Bio-transformation of chlorobenzenes by anaerobic mixed cultures and a pure bacterial strain

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Declaration

This dissertation was carried out at The Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany between April, 2010 and April, 2014 under supervision of PD Dr. Lorenz Adrian and Prof. Dr. Peter Neubauer. I herewith declare that the results of this dissertation were my own research and I also certify that I wrote all sentences in this dissertation by my own construction.

Signature **Date**

Deutsche Zusammenfassung

Chlorierte Benzole werden in vielen Prozessen angewendet und sind ubiquitär in der Umwelt nachweisbar. Sie stellen durch ihre Toxizität und ihre Persistenz eine Bedrohung für Umwelt und menschliche Gesundheit dar. Biologischer Abbau kann eine wichtige Rolle für ihren den Verbleib in der Umwelt spielen

Ein äquimolares Gemisch aus 1,2,3- und 1,2,4-Trichlorbenzol, Hexachlorbenzol und 1,3,5-Trichlorbenzol wurden von Mischkulturen umgesetzt, die aus Böden aus Vietnam und Deutschlang angereichert wurden. Die Mischkulturen transformierten ein Gemisch aus 1,2,3- und 1,2,4-Trichlorbenzol zu allen drei Dichlorbenzolen, Monochlorbenzol und Benzol. Hexachlorbenzol wurde dechloriert, ohne dass dabei das hochpersistente 1,3,5-Trichlorbenzol produziert wurde. Kulturen aus Vietnam dechlorierten außerdem 1,3,5-Tri- zu 1,3-Di- und Monochlorbenzol. Die Dechlorierungsmuster blieben über sieben Transfers stabil. Eine eine kurze Sauerstoffexposition des Inokulums veränderte das Dechlorierungsmuster einer Kultur nicht. Dagegen hemmte Vancomycin in einer Konzentration von 5 mg L^{-1}.

Mit *Desulfotomaculum guttoideum* strain VN1 wurde ein dechlorierender Reinstamm aus den Anreicherungskulturen isoliert. Der Reinstamm dechlorierte 1,2,3-Tri-, 1,2,4-Tri- und 1,2-Dichlorbenzol. Hexa-, 1,2,4-Tri-, alle Di- und Monobrombenzol wurden zu Benzol debromiert. Die optimale Wachstums-temperatur für Stamm VN1 war 30°C, der optimale pH bei 7.3. Der Stamm war tolerant gegenüber kurzzeitiger Sauerstoffexposition aber wuchs nicht in 0.5% NaCl, 0.2 mM Na_2S, 10 mg L^{-1} Gentamycin oder 10 mg L^{-1} Vancomycin. Stamm VN1 produzierte geringe Mengen H_2S aus Thiosulfat aber nicht aus Sulfat oder Sulfit. Vitamine oder Acetat waren für die Kultivierung nicht notwendig. Wasserstoff wurde zur CO_2–Reduktion zu Acetat aber nicht zur reduktiven Dehalogenierung verwendet. Pyruvat induzierte starkes Wachstum aber keine Dechlorierung. Glucose wurde nicht metabolisiert. Dagegen wurde Citrat als Elektronendonor für eine reduktive Dechlorierung verwendet, was auf eine kometabolische Nutzung der halogenierten Substrate hinweist.

Abstract

Chlorobenzenes are ubiquitous on Earth and are a big concern for the environment and human health due to their toxicity, their persistence and wide application in chemical processes. Biodegradation can play an important role to determine the fate of chlorobenzenes in the environment.

A mixture of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene and 1,3,5-trichlorobenzene were bio-transformed by mixed cultures enriched from dioxin-contaminated soil and sediments in Vietnam and Germany. Mixed cultures transformed a mixture of 1,2,3- and 1,2,4-trichlorobenzene to all isomers of dichlorobenzene, monochlorobenzene and benzene. Hexachlorobenzene was transformed without accumulating 1,3,5-trichlorobenzene. Only cultures from sediments in Vietnam could convert 1,3,5-trichlorobenzene to 1,3-dichlorobenzene and monochlorobenzene. Cultures were insensitive to oxygen but sensitive to cell wall antibiotics indicating that *Dehalococcoides* species were not responsible for dechlorination.

Desulfotomaculum guttoideum strain VN1, a pure strain was isolated from a 1,2,3- and 1,2,4-trichlorobenzene dechlorinating mixed culture. It dechlorinated 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene and 1,2-dichlorobenzene. Hexabromobenzene, 1,2,4-tribromobenzene, all isomers of dibromobenzene and monobromobenzene were debrominated to benzene. The optimal temperature and pH for strain VN1's growth were 30^0C and 7.3, respectively. The strain could grow and dechlorinate when briefly exposed to oxygen. However, it could not grow in medium spiked with 0.5% of NaCl or 0.2 mM of Na_2S or with vancomycin or gentamicin at a concentration 10 mg L^{-1}. Strain VN1 produced H_2S from thiosulfate but not from sulfate or sulfite. Vitamins and acetate were not necessary for VN1's growth and dechlorination. Hydrogen was an electron donor for CO_2 reduction to acetate but not for dechlorination. Strain VN1 required CO_2 as carbon source. Pyruvate supported strong growth of the bacterium but did not support dechlorination activity. Glucose was not used. Reversely, citrate was an electron donor for dechlorination and dechlorination occurred via co-metabolism.

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Abbreviations

	B
BES	2-Bromoethansulfonate
	D
DBB	Dibromobenzene
DCB	Dichlorobenzene
D. mccartyi	*Dehalococcoides mccartyi*
D. guttoideum	*Desulfotomaculum guttoideum*
	G
GC	Gas chromatograhy
GC/FID	Gas chromatography/ flame ionization detection
	H
HPLC	High performance liquid chromatography
	M
MBB	Monobromobenzene
MCB	Monochlorobenzene
	N
NTA	Nitrilotriacatate acid
	P
PBDEs	Polybrominated diphenyl ethers
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POPs	Persistent organic pollutants
	R
Rpm	Rounds per minute
	T
TiCi	Titanium(III) citrate
TiNTA	Titanium nitrilotriacetate
TrCDD	Trichlorodibenzo-*p*-dioxin
	V
V/v	volume/volume
	W
W/v	weight/volume

1. Introduction

Halogenated organic compounds are a big concern for the environment and one of the largest environmental pollutant groups. They are ubiquitous in the environment and can be easily found in water (Asplund et al. 1989; Christof et al. 2002; Fernández and Grimalt 2003; Häggblom et al. 2006; Pereira et al. 1988), in sediments (Fernández and Grimalt 2003; Häggblom et al. 2006; Pereira et al. 1988), in soil (Asplund et al. 1989; Fernández and Grimalt 2003; Häggblom et al. 2006) and in the air (Fernández and Grimalt 2003; Mohamed et al. 2002). Halogenated organic pollutants originate from natural and anthropogenic sources and they are toxic and persistent under aerobic and anaerobic conditions, some of them have a half-life up to 100 years (Paustenbach et al. 1992). The exposure with halogenated organic compounds can cause different diseases in humans. The fate of organohalide compounds in the environment is determined by many factors, among them biotic factor, namely microorganisms.

1.1 The presence of halogenated organic compounds in the environment

Halogenated organic compounds are organic compounds containing one or many halogen atoms namely chlorine, bromine, fluorine and iodine. They are considered as persistent organic pollutants (POPs) and originate from natural and anthropogenic sources. The natural production of halogenated organic compounds mainly comes from geothermal activity such as volcanic eruptions and forest fires (Gribble 2004; Keith and Telliard 1979) and biogenesis. Stoiber at al. 1971 and Pereira at al 1980 found many different halogenated organic compounds such as chlorinated propanes, chlorinated benzoates, polychlorinated biphenyls, pentachloroethane and tetrachoroethane in gas and ash of volcanic eruptions sites with rather high concentrations. Forest fires also contribute a significant amount of halogenated organic pollutants to the environment. According to a report of Sheffield 1985, approximately 60 kg of polychlorinated dibenzo-*p*-dioxin were produced every year via forest fires in Canada (Sheffield 1985). Moreover, a huge amount of several organohalogens is produced via biogenic production (Gribble 2009). A survey of Engvild 1986 reveals that there were just over 80 plants that

can produce halometabolites. According to research of De Jong et al. 1994, wood-rotting fungi produced up to around 75 mg of chlorinated anisyl per kg of wood. Besides, many reports also confirm that a lot of organohalide compounds are produced by organisms in marine ecosystems such as seaweeds, sponges, green and red algae, soft corals and ascidians (Asplund and Grimvall 1991; Gribble 1992; Gribble 1996; Hay and Fenical 1996) and surprisingly, just under two thousand natural organobromines are produced by marine organisms (Gribble 2009). It is estimated that the amount of chloromethane that was globally produced in the sea could reach over 5 million tons annually much higher than that of anthropogenic production with about 26 thousand tons per year (Harper 1985). Bacteria are also known to generate halogenated organic compounds (Van Pée 1996), over 60 Streptomyces species generate organohalogens (Nicolaou et al. 1999).

However, the most important source of halogenated organic compounds released into the environment is anthropogenic and the man-made production is much higher than that of nature (Neidleman and Geigert 1887). Most of the halogenated organic compounds are used for many industrial purposes as solvents and intermediates in chemical processes, pharmaceuticals, degreasing agents, plasticizers, flame retardants, pesticides, fungicides and seed protectant, as well as in the pulp and paper industry (Chaudhry and Chapalamadugu 1991; Fetzner 1998; Häggblom et al. 2006; Peters et al. 1987; Vetter and Gribble 2007). Burning municipal solid waste and fossil fuel and accidents of oil and petrol spills also released a huge amount of halogenated organic pollutants into the environment (Coates et al. 1997; Page et al. 1999). Beside unintentional releases of halogenated organic compounds into the environment, the intentional utilization of persistent organic pollutants also causes a big concern for the environment and human health. In the Vietnam War, the Agent Orange was used by the US army for ten years with approximately 21 million gallons of defoliants and herbicides containing around 336 kilograms of dioxins spayed on southern Vietnam (Dwernychuk 2005; Stellman et al. 2003). The amount of dioxins in Vietnam was much more than that of the well-known Seveso accident in Italy in 1976 with

around 20 kg of 2,3,7,8-dibenzo-*p*-dioxin (Bertazzi et al. 1989; Dwernychuk 2005; Pesatori et al. 2003; Pocchiari et al. 1979; Stellman et al. 2003; Young et al. 2008).

1.2 The impacts of halogenated organic compounds on human health

Organohalide compounds have adverse effects on the environment and human health due to their bioaccumulation in human and animal tissues, especially in fatty tissues, due to biomagnification in food chains and due to their carcinogenicity. The exposure with halogenated organic compounds can cause many different diseases such as cancer, diabetes, obesity, and interference with reproductive and immune systems (Kamrin and Fischer 1991; Kogevinas 2001). The study on adverse effects of hexachlorobenzene on Turkish's people by accidental exposure to this compound is considered as one of the well-known reports on the negative impacts of halogenated organic compounds on human health. In the period of 5 years (1955-1959), hexachlorobenzene was utilized quite popular in Turkey as pesticide, fungicide and seed protectant in agricultural activities which caused many diseases symptoms being diagnosed such as porhyria, pembe yara (pink sore), arthritis, bullae, hypertrichosis, and hyperpigmentation for around 3,000 to 4,000 people (Peters et al. 1987). Other diseases symptoms caused by hexachlorobenzene, for instance enlarged liver, dermatological lesions, osteoporosis and enlarged thyroid gland were also found (Gustafson et al. 2002). Moreover, there are several serious diseases triggered by dioxins, a large family of very toxic persistent organic pollutants, have been proven over some last decades. Typically, with many circumstantial evidences of dioxins–related diseases such as cancer, mutations, teratogenicity, diabetes, obesity, interruption of reproductive and immune systems being found in around 4000 Italian patients in the Seveso dioxins accident in Italy in 1976 and in Vietnamese victims of dioxins in the Vietnam War between 1961 and 1971 (Kogevinas 2001; Pocchiari et al. 1979; Stellman et al. 2003).

1.3 Diversity of dehalogenating bacteria

The fate of halogenated organic pollutants in the environment can be determined by many factors including their physical and chemical properties and many environmental factors such as pH, temperature, hydrolysis, dissociation, photo-oxidation, sorption, metabolic reactions, bioaccumulation and biodegradation (Chaplain et al. 2011; Chaudhry and Chapalamadugu 1991; Fuchs 2008; Holm et al. 2011; Indrebo et al. 2005; Mabey and Mill 1978; Maphosa et al. 2012; Titus et al. 2004; Zaidi and Imam 1999). Among these factors, biodegradation is a very important process in transformation of halogenated organic compounds with participation of aerobic and anaerobic microorganisms.

