Physiological predictions on the basis of phylogenetic affiliation alone are highly speculative; thus, attempts to infer physiological properties from rDNA sequences might fail, even when comparing species that are very closely related by 16S rRNA genes. However, studying relative changes in the abundance of 16S rDNA sequences in clone libraries, we demonstrated that distinct bacteria were enriched within the communities. The observed changes in community structure suggest a link between the 16S rDNA data and the physiological properties of the cultures. It is very likely that strain DCMB5 is involved in reductive dechlorination of chlorobenzenes and dioxins. It remains to be determined whether the pattern 6 bacterium participates directly in the dechlorination process or rather plays a role in cosubstrate utilization.

1,2,3-Trichlorobenzene was reductively dechlorinated to 1,3-dichlorobenzene in both enrichment cultures, although the addition of H_2 resulted in lower transformation rates compared to the culture amended with a mixture of organic acids. In principle, hydrogen is thought to be the prevailing electron donor for the reductive dechlorination process. Thus, it was surprising that substitution of organic acids with hydrogen in the two-phase cultures resulted in a decreased dechlorinating activity (Fig. 2). Similar inhibiting effects of higher hydrogen concentrations on the reductive dechlorination of dioxins and PCBs were also observed by Ballerstedt *et al*. (6) and Wiegel and Wu (52), respectively. The PCB-dechlorinating bacterium *o*-17 was even lost from the culture under a high H_2 partial pressure (16). The direct application of hydrogen at high initial levels might favor the growth

of acetogenic bacteria and methanogens, which are efficient competitors for hydrogen, especially at higher concentrations (32). This may limit access to hydrogen for the dehalogenating bacteria, but probably also interferes with the availability of other essential constituents of the medium such as vitamins and trace elements. Circumstantial evidence for an involvement of methanogens in cosubstrate or electron donor utilization (*i.e.* hydrogen and acetate) came from the observation that addition of BES as an inhibitor of methyl-coenzyme M reductase strongly enhanced the dechlorination activity. In our mixed cultures, the fermentation of organic acids might have supplied a suitably slow and continuous release of hydrogen. Clostridia and related bacteria, which are known to ferment pyruvate or fumarate were identified by 16S rDNA sequences (patterns 14, 16, 26) in one or both trichlorobenzene-enriched cultures. The continuous formation of hydrogen is beneficial for dehalogenating bacteria, which possess a high affinity for hydrogen at nanomolar concentrations (32) and can compete for it immediately after its release.

Since H_2 production from organic acids such as benzoate and acetate is thermodynamically favourable only at a low hydrogen partial pressure, this reaction must be coupled to the consumption of hydrogen by hydrogen-scavenging bacteria. Due to the low hydrogen threshold, dechlorinators constitute ideal hydrogenotrophic bacteria in such syntrophic relationships by increasing the energy yield for both partners. *Syntrophus*-like organisms were detected in the more active organic acids enrichment using our 16S rDNA approach. Strains of *Syntrophus* are capable of syntrophic benzoate-oxidation and have been isolated from a number of stable consortia (4, 27, 37, 49). In addition, *Syntrophus* sp. have been found in several organochlorine-contaminated aquifers (18) and dehalogenating consortia (36) and it was argued that they played an important role as syntrophic partners for reductive dechlorination. In our study, the *Syntrophus* species was completely lost in the H_2-fed culture.

Furthermore, in contrast to the more active organic acids culture, restriction pattern 5 could not be found in the clone library of the hydrogen-amended culture. Therefore, it can be speculated that with organic acids as electron donors, a well-balanced syntrophic association between hydrogen-utilizing bacteria (possibly dechlorinators) and organic acids-oxidizing, hydrogen-producing organisms existed, which resulted in a productive 1,2,3-trichlorobenzene dechlorination. These results may emphasize the involvement of a syntrophic network for such transformation reactions and might further underline the difficulties in isolating dioxin-dechlorinating bacteria from dechlorinating communities. Our results indicate that growth stimulation of PCDD dechlorinating bacteria is possible using trichlorobenzene. This might aid future attempts to enrich and isolate dioxin-dechlorinating bacteria from mixed cultures. It also highlights the potential of priming reductive dechlorination, although alternative less toxic compounds that are acceptable for application to dioxin contaminated sites remain to be discovered.