There are several reports on biodegradation of halogenated organic compounds by aerobic microorganisms. Many strains belonging to *Pseudomonas* species are known to have dehalogenation ability to transform a wide range of halogenated compounds. Spain and Nishino 1987 described *Pseudomonas* sp. strain JS6 which transformed 1,4-dichlorobenzene. One year later, Haigler at al. 1988 found another strain belonging to *Pseudomonas* species, strain JS100 was able to transform 1,2-dichlorobenzene and these authors in 1992 also reported on *Pseudomonas* sp. strain JS150 could dechlorinate wide range of chlorobenzenes (Haigler et al. 1992). Several other strains of *Pseudomonas* species can grow on many chlorinated benzenes as sole carbon and energy (Brunsbach and Reineke 1994; Oldenhuis et al. 1989; Oltmanns et al. 1988; Vogt et al. 2004). *Sphingomonas* species with many isolated strains were illustrated to dechlorinate 2,4-dichlorophenoxyacetate (2,4-D) and 1,4-dichlorobenzene (Löffler et al. 2003; Schraa et al. 1986). In addition, many polycyclic aromatic hydrocarbons were also transformed by some *Sphingomonas* strains such as pyrene by *Sphingomonas yanoikuyae* strain R1 (Kazunga and Aitken 2000), fluoranthene, fluorene, anthracene, phenanthrene by *Sphingomonas* sp. strains P2 and LB126 (Pinyakong et al. 2000; Pinyakong et al. 2003; Van Herwijnen et al. 2003; Van Herwijnen et al. 2003), fluoranthene and naphthalene by *Sphingomonas paucimobilis* strain EPA505 (Mueller et al. 1990; Story et al. 2001) and chlorinated dibenzo-*p*-dioxin by *Sphingomonas wittichii* strain RW1 (Nam et al. 2006).

Furthermore, several isolated strains in the genera of *Rhodococcus*, *Mycobacterium* and *Burkholderia* were reported on dehalogenation abilities of a variety of halogenated organic pollutants (Denef et al. 2005; Folsom et al. 1999; Kelley et al. 1993; Ross et al. 2002; Vogt et al. 2004; Wong et al. 2002).

When halogenated organic pollutants are discharged into the environment, they tend to migrate to soil, underground water, sediments, and clay layers where no oxygen is present and aerobes cannot develop their dehalogenation abilities. Therefore, anaerobes play a very important role in determining the fate of halogenated organic pollutants under anaerobic conditions and this is the main reason why anaerobic dehalogenating bacteria have been studied intensively over last three decades with many isolated pure strains belonging to different species. *Desulfomonile tiedjei* strain DCB-1 was known as the first isolated pure dechlorinating bacterium which obtained energy for growth from transformation of 3-chlorobenzoate to benzoate (De Weerd et al. 1990). Another pure strain of genus *Desulfomonile*, *Desulfomonile limimaris* strain DCB-M also grew on 3-chlorobenzoate as a terminal electron acceptor (Sun et al. 2001). In 2002, Sanford at al. reported a facultatively anaerobic isolate *Anaeromyxobacter dehalogenans* 2CP-1 that can reductively dechlorinate 2-chlorophenol; 2,6-dichlorophenol; 2,5-dichlorophenol and 2-bromophenol with many different electron donors such as acetate, hydrogen, succinate, pyruvate, formate, and lactate (Sanford et al. 2002). 2-chlorophenol and 2,6-dichlorophenol were also reductively dechlorinated by one isolate in the genus *Desulfovibrio*, *Desulfovibrio dechloracetivorans* SF3 using acetate, fumarate, lactate, propionate, pyruvate, alanine, or ethanol as electron donors (Sun et al. 2000). In addition, a diversity of dehalogenating bacteria was also found in genus *Sulfurospirillum* with strain *Sulfurospirillum halorespirans* which can reduce tetrachloroethene to *cis*-dichloroethene (Luijten et al. 2003) and strain *Sulfurospirillum multivorans* was described for reductive debromination ability of deca-brominated biphenyl ether to hepta- and octa-brominated biphenyl ether (He et al. 2006). With more than 15 pure dehalogenating isolated strains, *Desulfitobacterium* is considered as the largest dehalogenating group. Most of the

dehalogenating isolates belong to *Desulfitobacterium* that use chlorophenolic compounds as terminal electron acceptors. *Desulfitobacterium dehalogenans* JW/IU-DC1 was the first pure strain of this genus isolated and could transform many halogenated compounds including 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA), chlorinated and brominated phenols (Utkin et al. 1995). *Desulfitobacterium dichoroeliminans* strain DCA1 was the first pure culture that completely eliminated chlorines from 1,2-dichloroethane and vicinal dichloropropanes (De Wildeman et al. 2003).

Among anaerobic dehalogenating bacteria, *Dehalobacter* and *Dehalococcoides* are two important dehalogenating groups that grow by strictly obligate organohalide respiration with hydrogen as sole electron donor and a wide spectrum of halogenated organic compounds as terminal electron acceptors. For the *Dehalobacter* genus, there are 4 pure isolates which have been reported so far. *Dehalobacter restrictus* strain TEA was the first isolate published by Wild at al. 1996 which can transform both tetrachloroethene and trichloroethene to *cis*-l,2-dichloroethene. Similarly, reductive dechlorination of tetrachloroethene to *cis*-l,2-dichloroethene was also carried out by strain PER-K23 (Holliger et al. 1998). Another strain belonging to *Dehalobacter*, designated as strain TCA1 dechlorinated 1,1,1-trichloroethane to 1,1-dichloroethane and chloroethane (Sun et al. 2002). The latest isolate belonging to this genus is *Dehalobacter* species strain TCP1 that converted 2,4,6-trichlorophenol to 4-monochlorophenol (Wang et al. 2013).

The genus *Dehalococcoides* has received the most attention from environmental microbiologists over two decades. *Dehalococcoides mccartyi* strain 195 (formerly *Dehalococcoides ethenogenes*) was the first described to reductively dechlorinate tetrachloroethene to ethene (Maymó-Gatell et al. 1997). Besides tetrachloroethene dechlorination, *Dehalococcoides mccartyi* strain 195 can reductively dechlorinate other chloroethenes such as tetrachloroethene, *cis*-dichloroethene, 1,1-dichloroethene and 1,2-dichloroethane (Maymó-Gatell et al. 1999; Nijenhuis and Zinder 2005). Some chlorinated organic pollutants are also bio-transformed by this

bacterial strain including hexachlorobenzene, 1,2,3,4-tetrachlorodipenzo-*p*-dioxin, 2,3,4,5,6-pentachlorobiphenyl, 1,2,3,4-tetrachloronaphthalene but strain 195 cannot grow with vinyl chloride as terminal electron acceptor (Fennell et al. 2004). In contrast, another strain belonging to the genus *Dehalococcoides*, strain BAV1 completely reductively dechlorinates vinyl chloride to ethene, a chlorinated pollutant that cannot be degraded by *Dehalococcoides mccartyi* strain 195 except in the presence of a polychlorinated ethane for cometabolic requirement of transformation of vinyl chloride to ethene (He et al. 2003). Moreover, two other *Dehalococcoides* strains VS and FL2 also have the ability to transform vinyl chloride to ethene. However, strain FL2 also needs other chlorinated ethenes to cometabolically dechlorinate vinyl chloride (He et al. 2005; Müller et al. 2004). Reductive dechlorination of trichloroethene to ethene and tetrachloroethene to *trans*-1,2-dichloroethene were found in two strains of the *Dehalococcoides* species, strain GT and MB, respectively (Cheng and He 2009; Sung et al. 2006). *Dehalococcoides* strain CBDB1, is very sensitive to oxygen, strictly obligate organohalide respiring, and different from the *Dehalococcoides* species described above. This strain can dechlorinate perchloroethene and trichloroethene to *trans*-1,2-dichloroethene (Marco-Urrea et al. 2011). It can reductively dechlorinate many polychlorinated biphenyls in Aroclor 1260 (Adrian et al. 2009). Moreover, a wide spectrum of chlorinated and brominated benzenes and some dioxins namely 1,2,3-trichlorodibenzo-*p*-dioxin, 1,2,4-trichlorodibenzo-*p*-dioxin, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin were also degraded by this strain (Adrian et al. 2000; Bunge et al. 2003; Jayachandran et al. 2003; Wagner et al. 2012). Similarly, reductive dechlorination of 1,2,3-trichlorodibenzo-*p*-dioxin and 1,2,4-trichlorodibenzo-*p*-dioxin was also found in *Dehalococcoides* strain DCMB5 (Bunge et al. 2008).

Another pure bacterial strain belonging to the phylum *Chloroflexi*, designated as strain DF-1, which shares many similarities in microbial characteristics and dechlorination activities with *Dehalococcoides* strains. This isolate can reductively dechlorinate many polychlorinated biphenyl congeners and some chlorinated

benzenes with preferentially double-flanked chlorines (May et al. 2008; Wu et al. 2002).

1.4 Reductive dehalogenation patterns of persistent halogenated organic compounds

A persistent chlorinated pollutant receiving a lot of attention from environmental scientists is hexachlorobenzene. This organic compound is very recalcitrant with estimated half-life ranging from around 3 to 23 years and it is on the list of the12 persistent organic pollutants collected in the UN Stockholm Convention on Persistent Organic Pollutants (UNEP 1997). It was world-wide utilized as pesticide, fungicide and seed protectant for nearly three decades (Peters et al. 1987). Although its production and utilization was banned in most countries of the world because of its carcinogenicity and persistence (IPCS 1997), hexachlorobenzene is now still ubiquitously distributed in the environment and becomes a burden of the environment with an estimation of 10,000 to 26,000 tons in 2005 (Barber et al. 2005). There are many reports on reductive dechlorination of hexachlorobenzene by both mixed cultures and pure isolates under anaerobic conditions. To our knowledge, up to now all studies using mixed cultures to investigate hexachlorobenzene bio-transformation illustrate that this compound was degraded mainly to 1,3,5-trichlorobenzene except only one report on avoiding accumulation of 1,3,5-trichlorobenzene from hexachlorobenzene transformation by soil slurry cultures (Ramanand et al. 1993). The accumulation of 1,3,5-trichlorobenzene as the only or main end-product of hexachlorobenzene transformation was the consequence of preferential removal of doubly-flanked chlorines (Figure 1) (Chang et al. 1997; Chang et al. 1998; Fathepure et al. 1988). Similarly, some pure bacterial strains belonging to the phylum *Chloroflexi* such as *Dehalococcoides mccartyi* strain 195, *Dehalococcoides* strain CBDB1 and *Dehalobium* strain DF-1, which preferentially degrade doubly-flanked chlorines, can use hexachlorobenzene as terminal electron acceptor in the anaerobic obligate organohalide respiration. 1,3,5-trichlorobenzene was accumulated as the main end-product except hexachlorobenzene bio-transformation by CBDB1 with 1,4-

dichlorobenzene as the major final- product although 1,3,5-trichlorobenzene was found with rather high concentration (Figure 2) (Fennell et al. 2004; Jayachandran et al. 2003; Wu et al. 2002).

In contrast to knowledge on hexachlorobenzene dechlorination, there is very little information on reductive dechlorination of 1,3,5-trichlorobenzene by mixed or pure culture although 1,3,5-trichlorobenzene is widely used in industrial organic syntheses and considered as important starting material for explosive and many fine chemicals. Moreover, 1,3,5-trichlorobenzene is a very recalcitrant pollutant under aerobic and anaerobic conditions and the major accumulated end-product of reductive dechlorination of hexachlorobenzene by most dehalogenating bacteria. Only one publication reporting on a mixed culture obtained from municipal sewage plants in Germany growing on a mixture of 1,2,3- and 1,2,4-trichlorobenzene was able to reductively dechlorinate 1,3,5-trichlorobenzene to 1,3-dichlorobenzene and a small amount of monochlorobenzene (Hölscher et al. 2010).

Apart from the chlorinated benzenes, dioxins with many different related compounds are a big burden for the environment and human health due to their high toxicity, persistence and carcinogenicity. 1,2,3,4-tetrachorodibenzo-*p*-dioxin is much less toxic than the isomer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and other dioxins. However, the number of reports on reductive dechlorination of 1,2,3,4-tetrachorodibenzo-*p*-dioxin by mixed cultures and pure bacterial strains is the largest. The reason for this is that 1,2,3,4-tetrachlorodibenzo-*p*-dioxin cannot be transformed into the most toxic congeners substituted at the 2', 3', 7', and 8' positions but still has a fully substituted ring. 1,2,3,4-tetrachlorodibenzo-*p*-dioxin were transformed by many mixed cultures using different electron donors such as organic acid mixtures (pyruvate, acetate, benzoate and fumarate), lactate/propionate, butyrate plus yeast extract, but not hydrogen (Ahn et al. 2007; Ballerstedt et al. 1997; Bunge et al. 2001). Moreover, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin was also converted by two pure bacterial strains of *Dehalococcoides* species with hydrogen as sole electron donor: *Dehalococcoides* strain CBDB1 transforms 1,2,3,4-tetrachlorodibenzo-*p*-dioxin to 2,3-dichlorodibenzo-*p*-dioxin and

1,3-dichlorodibenzo-*p*-dioxin as intermediates and 2-monochlorodibenzo-*p*-dioxin as the main final-end product, (Figure 3) (Bunge et al. 2003). *D. mccartyi* strain 195 transformed 1,2,3,4-tetrachlorodibenzo-*p*-dioxin to 1,3-dichlorodibenzo-*p*-dioxin and 1,2,4-trichlorodibenzo-*p*-dioxin (Figure 4) (Fennell et al. 2004).

Brominated organic compounds are also used widely in chemical industries, especially they are used as flame retardants for commercial products and they are toxic and persistent in the environment. In comparison with chlorinated benzenes, brominated benzenes are easier to transform. Up to now only *Dehalococcoides* strain CBDB1 was evaluated for debromination ability against 1,2,4-tribromobenzene and all congeners of dibromobenzene and monobromobenzene (Figure 5) (Wagner et al. 2012).

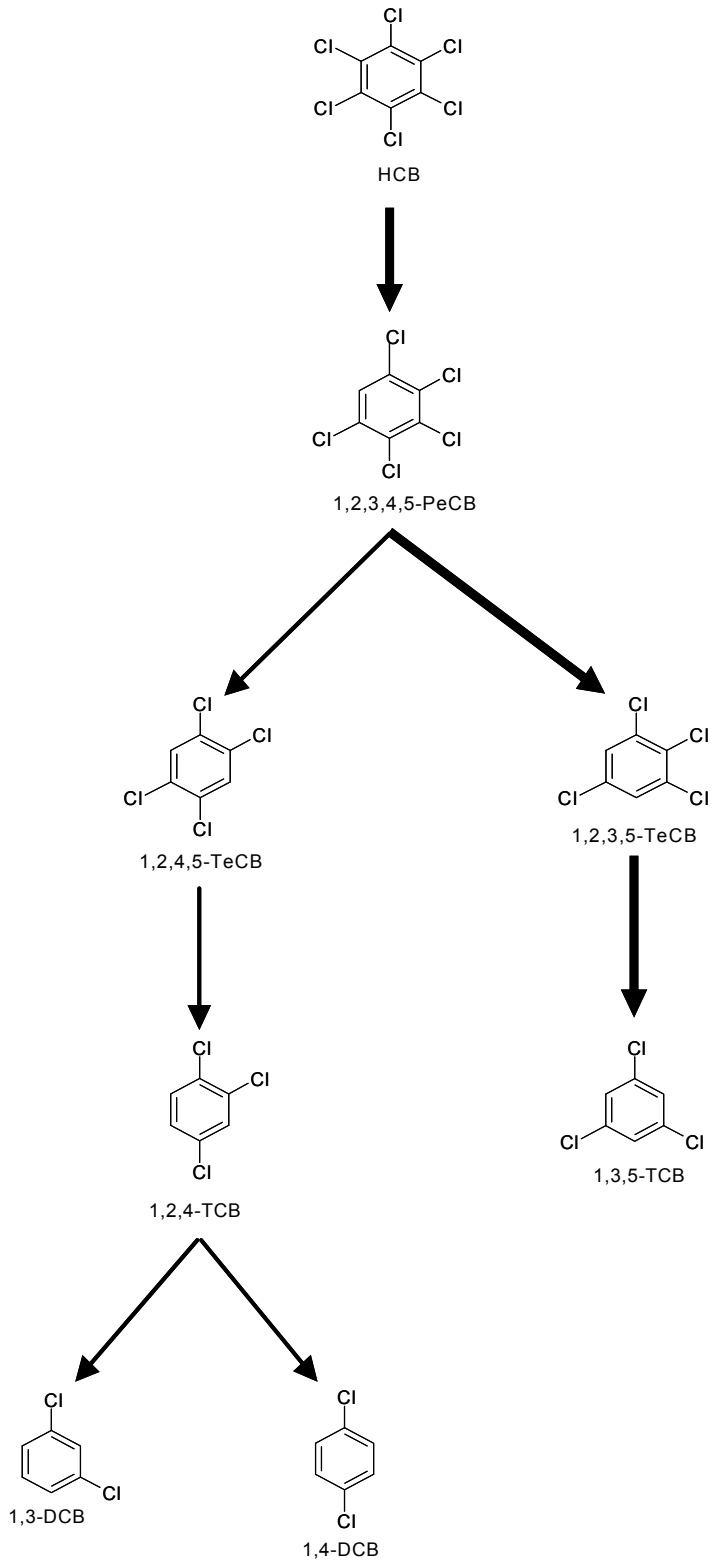

Figure 1: The pathway of reductive dechlorination of hexachlorobenzene by most previously described mixed cultures and *Dehalococcoides mccartyi* strain 195 and *Dehalobium* strain DF-1. Bold arrows indicate the main dechlorination pathway.

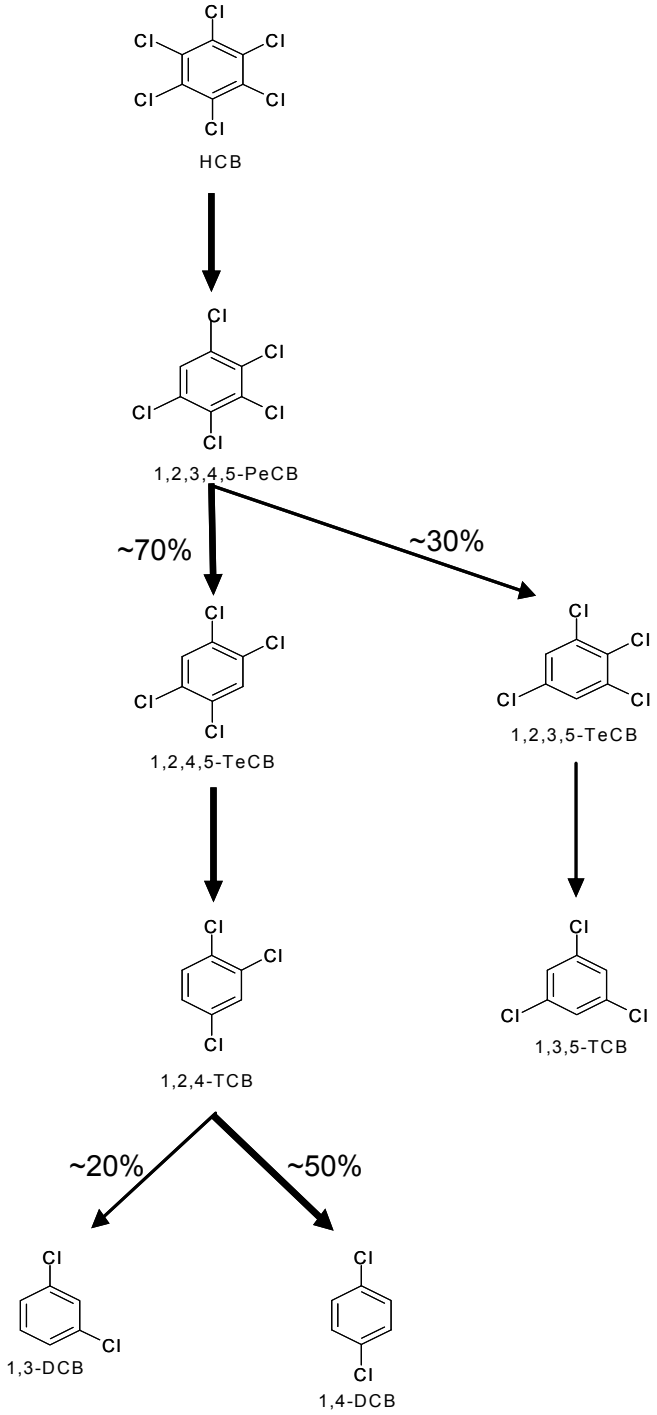

Figure 2: The pathway of reductive dechlorination of hexachlorobenzene by *Dehalococcoides* strain CBDB1. Bold arrows indicate the main dechlorination pattern (Jayachandran et al. 2003).

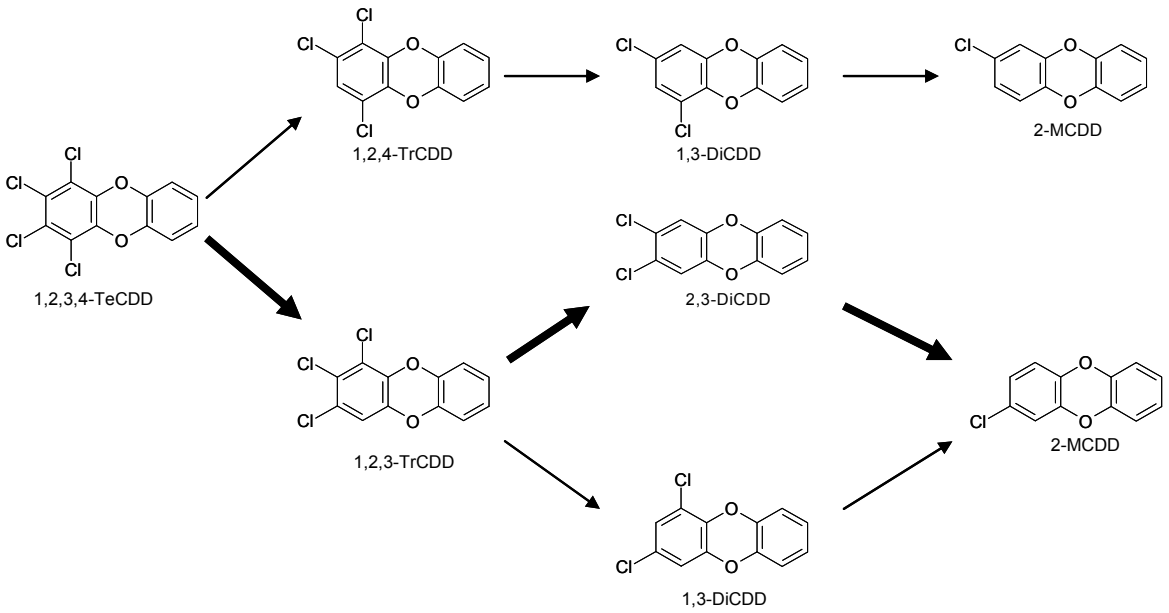

Figure 3: The pathway of reductive dechlorination of 1,2,3,4-TeCDD by *Dehalococcoides* strain CBDB1. Bold arrows indicate the main dechlorination pattern (Bunge et al. 2003).

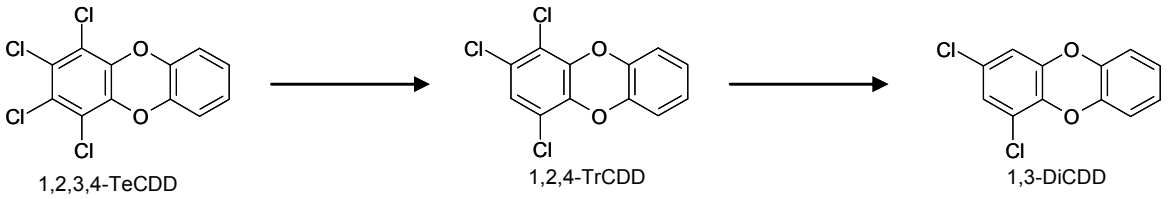

Figure 4: The pathway of reductive dechlorination of 1,2,3,4-TeCDD by *Dehalococcoides mccartyi* strain 195 (Fennell et al. 2004).