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Studying the Microbial Dynamics of a Trichlorobenzene-Dechlorinating Community by Single-Strand Conformation Polymorphism

Michael Bunge and Ute Lechner

Abstract

In this study, single-strand conformation polymorphism (SSCP) and sequencing of 16S rDNA fragments were conducted to describe the microbial dynamics of a 1,2,3-trichlorobenzene-dehalogenating community, which was cultivated in the presence of organic acids as electron donors and carbon sources. During reductive dechlorination of 1,2,3-trichlorobenzene applied via a hexadecane phase, the banding pattern, which was first dominated by clostridial sequences became more diverse. SSCP bands representing bacterial 16S rDNA sequences (such as Syntrophus and an organism of the Bacteroidetes phylum, Dehalococcoides and Trichococcus species) previously detected by restriction fragment length polymorphism (RFLP) after 8 months of cultivation, became already visible after 4 to 7 weeks. Our approach demonstrated the presence of a Trichlorobacter species, which had not previously recognized by RFLP and sequencing. The corresponding 16S rDNA fragment became more predominant during the course of 1,2,3-trichlorobenzene dechlorination and argues for an important role of these bacteria within the dechlorinating community.

INTRODUCTION

We have recently used a two-phase hexadecane-water system for the cultivation of 1,2,3-trichlorobenzene- (1,2,3-TrCB) dechlorinating communities (2). The most-probable-number technique has been applied to describe the effect of 1,2,3-trichlorobenzene (1,2,3-TrCB) on growth of the dioxin-dehalogenating population. The number of 1,2,4-trichlorodibenzo-*p*-dioxin- (1,2,4-TrCDD) dechlorinating bacteria has been shown to increase during dehalogenation of 1,2,3-TrCB to 1,3-dichlorobenzene (1,3-DiCB). Clone libraries of 16S rRNA genes obtained from cultures incubated with 1,2,4-TrCDD and 1,2,3-TrCB, respectively, were analyzed and compared by restriction fragment length polymorphism (RFLP) in this recent study (2). RFLP analysis reflected the enrichment of several bacteria during trichlorobenzene cultivation including a *Dehalococcoides* species and a bacterium of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group. However, until recently we were unable to characterize the microbial dynamics over time.

In this report we provide additional information regarding the community structure at specific time points by using single-strand conformation polymorphism (SSCP). SSCP analysis allows one to distinguish between single-strand DNA of the same length but of different nucleotide sequences on the basis of differences in melting behaviour. In a modified protocol by Schwieger and Tebbe (9), double-stranded PCR products are first converted into single strands by lambda exonuclease digestion of phosphorylated strands. Subsequently, the non-phosphorylated strands are electrophoretically separated in non-denaturing polyacrylamide gels. The application of SSCP to 16S rRNA genes provides a promising tool for analyzing microbial communities (7-10, 13, 14).

The aim of this work was to investigate the dynamics of a microbial community following the addition of 1,2,3-TrCB dissolved in hexadecane. The occurrence and

fluctuations of specific bacterial taxa is discussed in the context of their frequency in clone libraries obtained previously from the same two-liquid-phase culture.

MATERIALS AND METHODS

Cultivation and trichlorobenzene dechlorination. A detailed description of the cultivation methods has been presented elsewhere (2). Briefly, a 1,2,4-TrCDD dehalogenating mixed culture derived from Spittelwasser sediments was spiked with a nominal concentration of 10 mM of 1,2,3-TrCB in a two-phase-system with hexadecane. The culture was amended with a mixture of organic acids (5 mM acetate and 2.5 mM each of formate, benzoate, pyruvate, and fumarate). Based on chloride release measurement, the enrichment converted 5.5 mM of 1,2,3-TrCB to 1,3-DiCB within 244 days of incubation.