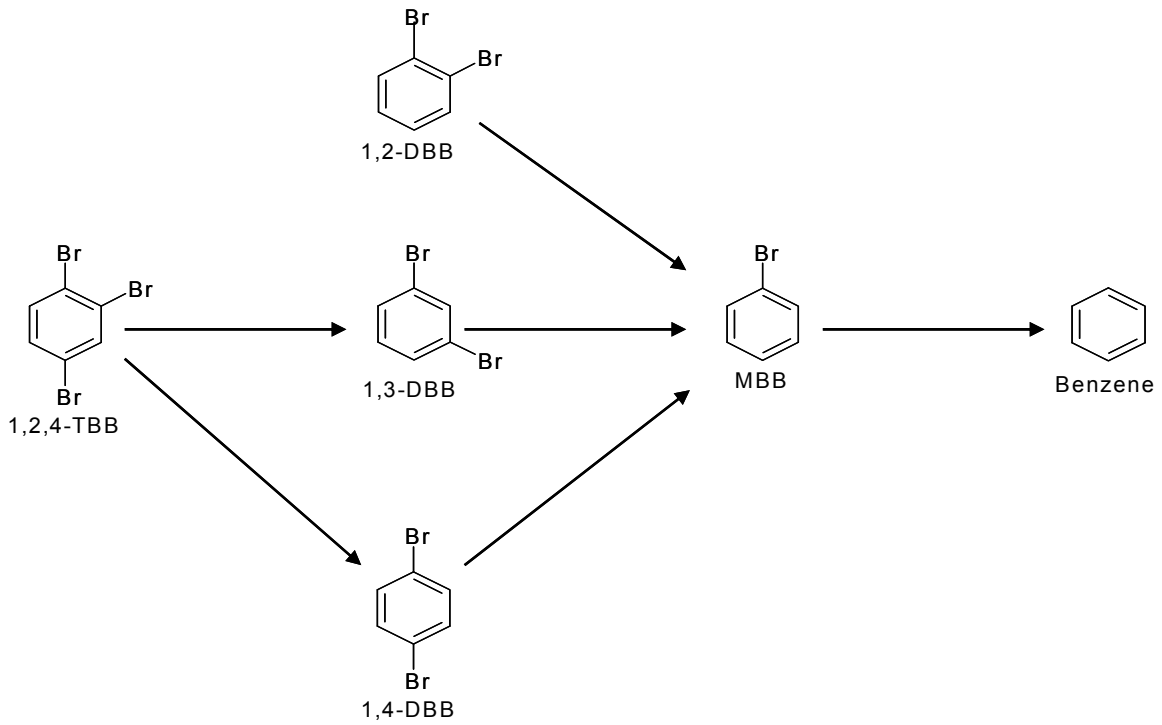

Figure 5: The pathway of reductive debromination of 1,2,4-tribromobenzene, all congeners of dibromobenzene and monobromobenzene by *Dehalococcoides* strain CBDB1 (Wagner et al. 2012).

1.5 Thesis objectives

From the environmental problems abovementioned, the PhD project focused on the following objectives:

1. Enrichment of mixed cultures amended with hexachlorobenzene from sediments and soil in Germany and Vietnam that transform hexachlorobenzene but avoid accumulating 1,3,5-trichlorobenzene which was the main final end-product of hexachlorobenzene transformation by most previous mixed cultures.

2. Enrichment of mixed cultures from sediments and soil that transform 1,3,5-trichlorobenzene when this recalcitrant chlorobenzene is used as initial electron acceptor.

3. Investigation of bio-transformation of a mixture of chlorobenzenes with better water-solubility. For that a mixture of 1,2,3- and 1,2,4-trichlorobenzene was used as electron acceptor for mixed cultures originating from sediments and soil.

4. Isolation and identification of a pure strain of chlorobenzene-dechlorinating bacteria from mixed cultures and describe their bio-characterizations.

5. Study on reductive dehalogenation of some chlorobenzenes and bromobenzenes by the pure strains.

2. Materials and Methods

2.1 Chemicals

All chemicals used were of analytical grade. Hexachlorobenzene and all isomers of trichlorobenzenes (1,2,3-; 1,2,4- and 1,3,5-trichlorobenzene) 99.9% analytical standards were purchased from Sigma-Aldrich (Steinheim, Germany). Vancomycin hydrochloride and gentamicin sulfate were supplied by AppliChem GmbH, Darmstadt, Germany and Sigma-Aldrich, respectively. N_2 and H_2 were obtained in 99.999% and CO_2 in 99.8% (v/v) quality. Trace oxygen was eliminated by a reduction column (Ochs, Bovenden, Germany). Titanium chlorine 15% was bought from Merck, Darmstadt, Germany. Sodium pyruvate and D(+)-Glucose obtained from by AppliChem GmbH, Darmstadt, Germany and Merck, Darmstadt, Germany, respectively. SYBR Green I was from Invitrogen Company, Oregon, USA. All primers were supplied by Eurofins MWG, Ebersberg, Germany. Low melting temperature agarose was bought from Lonza, Rockland, ME, USA. Electrophoresis gel was provided by Biozym Scientific, Oldendorf, Germany. The kit for DNA extraction was provided by Macherey-Nagel, Düren, Germany, whereas the kit for purification of DNA was supplied by Promega, Madison, USA.

2.2 Methods

2.2.1 Collecting samples

Samples were collected from two countries Germany and Vietnam. In Germany, inocula were obtained from anaerobic sediments of a town lake in Leipzig (Arthur-Bretschneider-Park $51^0$21'59.60"N, $12^0$22'53.61"E). Anaerobic sediments and dioxin–contaminated soil samples were collected from Hue, Vietnam, namely Nam Pho Canal ($16^0$29'39.59"N, $107^0$35'47.14"E) for the former and Ashau, the former American army airbase in A Luoi district ($16^0$07'17.36"N, $107^0$19'58.66"E) for the latter. For sediments, samples were fully collected in 500-mL bottles and closed tightly under water to ensure no oxygen penetration. With soil, samples were taken by making pieces with around 1000 cm^3 in volume. Samples were kept at 4^0C during the time of transporting and stored in a cooling room at 4^0C until

inoculation. Strictly anaerobic conditions for samples were ensured during transporting, storing and spiking.

2.2.2 Inoculating and transferring sub-cultures

Samples were inoculated with 5% (w/v) to a purely synthetic medium in 60-mL serum bottles with sodium bicarbonate as pH buffer, vitamins, trace elements, 5 mM acetate and titanium (III) citrate as a carbon source and reducing agent, respectively. The flasks were sealed with Teflon-lined butyl-rubber-septa and aluminum crimp caps and the headspace was flushed with N_2/CO_2 (80:20%, v/v). Hexachlorobenzene was added directly as crystals to the medium (approximately 10 mg per 30 mL of liquid medium) before the flasks were sealed and autoclaved, whereas 1,3,5-trichlorobenzene was added from a 2 M solution in acetone to the culture to a final concentration of 30 µM. A mixture of 1,2,3- and 1,2,4-trichlorobenzene was made by dissolving directly 1,2,3-trichlorobenzene in crystalline form into the liquid pure 1,2,4-trichlorobenzene to obtain an equimolar solution. This solution was applied to the cultures to obtain 80 µM final concentration. “Biogon” gas containing N_2 and CO_2 (4:1 v/v) was applied to the cultures for 10 seconds and hydrogen was added as electron donor (0.4 bar to give a total pressure of 1.4 bar). The cultures were incubated at 30^0C in the dark without shaking. All experiments were conducted in triplicate. Control experiments were done in medium containing electron donor and electron acceptor but no inoculum or they contained autoclaved inoculum.

For transferring subcultures, the most active culture among a triplicate, which produced highest concentration of products, was selected for transferring to the next generation. Cultures were regularly transferred to fresh medium using 5% (v/v) inoculum. Hexachlorobenzene cultures were transferred after at least 2 months, 1,3,5-trichlorobenzene and a mixture of 1,2,3- and 1,2,4-trichlorobenzene cultures were transferred when 75% or more of the added electron acceptor was transformed. A total of seven transfers were carried out.

2.2.2.1 Preparation of purely synthetic medium

The content per liter of basal medium included:

800 mL fresh Milli-Q water

20 mL mineral solution

10 mL trace metal solution

5 mL sodium acetate solution 1 M

50 µL resazurin (redox indicator) (5 drops)

Milli-Q water was added to 1 liter.

The medium was degassed in the airlock of the anaerobic chamber with vacuum for 1 hour by applying vacuum and nitrogen in turn for three times. Then the medium continued to be purged by Nitrogen at 0.3 bar for at least half an hour before being dispensed to clean 60-mL flasks in the anaerobic tent. Next the flasks were sealed with thick grey Teflon-lined butyl-rubber-septa and aluminum crimp caps and then were sterilized by autoclaving at 121^0C for 40 minutes. After autoclaving, the autoclaved sodium bicarbonate solution 1 M as pH buffer, titanium (III) citrate 0.1 M as reducing agent and vitamins were added to the medium with concentration at 1%. The flasks were stored in the dark overnight to ensure that trace oxygen in the medium would be removed completely by titanium (III) citrate before spiking with inocula.

2.2.2.2 Preparation of stock solutions

Mineral solution (Widdel and Pfennig 1981)

Stock solution (50x):

KH_2PO_4	10.0 g L^{-1}
NH_4Cl	13.5 g L^{-1}
NaCl	50.0 g L^{-1}
$MgCl_2.6H_2O$	20.5 g L^{-1}
KCl	26.0 g L^{-1}
$CaCl_2.\ 2H_2O$	7.5 g L^{-1}

The solution was sterilized by autoclaving and added 20 mL per liter of medium

Trace metal solution (Tschech and Pfennig 1984)

Stock solution (1000x):	H_2O	500 mL
	NTA	12.8 g
	$FeCl_2.4H_2O$	2.0 g
	$ZnCl_2$	70.0 mg
	$MnCl_2.2H_2O$	80.0 mg
	H_3BO_3	6.0 mg
	$CoCl_2.6H_2O$	190.0 mg
	$CuCl_2.2H_2O$	2.0 mg
	$NiCl_2.6H_2O$	24.0 mg
	$Na_2MoO_4.2H_2O$	36.0 mg
	NaOH	ad pH 6.0
	H_2O	ad 1000 mL

The solution was sterilized by autoclaving and added 10 mL per liter of medium

Redox indicator: Resazurin

Stock solution of 5 mg mL^{-1}. Final concentration of 0.5 mg L^{-1} by adding 5 drops to 1 liter of medium.

Sodium bicarbonate solution (Widdel and Pfennig 1981)

Sodium bicarbonate ($NaHCO_3$) was used as a pH buffer. The stock solution was prepared by dissolving 7.06 gram of $NaHCO_3$ in 84 mL of sterilized anoxic water and purging headspace of the flask with CO_2 for 5 times during dissolving $NaHCO_3$. The solution was autoclaved at 121^0C for 40 minutes before using. It was applied 10 mL per liter of medium.

Vitamins solution (Pfennig 1978)

Stock solution (1000x):

4- Aminobenzoic acid	20.0 mg L^{-1}
D (+)- Biotin	5.0 mg L^{-1}
Nicotinic acid	50.0 mg L^{-1}
Ca-D (+) Pantothenate	25.0 mg L^{-1}

Pyridoxine hydrochloride	75.0 mg L^{-1}
Thiamine chloride-di-hydrochloride	50.0 mg L^{-1}
Cyanocobalamin	50.0 mg L^{-1}

The solution was sterilized by a 0.2-µm filter and stored in the fridge at 4^0C. It was added 10 mL per liter of medium.

Ti (III) citrate solution 0.1 M (Zehnder and Wuhrmann 1976)

200 mL of Ti(III) citrate solution (0.1 M, in regard to Ti(III)) was prepared from 15% (g/v) Ti(III)Cl_3 (1 M) by combination of 40 mL of tri-sodium citrate 1 M and 20.6 mL of Ti(III)Cl_3 1 M in a two arm bottle containing 120 mL of sterilized anoxic water. The pH of the solution was adjusted to 7.0 by adding powder of sodium carbonate (Na_2CO_3) and sterilized anoxic water was filled to 200 mL. Nitrogen was applied to the solution to prevent the reaction between oxygen and titanium (III) which would decrease the concentration of Ti(III). The solution was sterilized using 0.2-µm filters and was applied 10 mL per liter of medium.

2.2.3 Testing the presence of *Dehalococcoides mccartyi* strains in the mixed cultures

2.2.3.1 Testing the presence of *Dehalococcoides mccartyi* strains in the mixed cultures by oxygen exposure and vancomycin

For testing the presence of *Dehalococcoides mccartyi* strains in mixed cultures, cultures were exposed to oxygen and by adding vancomycin. To briefly expose bacteria to oxygen, an inoculum was taken up with a sterile 3-mL syringe and air bubbles were taken up through the liquid of the culture until the redox indicator resazurin in the sample turned pink. After 20 seconds to 1 minute of waiting, the air was pressed out and the inoculum was injected into a culture flask containing reduced fresh medium. To test the sensitivity to cell wall antibiotics, vancomycin was applied to the cultures at a concentration of 5 mg L^{-1}. Exposure to oxygen and additions of vancomycin were conducted from the second transfer onwards and stopped if a culture was inactive.

2.2.3.2 Testing the presence of *Dehalococcoides mccartyi* strains in the mixed cultures by PCR

To screen for the presence of *D. mccartyi* strains, the *Dehalococcoides*-targeted primers 5'-AAGGCGGTTTTCTAGGTTGTCAC-3' and 5'-CGTTTCGCGGGGCAGTCT-3' (Löffler et al. 2000) were used in PCR amplifications of DNA samples extracted from cultures with hexachlorobenzene and 1,3,5-trichlorobenzene. PCR reactions (final volume of 20 µL) contained 10 µL of 2 x Sensi-Mix SYBR Kit PCR Master Mix (Bioline, London, England), 1 µL of each primer, 1 µL of DNA template and deionized water up to 20 µL. PCR cycling conditions included an initial enzyme activation step at 95^0C for 10 minutes, followed by 30 cycles of 95^0C for 30 seconds, 58^0C for 30 seconds and 72^0C for 45 seconds. A final extension of 72^0C for 5 minutes was included. Genomic DNA from *D. mccartyi* strains 195 and CBDB1 were used as positive controls. The PCR products were checked for correct sizes on a 1 % (w/v) agarose gel.

2.2.4 Isolation of pure strains

2.2.4.1 Preparing deep agarose dilution cultivation tubes ("shakes")

Low melting temperature agarose 1% (w/v) was boiled completely and dispensed into 15-mL tubes with 2 mL and the tubes were closed with butyl rubber caps and stoppers in the anaerobic tent and were autoclaved for 121^0C for 40 minutes before using. For isolation dechlorinating bacteria in mixed cultures enriched with hexachlorobenzene, hexachlorobenzene was added to shakes before autoclaving. When cultures were set up the agarose was molten at 80^0C in a block heater and then cooled down to 60^0C before filling with 10 mL of purely synthetic medium, preheated to 35°C. After this, the shakes with basal medium were kept in a water bath at 30-32^0C and 0.5 mL of the most active culture was transferred to a dilution series of 5 shakes for isolating bacteria. 0.5 mL of the most active culture was spiked to the first shake and 0.4; 0.3; 0,2 and 0.1 mL of suspension from the first, the second, the third and the fourth shake as inocula were transferred to the second, the third and the fourth and the fifth shake, respectively. Shakes were

solidified in ice-containing water at 4^0C for 3-4 minutes. Finally, the shakes were incubated in the dark at 30^0C.

2.2.4.2 Picking up colonies

Colonies with different shapes, sizes and colours in the highest dilution tubes were targeted for picking. Thinly elongated Pasteur pipettes were used for picking up colonies with reduced medium and the picked up colonies were transferred into 2 mL screw vials containing sterilized and reduced PBS buffer (see below) and 2 mm magnet stirring bars. The colonies in the vials were dispersed completely by stirring before a volume of 0.5 mL was transferred to a new series of the shakes containing 2 mL of low melting temperature agarose and 10 mL of basal medium. Hydrogen as an electron donor and hexachlorobenzene or trichlorobenzenes as electron acceptors were also applied to the shakes. Shakes were solidified in cold water for 5 minutes before incubated in the dark at 30^0C. Colonies were picked up and transferred for at least 5 times to check the purity and stopped if the results GC of the shakes showed that the activity was lost. Finally, pure colonies were transferred to purely synthetic liquid medium (Figure 6).

The content of PBS buffer was as following:

Milli-Q water	150 mL
NaCl	1.519 g
K_2HPO_4	0.3484 g
KH_2PO_4	0.2722 g

pH was around 6.6 and was adjusted to 7.2 with 20% NaOH

Water was added to 200 mL

Three drops of resazurin were added as an oxygen indicator.

The buffer was filled into 2-mL screw vials in a way that a drop of liquid was on the top of the vial. The vial contained a 2 mm magnet stirring bar and was sterilized by autoclaving at 121^0C for 40 minutes. After autoclaving, trace of oxygen of the PBS buffer in the vials was reduced by adding three drops of Ti (III) citrate 0.1 M.

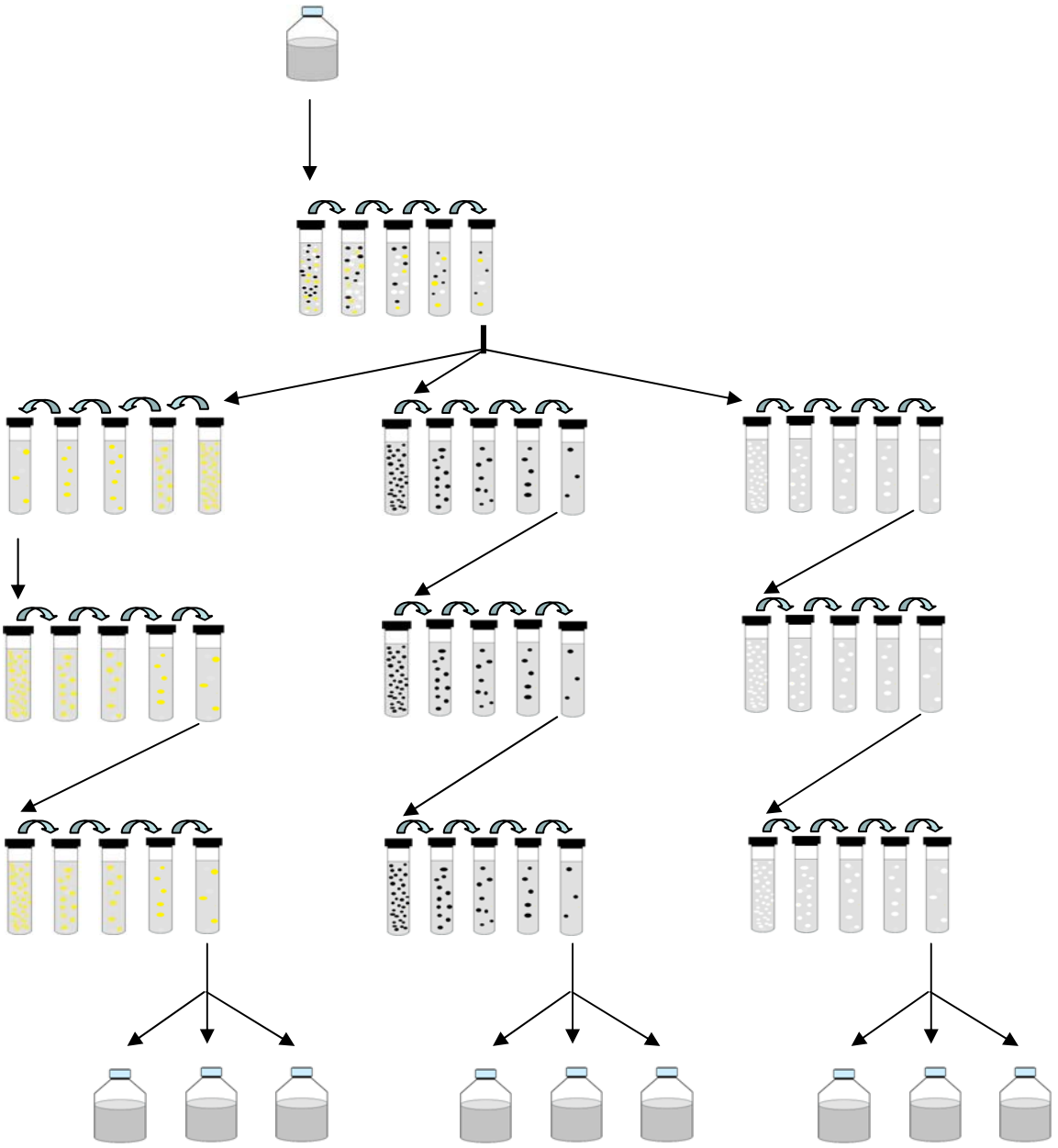

Figure 6: Isolation procedure of chlorobenzene-dechlorinating bacteria by a series of picking-up colonies and transferring into shakes containing low melting temperature agarose.

2.2.5 Analysis of halogenated organic compounds by GC-FID

Chlorobenzene concentrations were measured by headspace GC/FID. Samples were prepared by adding 1 mL of bacterial suspension and 1 mL of 1 M NaCl to a 10 mL-GC headspace vial which was then sealed with a Teflon-lined butyl-rubber-

septum and an aluminum crimp cap. A 5890 Hewlett Packard gas chromatograph equipped with a capillary column (HP-5, 5% phenyl methyl siloxan, Agilent, length: 30 m; inner diameter: 320 µm; film thickness: 0.25 µm) was used for analyzing chlorobenzenes. The column temperature was initially set to 55^0C for 1 min. Then the temperature was increased by 10^0C min^{-1} to 90^0C, and then increased by 6^0C min^{-1} to 130^0C. Finally, the temperature was increased to 220^0C with a rate of 30^0C min^{-1}.

For brominated benzenes analysis, headspace GC/FID was employed. Samples were prepared the same as described for chlorinated benzenes above. A 7820 gas chromatograph (Agilent) combined with a PAL autosampler was used. It contained a capillary column (ZB-1, Phenomenex, length: 60 m; inner diameter: 320 µm; film thickness: 1 µm). The column temperature was initially set to 55^0C for 1 min. Then the temperature was increased by 15^0C min^{-1} to 200^0C. Finally, the temperature was increased to 250^0C with a rate of 8^0C min^{-1}.

2.2.6 Analysis of acetate by GC-FID

The concentration of acetate in the cultures was analysed by GC/FID. 1 mL of sample was added to a 1.5-mL Eppendorf tube and the tube was centrifuged at 12.000 rpm for 10 minutes to remove cells and cell debris. Then 500 µL of suspension was transferred from the supernatant to a 2-mL HPLC vial containing 500 µL of HCl 0.5 M and the vial was sealed with a septum and an aluminum crimp cap. A 5890 Hewlett Packard gas chromatograph equipped with a capillary column type FFAP (free fatty acid phase) (Macherey-Nagel capillary Permabond–FFAP 723116.25, length: 25 m; inner diameter: 250 µm; film thickness: 0.25 µm) was used for analyzing acetate. The column temperature was initially set to 50^0C for 1 min. Then the temperature was increased by 25^0C min^{-1} to 180^0C and this temperature was held for 1 minute.

2.2.7 Cell counting

The growth of cells number was monitored by counting cells on low melting agarose coated slides (Adrian et al. 2007). 20 µL of liquid culture was stained with

1 µL of SYBR Green I 1% solution (Invitrogen Company, Oregon, USA) for 20 minutes and then 18 µL of this stained culture was put on a slide and covered by a 20 mm x 20 mm thin cover glass. Each slide was counted at least 15 different points on the fluorescent microscope.

2.2.8 Identification of the 16S rRNA genes of dechlorinating bacteria

2.2.8.1 DNA extraction

For DNA extraction a kit and protocol from Macherey-Nagel (Ref 740952.250) was used according to the instructions of the manufacturer. The DNA sample was stored at 4^0C in the fridge until amplification.

2.2.8.2 Amplification of the 16S rRNA gene

The 16S rRNA gene of strain VN1 was amplified by PCR reactions. The PCR reactions contained 16.875 µL of DNA-free water 2.5 µL of standard tag buffer (Bioline, London, England), 2.5 µL of dNTPs, 1 µL of forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') 5 pmol μL^{-1}, 1 µL of reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') 5 pmol μL^{-1} (Paju et al. 2003), 0.125 µL of Tag DNA polymerase (Bioline, London, England) and 1 µL of DNA template. PCR cycling conditions included an initial enzyme activation step at 95^0C for 30 seconds, followed by 31 cycles of 95^0C for 30 seconds, 52^0C for 30 seconds and 68^0C for 65 seconds. A final extension of 68^0C for 5 minutes was included. A 1 kbp ladder was used for checking of the working ability of ethidium bromide staining solution and of the size of bands of PCR products. The PCR products were checked for correct sizes on a 1% (w/v) agarose gel stained with ethidium bromide.

2.2.8.3 Purification of the PCR product

To remove primers and PCR reagents, the PCR product was cleaned with a commercial PCR purification kit (Promega) following the instructions of the supplier.

2.2.8.4 Sequencing of DNA

DNA was sequenced by the GATC Company (Köln, Germany) using 5 µL of DNA of samples after purification and 5 µL of forward primer 27F 5 pmol μL^{-1} or 5 µL of reverse primer 1492R 5 pmol μL^{-1}.