DNA extraction, primers and PCR conditions. The enrichment cultures were sampled anaerobically using syringes. Community DNA was isolated from one ml of culture at several time points as described previously (2). For SSCP analysis, 16S rRNA genes were amplified from total DNA using a *nested* PCR approach. In the first PCR, bacterial domain specific primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTA CGACTT-3') (12) were used to amplify 16S rRNA genes from total DNA extracted from one culture at the time points indicated in Fig. 1. The PCR mixtures used for the first amplification contained approximately 50 ng of template DNA, each deoxyribonucleoside triphosphate (dNTP) at a concentration of 0.25 mM, 1 x *Taq* PCR reaction buffer (Promega), 2.0 mM $MgCl_2$, 0.025 U of *Taq* DNA polymerase (Promega) per μl reaction volume, and each primer at a concentration of 0.21 μM. The PCR program for primers fD1 and rP2 consisted of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The last 10 elongation steps were

performed with a 20 s time increment per cycle. The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and eluted with 40 μl of 10 mM Tris-HCl buffer, pH 8.5. The second *nested* PCR amplification (final volume, 100 μl) was performed using components of the first amplification at the same concentrations but 2 μl of the first amplicons were used as the template with the primer Com1 (5'-CAGCAGCCGCGGTAATAC-3', (9); *E. coli* positions 519-536 (1)) and the 5'-terminally phosphorylated primer 907R_PH (5'-CCGTCAATTCCTTTRAGTTT-3', (6); *E. coli* positions 926-907 (1)). The primer set targeted conserved sequences flanking variable regions V4 and V5 in the 16S rDNA molecule and yielded an amplicon of about 400 base pairs. The mixture was heated at 94°C for 3 min. It was then subjected to 35 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The reaction was terminated with a final extension step at 72°C for 4 min. The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and eluted with 50 μl sterile deionized water. The concentrations were determined using the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Freiburg, Germany).

Single Strand Conformation Polymorphism. In order to obtain single-stranded DNA from the amplicons, the phosphorylated strand was removed by lambda exonuclease digestion (Epicentre, Madison, WI); 20 U of Lambda Exonuclease was mixed with 5.2 μl of 10 x lambda exonuclease reaction buffer and 45 μl of the resuspended PCR product in a total volume of 52 μl and incubated at 37°C for 3 h. The single strands were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and eluted with 30 μl of 10 mM Tris-HCl, pH 8.5. Aliquots of the final products (12.5 μl, about 2,000 ng) for each of the sampling points were mixed with 8 μl of loading buffer (95 % deionized formamide (Carl Roth, Karlsruhe, Germany), 0.025 % bromophenol blue, 0.025 % xylene cyanole (Sigma-Aldrich, Steinheim,