2.2.9 Gram staining

Gram staining was done according to the following protocol:

- Making a smear of bacteria on a slide and fixing bacteria on the slide by a brief exposure of the backside of the slide to a flame.
- The smear was covered with few drops of gentian violet (a mixture of methyl violet and crystal violet).
- Then the slide was washed with water after one minute of exposure to the staining solution.
- The smear continued to be treated with few drops of lugol solution for a minute. The slide was washed by water again.
- Then the slide was decolorized by a mixture of acetone-ethyl alcohol (1:1) for less than 30 s. The slide was then intermediately washed with water.
- The smear was finally treated with a few drops of safranin for 30 s. The slide was then washed again and dried in air and heat-fixed before examining it under microscope.
- Positive controls for gram-positive and gram-negative bacteria were carried out on the same slide with *Escherichia coli* for gram-negative and *Staphylococcus aureus* for gram–positive cells.

2.2.10 Temperature, NaCl and Na_2S tests with strain VN1

To investigate the optimal temperature for strain VN1's dechlorination ability and growth, the flasks containing fresh medium spiked with strain VN1 and substrate was incubated at different temperature points from 5^0C to 60^0C, namely 5^0C; 20^0C; 30^0C 40^0C; 50^0C and 60^0C. A control series without inocula but spiked with substrate was also incubated with the aforementioned tested temperatures to check abiotic degradation.

The tolerance of VN1 to NaCl was tested by setting up cultures of VN1 containing different concentrations of NaCl, namely 0.5%; 1%; 1.5%; 2%; 3%; 3.5% and 4%. Moreover, Na_2S with the concentrations of 0.2 mM; 0.4 mM; 0.6 mM; 0.8 mM and 1 mM was also applied to VN1 cultures to examine the ability of VN1's tolerance to Na_2S.

2.2.11 Antibiotics tests with strain VN1

Gentamicin and vancomycin were tested for strain VN1's antibiotics tolerance. The two antibiotics were applied with concentrations 5 mg L^{-1}; 10 mg L^{-1} and 20 mg L^{-1}. Gentamicin was tested with two ways. With the first approach, gentamicin was applied directly to the cultures under anaerobic conditions. The second approach was that a mixture of inoculum and gentamicin was exposed with oxygen in an empty sterilized flask for approximate 3 minutes. The mixture was shaken during oxygen exposure in order to enhance the effect of this antibiotic on bacteria because gentamicin works well in the presence of oxygen. Then the mixture was spiked in fresh medium under anaerobic conditions. Besides, a mixture of gentamicin and vancomycin with 5 mg L^{-1} each was also tested due to the effect of gentamicin will be increased in combination with vancomycin.

2.2.12 Changes of pH during incubation and the effects of pH on dechlorination ability

Changes of pH of the sodium bicarbonate buffer adjusted cultures were investigated by extracting around 7 mL of cultures as the same time of sampling for GC analysis and cell counting. pH values were measured by pH meter (Toledo). To ensure enough volume of the cultures for many times of pH and GC measuring, the experiment was set up with 60-mL medium in a 120 mL flask instead of 30-mL of medium. Besides, different initial pH levels of medium (6.5; 7.0; 7.5; 8.0 and 8.5) adjusted with NaOH 5 M were also tested for dechlorination ability and growth of strain VN1. The pH of the medium after autoclaving and adding vitamins and titanium (III) citrate 0.1 M was around 5.8 and was adjusted by solutions of NaOH 5 M. The adjustment of pH was carried out in an autoclaved 220-mL flask containing 200 mL of sterilized fresh medium and the solution of

NaOH 5 M was added to the medium via a sterilized syringe connected a 0.2-µm sterile-filter. The targeted pH levels were obtained after several times of extraction for measuring. Finally, the medium was dispensed by a 60-mL sterile syringe to 120-mL autoclaved flasks with 60 mL when the pH reached targeted level (Figure 7). For the medium with pH=7.3 was considered as positive control which pH was only adjusted by $NaHCO_3$ buffer 1 M.

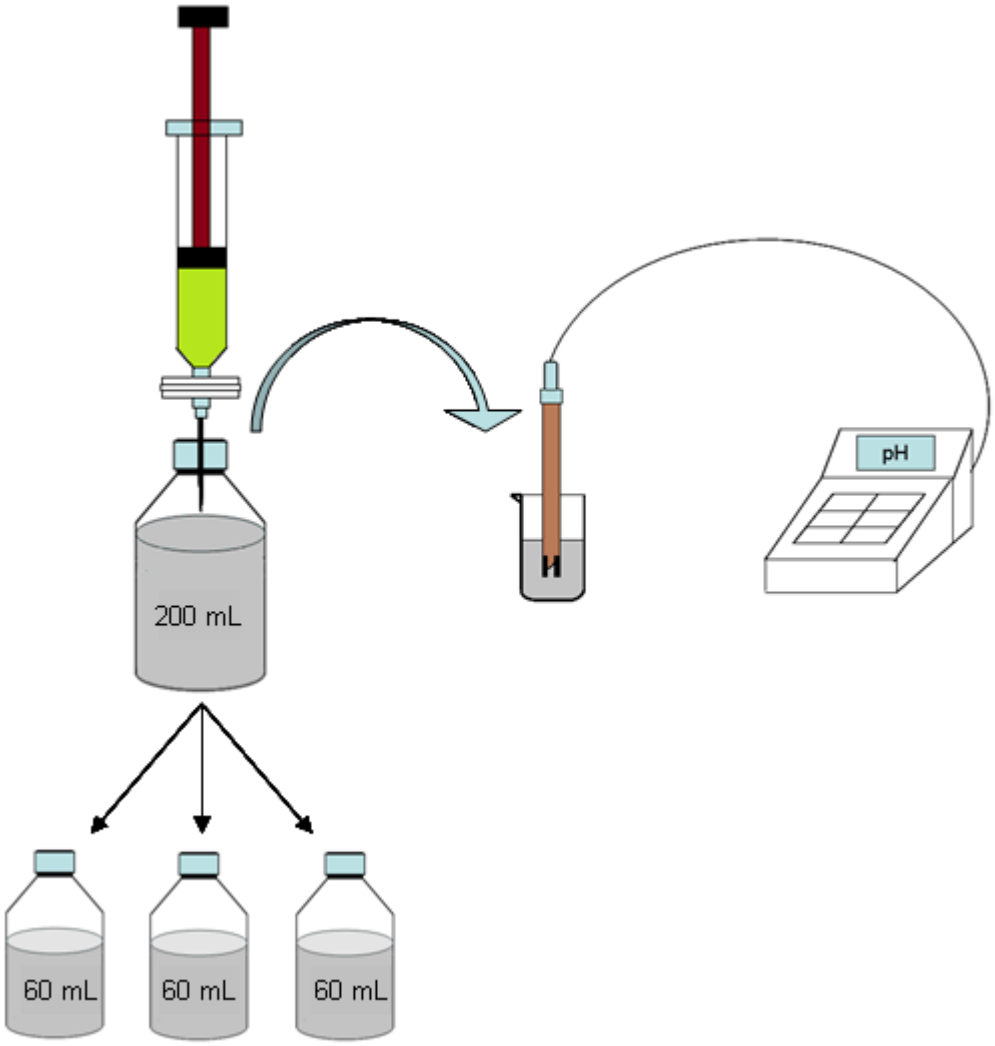

Figure 7: Adjustment of pH of medium for investigating the effects of pH on the growth and dechlorination of strain VN1.

2.2.13 Analysis of H_2S concentration

Desulfotomaculum guttoideum strain VN1 was tested for its sulfate-reducing ability by setting up experiments of strain VN1 cultures spiked with sodium sulfate (Na_2SO_4), sodium thiosulfate ($Na_2S_2O_3$) or sodium sulfite (Na_2SO_3) with a final concentration at 10 mM as electron acceptors and hydrogen or pyruvate 4 mM as electron donors. H_2S, a product of reducing sulfate, was measured by following steps (Cline 1969):

- Adding 1 mL of zink acetate solution 3% (w/v) to a 15-mL tube.
- Adding 4 mL of Milli-Q water to the tube.
- Adding 100 µL of culture to the tube.
- Vortexing for 10 seconds.
- Adding 400 µL of mixed diamine reagent including in *N,N*-dimethyl-*p*-phenylenediamine sulfate and ferric chloride ($FeCl_3.6H_2O$) with factor F= 0.0052 to the tube.
- Vortexing for 3 seconds.
- Incubating the tube in the dark place for at least 15 minutes and vortexing for 5 seconds before measuring.
- Measuring the sample at 670 nm on spectrophotometer

The concentration of H_2S can then be calculated by the following formula:

$$CMSulfide[\mu M] = \frac{A * Vsample(\mu L)}{F * 1000(\mu L)}$$

CM: concentration of sulfide; F= 0.0052; V: volume of sample; A: Absorbance value of sample at 670 nm

2.2.14 Sulfate-reducing bacterium test by using molybdate as an inhibitor

Together with the analysis of the H_2S concentration produced from Na_2SO_4 and $Na_2S_2O_3$, the sulfate-reducing capacity of *Desulfotomaculum guttoideum* strain VN1 was also evaluated by supplementing sodium molybdate, a selective inhibitor of sulfate-reducing bacteria, to the cultures at a concentration of 0.5 mM (Wild et al. 1996).

2.2.15 Requirements of energy sources and nutrients of strain VN1

To elucidate requirements of energy sources and nutrients for VN1, changes of different components of basal medium and additions of other substances were applied. For vitamin requirements, the medium was not supplemented with vitamins solution whereas other components of medium were remained and the same was repeated with acetate. Similarly, hydrogen was not supplied when experiments were set up to exam this gas in the role of electron donor and acetogenic bacteria of strain VN1. Moreover, the trace of hydrogen in the headspace of the cultures transferred from parental cultures and from anaerobic tent when dispensing the medium was eliminated by a 10-minute period of nitrogen gas exchange. Elimination of components in the medium by replacement with other compounds was employed, for instance using TiNTA instead of TiCi as reducing agent in the experiments to check effects of citrate on the growth and dechlorination ability. Sodium citrate was applied to the medium with final concentration 4 mM to check the role of citrate as an electron donor by strain VN1. In addition, glucose or pyruvate was also added to the medium with concentration at 4 mM each to investigate if these compounds could be used as electron donor for the dechlorination activity of VN1. All of the experiments were transferred at least three times to eliminate tested factors transferred from parental cultures.

3. Results

3.1 Dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by mixed cultures

Sediments from a lake in Leipzig, Germany and sediments from Hue canal, Vietnam and dioxin-contaminated soil in Hue, Vietnam were tested with a mixture of 1,2,3- and 1,2,4-trichlorobenzene as electron acceptors with approximately 80 µM each. The cultures were inoculated with 5% (w/v). Two of five samples of dioxin-contaminated soil were active with a mixture of 1,2,3- and 1,2,4-trichlorobenzene after nearly one month of incubation. The mixture of trichlorobenzene was also degraded by three of ten samples of Leipzig's lake sediment. Interestingly, all of the cultures amended with sediments from Hue canal dechlorinated the mixture of two isomers of trichlorobenzene after 4 weeks of incubation. The products of reductive dechlorination of all cultures were all of the isomers of dichlorobenzenes (1,4-; 1,3- and 1,2-dichlorobenzene) and monochlorobenzene. Monochlorobenzene and 1,4-dichlorobenzene were the main final end products of reductive dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by mixed cultures enriched from dioxin-contaminated soil in Vietnam (Figure 10) and mixed cultures enriched from canal sediment in Hue Vietnam (Figure 9) while monochlorobenzene, 1,2- and 1,3-dichlorobenzene were major final end-products of cultures amended with sediment from Leipzig (Figure 8). Especially, benzene was found in one culture from Hue canal after two months of incubation and its concentration went up to approximately 130 µM when the culture was further incubated for two years (Table 1). The concentration of benzene increased together with an increase of concentration of monochlorobenzene during the period of over two years. Reductive dechlorination did not occur in bottles with autoclaved inocula or in bottles without inocula. Inocula obtained from different sampling points but the same location mostly had the same behavior of reductive dechlorination activities such as pathways and rates, intermediates and final end-products.