Germany), 10 mM NaOH), denatured for 3 min at 95°C in a PCR thermocycler, and cooled immediately on ice. The samples were loaded onto a polyacrylamide gel using applicator strips (Serva Electrophoresis, Heidelberg, Germany). The polyacrylamide gels (total volume, 50 ml, consisting of 25 ml deionized water, 10 ml 5 x TBE, and 15 ml of 2 x MDE gel solution, Cambrex Bio Science, Baltimore, MD) were poured between acryl-glide coated (Amresco, Solon, OH) glass plates with a carrier film (polybond films, Biometra, Göttingen, Germany). The pH was adjusted to 7.8 by the addition of H_2SO_4. The polymerization (about 6 h) was started with 50 μl APS (40 % ammonium persulfate) and 20 μl TEMED (N, N, N', N'-tetramethylethylenediamine). The samples were electrophoresed horizontally in 1 x TBE buffer for 14 h at a constant voltage of 400 V and a temperature of 20°C in a TGGE Maxi System (Biometra). A mixture of single strands obtained under the same conditions as the samples but derived from a different two-phase enrichment culture with 1,2,3-TrCB was used as an internal standard to compare different SSCP gels. Gels were stained using 2 x SYBR Gold (final concentration, in 1 x TBE, pH 8.0) (Molecular Probes, Eugene, OR) according to the manufacturers instructions and analyzed by the Gel Doc 2000 documentation system and the Quantity One 4.2 software (Bio-Rad, Hercules, CA). Selected bands were excised, and the corresponding DNA was recovered from one band by crushing the gel fragment in 75 μl of sterile water. The mixtures were heated for 1 h at 80°C and incubated overnight at 4°C. Ten μl of the supernatants of a centrifugation step (13,000 x g for 15 min) were used for reamplification by PCR (final volume, 100 μl) using the components of the reaction mixture and a temperature program as described above, except the primer pair Com1/907R was unlabelled. Amplified products were purified using the QIAquick PCR purification Kit (Qiagen) and sequenced as described (2) with the primers Com1 and 907R.

RESULTS AND DISCUSSION

Single Strand Conformation Polymorphism (SSCP). In order to study the dechlorinating community over time, the enrichment culture was sampled periodically and monitored by single-strand conformation polymorphism (SSCP) (Fig. 1). SSCP analysis of PCR products, comprising about 400 nucleotides, revealed a relatively low number of dominant bands in the dechlorinating community and argues for a comparatively small number of dominant bacterial species. This is consistent with data obtained by RFLP (2) although it does not exclude the possibility that numerous other bacteria occur in smaller numbers in the community and therefore were represented only by weak or even invisible bands. Furthermore, it does not rule out the possibility that these bacteria also represent actively dechlorinating species.

Changes in SSCP profiles and sequence analysis of 16S rDNA fragments isolated from SSCP bands. Although SSCP is not quantitative, a relative increase in band intensity indicates a relative increase in the amount of corresponding 16S rDNA, which is consistent with a relative increase in cellular growth. Comparison of the SSCP profiles obtained at each sampling time showed the disappearence of some bands and the appearence of other bands indicating shifts in the community. Some of the single strands could be recovered from the SSCP gel. Bands were excised, the opposite strands were regenerated and the products were reamplified by PCR. The sequences were used to gain insight into the identity of representative members of the dehalogenating community. We analyzed a total of 20 bands from the SSCP profile as depicted in Fig. 1 and Table 1.

Comparison of single strands obtained at day zero showed two dominant bands (Fig. 1, bands 7a and 8a), which were not visible in the patterns from day 28 and thereafter, indicating that they are not directly involved in the dechlorination process of 1,2,3-trichlorobenzene. Nonetheless, they may play a role in early stages of cosubstrate utilization. Both of these

bacteria are related to Clostridia (Table 1). In the case of band 7a, the corresponding DNA fragment was highly similar to an uncultured low G+C Gram-positive from a trichloroethene-contaminated site (clone ccslm 2120, AY133085).