Age (days)	Benzene (μM)	MCB (μM)	1,2-DCB (μM)	1,3-DCB (μM)	1,4-DCB (μM)	1,2,4-TCB (μM)	1,2,3-TCB (μM)
0	0.0	0.0	0.0	0.0	0.0	80.0	80.0
33	0.0	14.5	1.1	0.0	2.1.0	67.5	65.6
63	2.1	43.2	3.0	2.4	4.4	27.3	35.5
91	2.3	52.1	4.4	8.1	7.7	11.7	17.8
130	2.9	67.8	3.0	6.9	11.3	0.0	0.0
130 *	2.9	67.8	3.0	6.9	11.3	80.0	80.0
165	4.8	87.0	4.3	6.1	5.9	65.5	71.2
186	7.7	108.9	6.7	8.8	12.6	40.7	44.3
255	11.3	155.4	12.0	17.8	14.4	20.9	24.9
289	12.2	179.1	19.9	21.2	23.1	0.0	0.0
289 *	12.2	179.1	19.9	21.2	23.1	80.0	80.0
333	15.2	221.1	17.6	25.1	24.2	43.0	51.1
390	18.9	290.7	12.6	29.2	43.4	0.0	0.0
390*	18.9	290.7	12.6	29.2	43.4	80.0	80.0
424	24.8	355.1	16.4	44.1	49.9	0.0	0.0
424*	24.8	355.1	16.4	44.1	49.9	80.0	80.0
482	34.6	481.1	19.6	55.9	77.8	0.0	0.0
482*	34.6	481.1	19.6	55.9	77.8	80.0	80.0
533	45.9	639.9	17.4	66.6	79.2	0.0	0.0
533*	45.9	639.9	17.4	66.6	79.2	80.0	80.0
590	62.2	788.8	11.3	74.4	67.4	0.0	0.0
590*	62.2	788.8	11.3	74.4	67.4	80.0	80.0
637	76.3	933.3	17.8	70.8	78.2	0.0	0.0
637*	76.3	933.3	17.8	70.8	78.2	80.0	80.0
670	97.6	1001.7	16.9	66.1	84.1	0.0	0.0
670*	97.6	1001.7	16.9	66.1	84.1	80.0	80.0
701	116.6	1131.4	17.2	55.5	89.9	0.0	0.0
701*	116.6	1131.4	17.2	55.5	89.9	80.0	80.0
774	129.6	1312.9	11.7	62.1	91.2	0.0	0.0

Table 1: Dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by a mixed culture obtained from sediment in Hue, Vietnam after over two years of consecutive incubation. The asterisks (*) indicate the time points of additional amendment with a mixture of 1,2,3- and 1,2,4-trichlorobenzene.

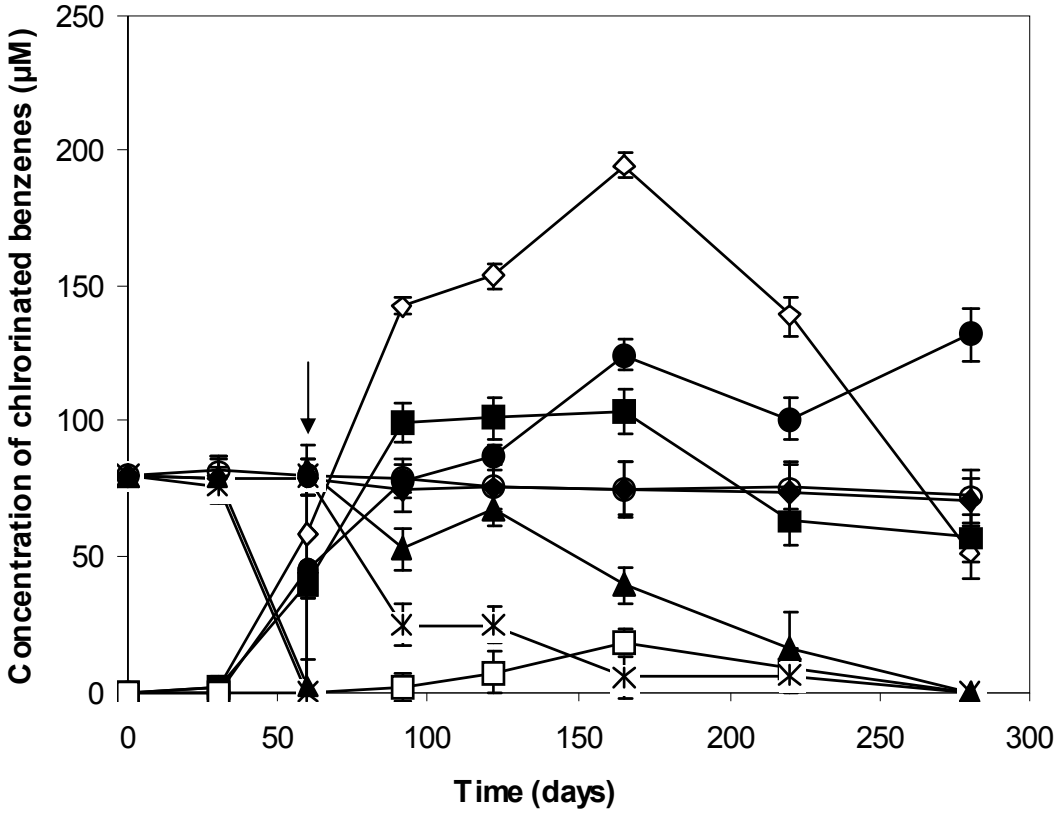

Figure 8: Dechlorination products from a mixture of 1,2,3- and 1,2,4-trichlorobenzene by a mixed culture from Leipzig lake's sediment. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with a mixture of 1,2,3- and 1,2,4-trichlorobenzene; (filled diamond) 1,2,3-trichlorobenzene of negative control with autoclaved cells and a mixture of 1,2,3- and 1,2,4-trichlorobenzene; the downwards arrow indicates the time point of additional amendment with a mixture of 1,2,3- and 1,2,4-trichlorobenzene. These values represent calculated, not measured concentrations. The error bars represent the standard deviation of the measurements of triplicate.

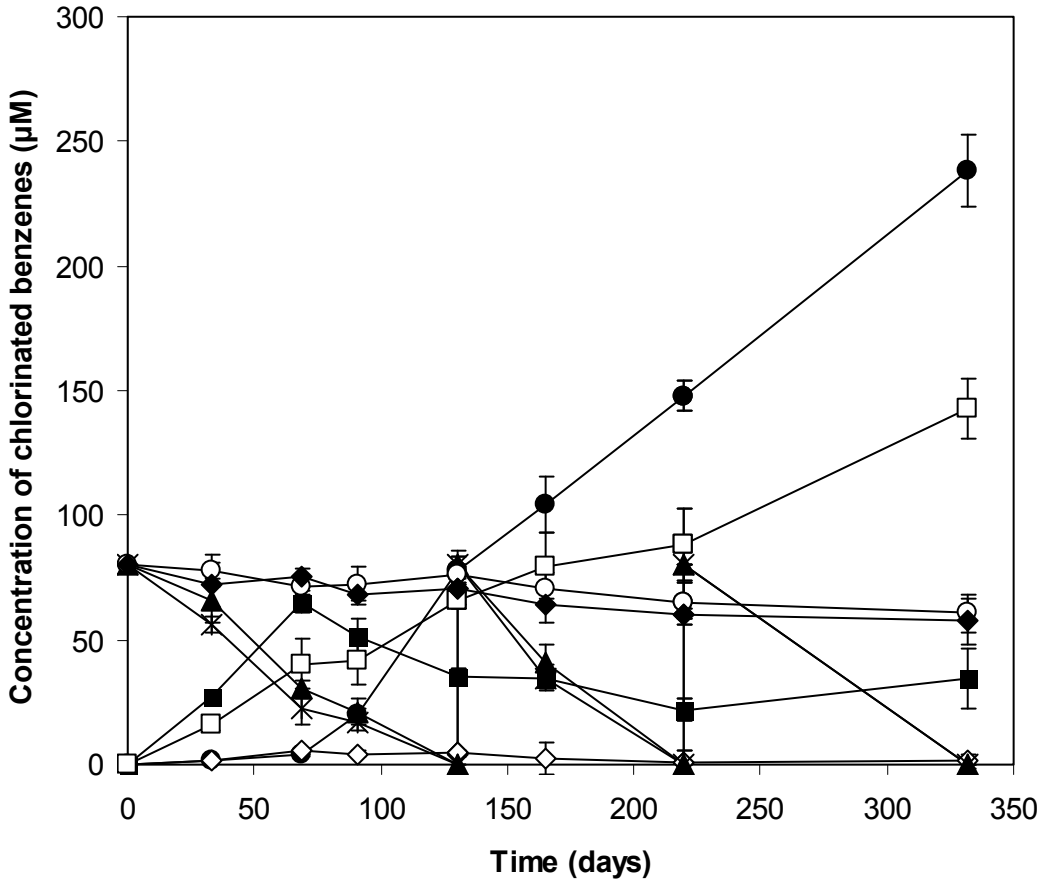

Figure 9: Dechlorination products from a mixture of 1,2,3- and 1,2,4-trichlorobenzene by a mixed culture from Hue canal's sediment. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with a mixture of 1,2,3- and 1,2,4-trichlorobenzene ; (filled diamond) 1,2,3-trichlorobenzene of negative control with autoclaved cells and a mixture of 1,2,3- and 1,2,4-trichlorobenzene; the downwards arrow indicates the time point of additional amendment with a mixture of 1,2,3- and 1,2,4-trichlorobenzene. These values represent calculated, not measured concentrations. The error bars represent the standard deviation of the measurements of triplicate.

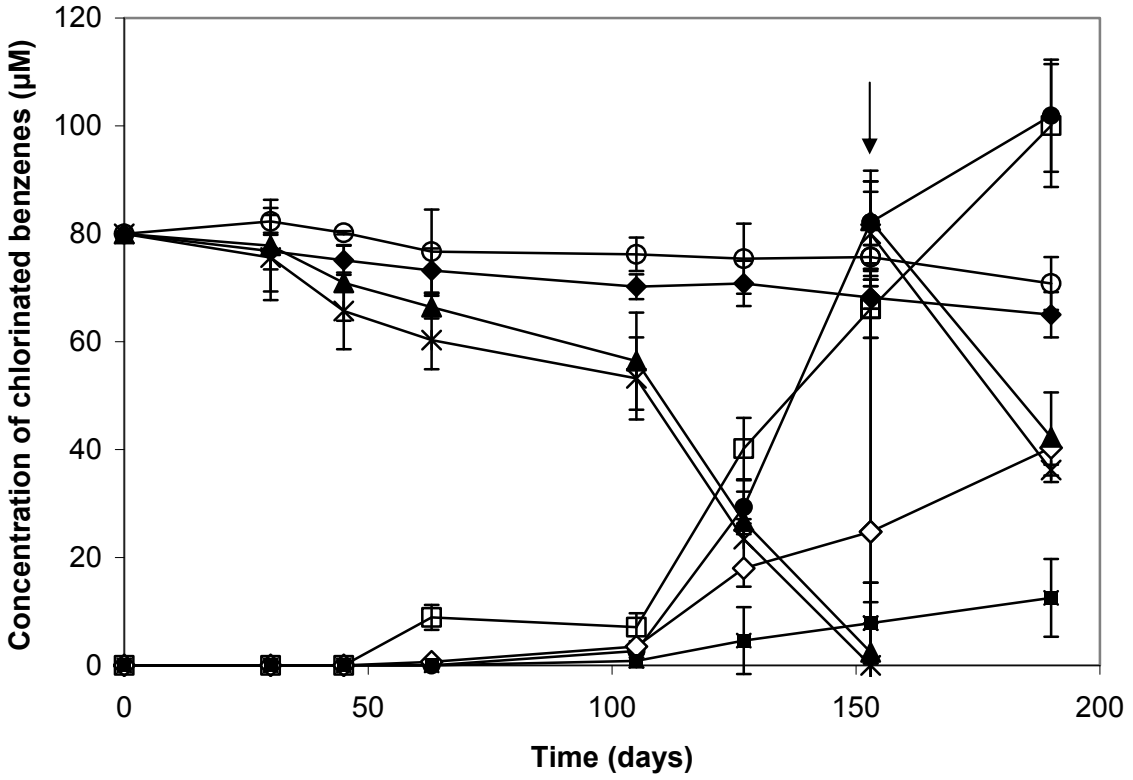

Figure 10: Dechlorination products from a mixture of 1,2,3- and 1,2,4-trichlorobenzene by a mixed culture from dioxin-contaminated soil in Hue. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with a mixture of 1,2,3- and 1,2,4-trichlorobenzene; (filled diamond) 1,2,3-trichlorobenzene of negative control with autoclaved cells and a mixture of 1,2,3- and 1,2,4-trichlorobenzene; the downwards arrow indicates the time point of additional amendment with a mixture of 1,2,3- and 1,2,4-trichlorobenzene. These values represent calculated, not measured concentrations. The error bars represent the standard deviation of the measurements of triplicate.