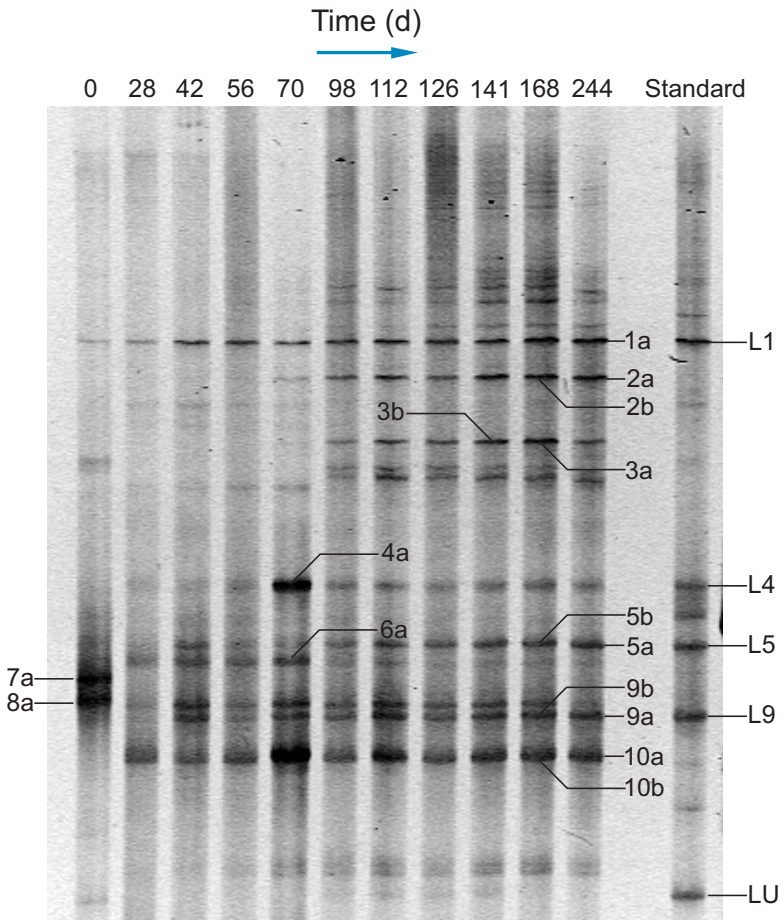

Figure 1 SSCP analysis of 16S rDNA fragments from the 1,2,3-trichlorobenzene dechlorinating culture grown with a mixture of organic acids. 16S rRNA genes were obtained from the culture and amplified with bacterial group-specific primers. Each lane represents the community structure at each time point. SSCP revealed changes in relative band intensities indicating shifts in the predominant species. The numbers above the gel indicate the time of incubation. The numbers within the gel represent bands that were excised, reamplified and sequenced, and correspond to sequences designated 1 to 10 in the text. Standard: SSCP profile from a two-phase-liquid enrichment culture with 1,2,3-trichlorobenzene dissolved in hexadecane obtained from Leine sediment (near Bitterfeld, Germany). The phylogenetic affiliation of standard fragments is shown in legend of Table 1.

Meanwhile, another population (represented by band 6a) was more prominent at 28 days and steadily increased thereafter but became less prominent after 70 days. The sequence of this SSCP fragment was 98 % identical to *Sedimentibacter saalensis* ZF2, matching RFLP sequence type 4 (2). This result indicates that the relative detection frequency (1.5 %) in the clone library of the culture at 244 days underestimated its temporal contribution to the community. The SSCP fragment from band 4a was also identified as a *Clostridium* species, closely related to *Clostridium cadaveris* and *Clostridium putrefaciens* and could be assigned to RFLP pattern 26.

The SSCP fragment from bands 10a and 10b was similar to the 16S rDNA sequence of *Trichococcus pasteurii* KoTa2^{T} (5), strongly suggesting that it was derived from one bacterium that is represented by RFLP pattern 2 (2). The corresponding bands exhibited the strongest signal observed on the gel suggesting the dominance of this organism in the culture. These results confirm the findings of our previous study, which recognized its RFLP pattern as the most predominant pattern in the clone library of the culture (up to 49 %) (2).

All of the organisms discussed above are Gram-positive bacteria. It was shown in our recent study, that inhibition of Gram-positive bacteria by vancomycin treatment appeared to negatively affect the dehalogenation of trichlorobenzene when compared to an untreated parallel culture. However, the culture supplied with the inhibitor retained moderate dehalogenating activity, suggesting that Gram-positive bacteria are not essential and can be replaced by other bacteria. In this regard, the role of *Syntrophus* species should be addressed. The DNA of bands 1a and of the corresponding band in the marker lane (L1) showed high sequence similarity to the 16S rDNA of *Syntrophus gentianae* HQgö1 (X85132) and *Syntrophus aciditrophicus* (U86447). Curiously, DNA sequences corresponding to bands 2a and 2b were also very similar to *Syntrophus gentianae*.

Table 1 Phylogenetic affiliations of predominant DNA fragments from the 1,2,3-trichlorobenzene-dechlorinating culture based on partial 16S rRNA gene sequence from bands excised from the SSCP gel shown in Fig. 1. 16S rDNA sequences from bands of the internal standard lane showed the following phylogenetic affiliations (GenBank accession numbers and similarities are given in parentheses): L1, *Syntrophus aciditrophicus* (U86447, 99.4 %); L4, uncultured bacterium clone MW2-28 (AY122598, 99.1 %); L5, uncultured bacterium clone WCHB1-69 (AF050545, 100 %); L9, *Dehalococcoides* sp. FL2 (AF357918.2, 99.4 %); LU, *Sulfurospirillum* sp. EK7 (AJ535704, 99.6 %). An assignment of the sequences to RFLP patterns from reference (2) is also provided.

Band	Phylogenetic relatives	Phylum	Similarity to relatives (%)	RFLP pattern (see reference 2)
1a	*Syntrophus gentianae* (X85132) *Syntrophus aciditrophicus* (U86447)	*Proteobacteria*	100 100	pattern 1
2a	*Syntrophus gentianae* (X85132) *Syntrophus buswellii* (X85131)	*Proteobacteria*	100 100	pattern 1
2b	uncultured bacterium clone BA039 (AF323763) *Syntrophus gentianae* (X85132)		99.3 99.0	
3a	uncultured bacterium clone ZZ12C11 (AY214185) *Trichlorobacter thiogenes* (AF223382)	*Proteobacteria*	99.4 99.1	
3b	uncultured bacterium clone ZZ12C11 (AY214185) *Trichlorobacter thiogenes* (AF223382)		99.3 99.0	
4a	uncultured bacterium clone MW2-28 (AY122598) *Clostridium cadaveris* (M59086)	*Firmicutes*	100 95.4	pattern 26
5a	uncultured bacterium clone WCHB1-69 (AF050545) uncultured bacterium clone IIIB-28 (AJ488099)	*Bacteroidetes*	99.7 99.2	pattern 6
5b	uncultured bacterium clone WCHB1-69 (AF050545) uncultured bacterium clone IIIB-28 (AJ488099)		99.0 97.0	
6a	uncultured bacterium clone TCE 41 (AF349757.2) *Sedimentibacter saalensis* ZF2 (AJ404684)	*Firmicutes*	98.7 98.3	pattern 4
7a	uncultured bacterium clone ccslm 2120 (AY133085) uncultured bacterium clone HC-31 (AY168741)	*Firmicutes*	98.1 98.1	
8a	uncultured bacterium clone p55-a5 (AF371690) *Clostridium glycolicum* (AY007244)	*Firmicutes*	85.5 85.1	
9a	uncultured bacterium clone WCHB1-29 (AF050544) uncultured bacterium clone SHA-7 (AJ249109)	*Bacteroidetes*	93.7 92.7	
9b	bacterium VS (AY323233) *Dehalococcoides* sp. FL2 (AF357918.2)	*Chloroflexi*	100 100	pattern 5
10a	*Trichococcus pasteurii* (X87150) *Trichococcus collinsii* (AJ306612)	*Firmicutes*	100 100	pattern 2
10b	*Trichococcus pasteurii* (X87150) *Trichococcus collinsii* (AJ306612)		98.3 98.3	

Obviously, the intensities of the no.2 bands appeared to increase more rapidly during cultivation as compared to the no.1 bands. At this stage, we cannot distinguish whether bands 1 and 2 describe different organisms or multiple *rrN* operons of one organism, which might lead to overestimation of the number of *Syntrophus* bacteria both in SSCP fingerprinting and in

cloning strategies such as RFLP analysis. An artifact caused by the conditions used in SSCP analysis could also have influenced the banding pattern. For example, the same single-strand molecule has been shown to fold into more than one conformation under certain conditions (11).

The population represented by bands 5a and 5b appeared to be enriched during the course of dehalogenation. The sequence of the fragments was 97 to 99 % identical to the 16S rDNA of clone IIIB-28 (AJ488099) derived from a bacterial consortium that removes predominantly singly flanked chlorine substituents from chlorobenzenes. The enrichment of a phylogenetically similar bacterium within the trichlorobenzene-dechlorinating community was evident from an increased abundance of the RFLP pattern 6 in the clone library of the culture after 244 days of incubation (2).

SSCP bands at the same position in the gel have the same melting behaviour, but not necessarily the same sequence. Sequencing of an SSCP fragment requires elution of the fragment from the gel and subsequent reamplification. Co-migration of DNA fragments can be a problem for retrieving clean sequences from individual bands. This was apparent from direct sequencing of excised band 9a, which revealed mixed sequences and a high background indicating that the DNA extracted from one position in the gel consisted of more than one fragment. Manual re-checking of the original data from band 9a showed a sequence highly similar to uncultivated bacteria of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group (94-% similar to clone WCHB1-29 derived from a chlorinated-solvent-contaminated aquifer (4)). In contrast, DNA obtained from band 9b and from L9 identified a *Dehalococcoides* sp. as the corresponding bacterium that could be assigned to RFLP pattern 5 (Table 1 and reference (2)). These results suggest that an increase in the band of *Dehalococcoides*, which was remarkably

enriched in the culture (2), was masked by the comigrating band corresponding to the CFB organism.

Sequence obtained from bands 3a and 3b, which appeared to be notably enriched, displayed sequence similarity to the δ–Proteobacterium *Trichlorobacter thiogenes* K1 (99 %) (3). De Wever *et al*. (3) reported a novel sulfide-sulfur cycle coupled with oxidation of acetate and reductive dechlorination of trichloroacetic acid (TCA) in strain K1. It was argued that *Trichlorobacter thiogenes* strain K1 has an extremely narrow electron donor and acceptor specificity. Nevertheless, chlorinated aryl compounds have not yet been tested or published with this strain. Because both sulfide and acetate were components of the medium, it is possible that a similar sulfide-sulfur cycle might take place in our chlorobenzene-dehalogenating communities. RFLP analysis and sequencing failed to identify a *Trichloro-bacter*-like 16S rDNA sequence in the clone library from the culture (2) although these sequences would possess a unique *Msp*I/*Rsa*I RFLP pattern according to *in silico* digestions and should be distinguishable from other "Deltaproteobacteria" (such as *Syntrophus*). This result indicates the need for an additional 16S rDNA-targeting fingerprint method for the description of microbial communities. However, other limitations of the techniques should be taken into account. Bias can be introduced at various stages in both techniques, particularly during cell lysis, extraction of intact nucleic acid, and PCR amplification. Clone library construction might also be associated with biases (*e.g.* different cloning efficiency and stability of 16S rDNA in clones) that could affect the outcome of the patterns and the interpretion of microbial diversity. In addition, the efficiency of detection and preferential amplification of some 16S rDNA sequences might depend on the type of primer set used (*i.e.*, degree of specificity). Although the primers Com1 and 907R used in this study bind to conserved regions of the 16S rDNA and have been shown to be well suited for SSCP purposes (8), they do not

universally correspond to all species of the *Bacteria*. Conversely, *nested* PCR has been shown to increase the sensitivity and our *nested* PCR approach might also influence the detection limit of the SSCP analysis. Despite these limitations, the results of both community composition surveys are in good agreement. The less laborious SSCP method allowed us to monitor changes in the community over time and to detect the appearence of a novel member.

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Halle, den 30. November 2003

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe und keine anderen als die in den aufgeführten Quellen und Verweisen angegebenen Hilfsmittel verwendet habe. Die aus den benutzten Werken stammenden wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Halle, den 30. November 2003

Michael Bunge