The cultures were transferred seven times in the same conditions of medium and electron acceptor with 5% (v/v) inocula from the most active parental cultures in triplicate. The results of these experiments show that the pathways of reductive dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene remained unchanged over seven sediment or soil free subcultures (Figure 14 A, 15 A and 16

A). Here, the figures describe the relative concentrations of 1,2,3- and 1,2,4-trichlorobenzene and all their products over all measurements of seven subcultures. The set-up of the figures was based on the mean degree of chlorination. All cultures containing 1,2,3- and 1,2,4-trichlorobenzene had a mean degree of dechlorination of 3 at the time zero of incubation (meaning that each benzene had three chlorine substituents). When the value of 3 dropped to 2 or 1, this means that all trichlorobenzenes were transformed to dichlorobenzenes and monochlorobenzene, respectively. Results of all cultures from one inoculum source were put into one graph. Interestingly, with mixed cultures amended with sediment from Hue canal, benzene was found after 3- 6 months of incubation in most of the third generation of sub-cultures onwards. However, the concentration of benzene in most cases increased very slowly over time and was below 10 µM. Experiments were not carried out to find evidence of anaerobic biotransformation of benzene to the nontoxic products, CO_2 or CH_4 (Liang et al. 2013).

To elucidate the pathway of reductive dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzenes, 1,2,3- and 1,2,4-trichlorobenzene were tested as single compound with subcultures originating from the lake's sediments in Leipzig and sediments from Hue's canal and dioxin-contaminated soil. For 1,2,3-trichlorobenzene, monochlorobenzene and 1,2-dichlorobenzene were products of transformation by cultures from subcultures originating from Leipzig sediment (Figure 11). Monochlorobenzene and 1,2- and 1,3-dichlororbenzenes were found in the cultures originating from Hue canal and dioxin-contaminated soil in Hue and monochlorobenzene was the main product of both cultures (Figure 12 and Figure 13, respectively). With 1,2,4-trichlorobenzene as a sole electron acceptor, monochlorobenzene and all three isomers of dichlorobenzenes appeared in subcultures from the three different sources except 1,2-dichlorobenzene in subcultures stemming from soil. Monochlorobenzene and 1,4-dichlorobenzene were the main products of transformation of 1,2,4-trichlorobenzene by subcultures from soil and Hue canal's sediment. Subcultures from Leipzig lake's sediment transformed 1,2,4-trichlorobenzene to monochlorobenzene and 1,3-

dichlorobenzene. Interestingly, benzene was found in the subcultures from Hue canal's sediment. There was however no evidence of benzene in any of the subcultures from the three original inocula amended with monochlorobenzene as sole electron acceptor after six months of incubation.

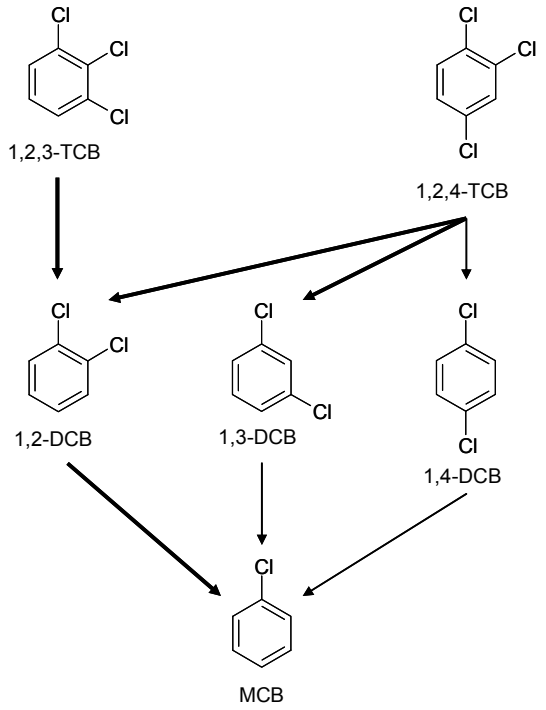

Figure 11: Proposed reductive dechlorination pathway of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by mixed cultures from sediments in, Leipzig, Germany. The bold black arrows indicate the main pathway.

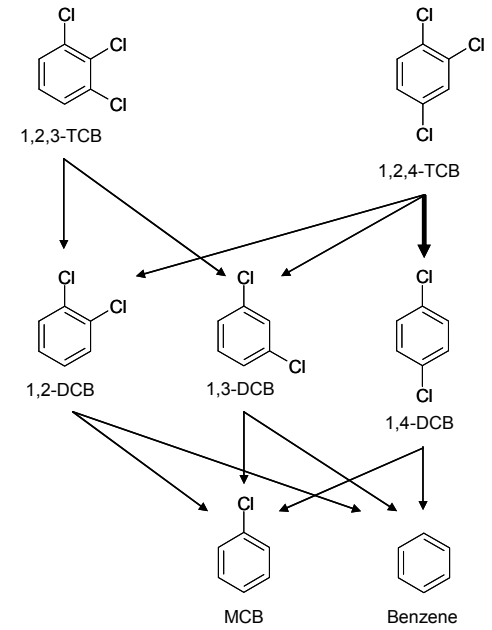

Figure 12: Proposed reductive dechlorination pathway of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by the mixed cultures from Hue canal's sediments in Vietnam. The bold black arrow indicates the main pathway.

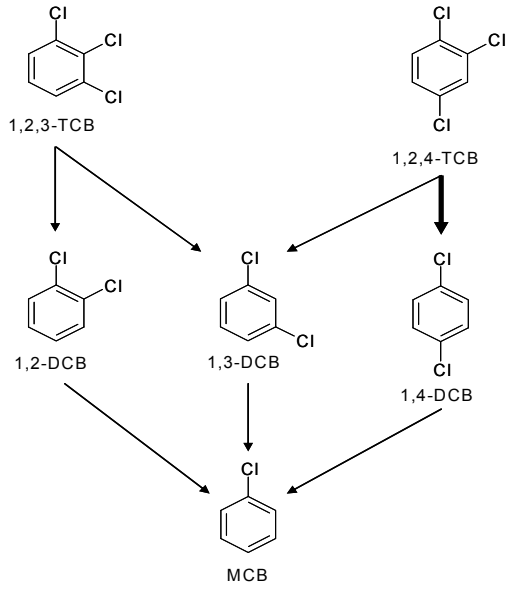

Figure 13: Proposed reductive dechlorination pathway of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by mixed cultures from dioxin–contaminated soil in Hue, Vietnam. The bold black arrow indicates the main pathway.

For the cultures from Leipzig sediment, dechlorination pattern tended to prefer to remove singly flanked chlorine substituents than to remove doubly flanked chlorine substituents. Firstly, both 1,2,3- and 1,2,4-trichlorobenzene were dechlorinated predominantly to 1,2-dichlorobenzene and monochlorobenzene. 1,2-dichlorobenzene was mainly produced from 1,2,3-trichlorobenzene which is proved by 1,2-dichlorobenzene being detected as only intermediate of subcultures supplemented with only 1,2,3-trichlorobenzene and a trace amount of 1,2-dichlorobenzene in subcultures with sole 1,2,4-trichlorobenzene. 1,3-dichlorobenzene was found with rather high concentration which resulted from transformation of singly flanked chlorine of 1,2,4-trichlorobenzene not from 1,2,3-trichlorobenzene. The result was confirmed by absence of 1,3-dichlorobenzene in cultures with only 1,2,3-trichlorobenzene as electron acceptor and its predominance in cultures amended with sole 1,2,4-trichlorobenzene. A trace amount of 1,4-dichlorobenzene was produced from 1,2,4-trichlorobenzene. After both trichlorobenzenes were mostly dechlorinated, 1,2- and 1,3-dichlorobenzene were dechlorinated to monochlorobenzene.

In contrast, cultures enriched from soil and sediments from Vietnam preferentially removed doubly flanked and isolated chlorine substituents although also singly flanked chlorines were removed. Bacteria which preferentially eliminate doubly flanked chlorine played a determined role to transfer 1,2,3- and 1,2,4-trichlorobenzene mainly to monochlorobenzene and 1,4-dichlorobenzene. 1,2- and 1,3-dichlorobenzene appeared in cultures with low concentrations. When 1,2,3- and 1,2,4-trichlorobenzene was nearly used up, bacteria which preferentially remove isolated chlorines transformed most 1,4-dichlorobenzene to monochlorobenzene.

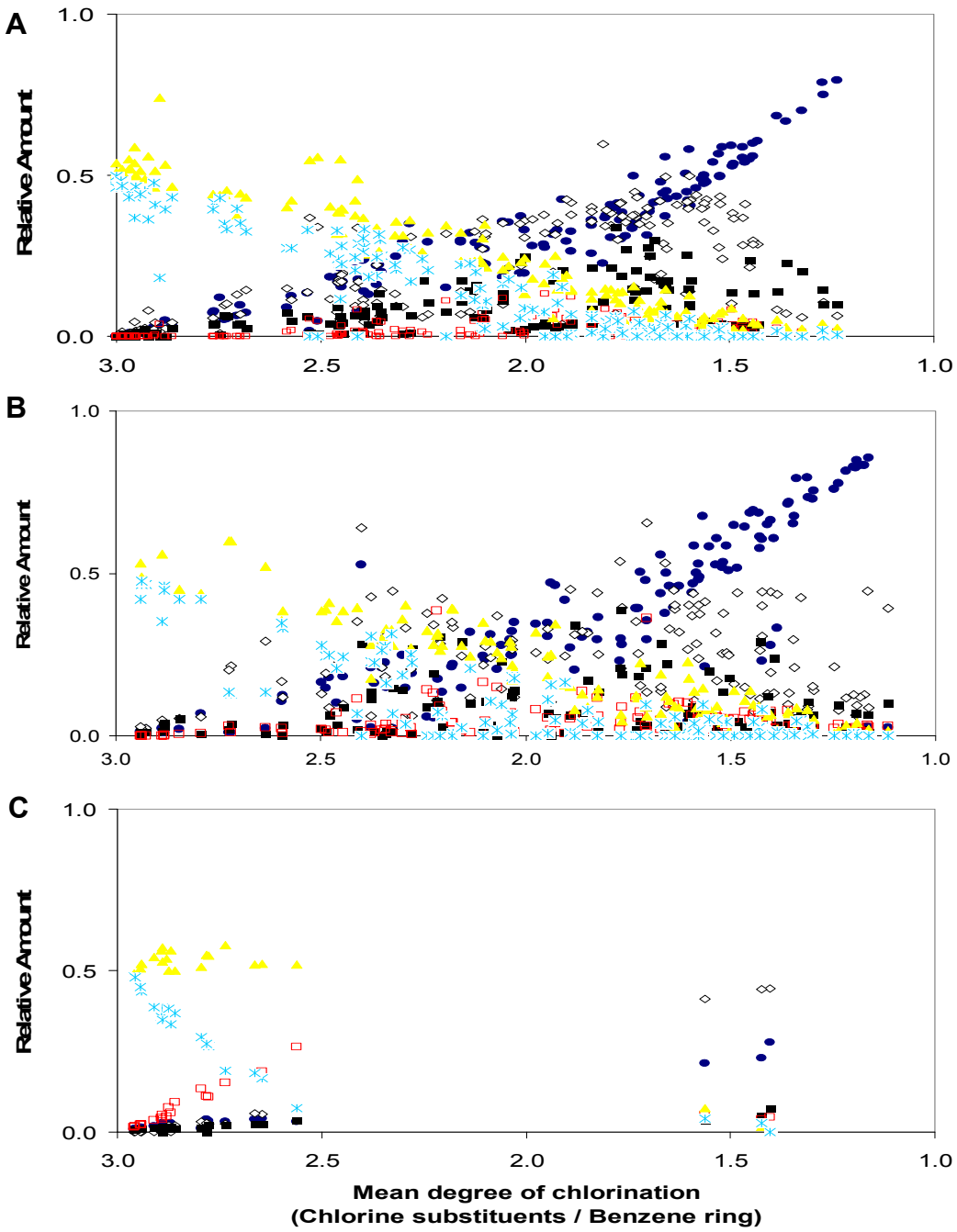

Figure 14: Chlorobenzene dechlorination patterns from a mixture of 1,2,3- and 1,2,4-trichlorobenzene in seven consecutive transfers of the mixed culture enriched from Leipzig sediment. **Panel A:** Standard cultures; **Panel B:** Cultures in which the inoculum was briefly exposed to oxygen; **Panel C:** Cultures with vancomycin. Symbols: (yellow filled triangle) 1,2,3-trichlorobenzene; (cyan asterisk) 1,2,4-trichlorobenzene; (red open square) 1,4-dichlorobenzene; (black filled square) 1,3-dichlorobenzene; (black open diamond) 1,2-dichlorobenzene; (blue filled circle) monochlorobenzene.

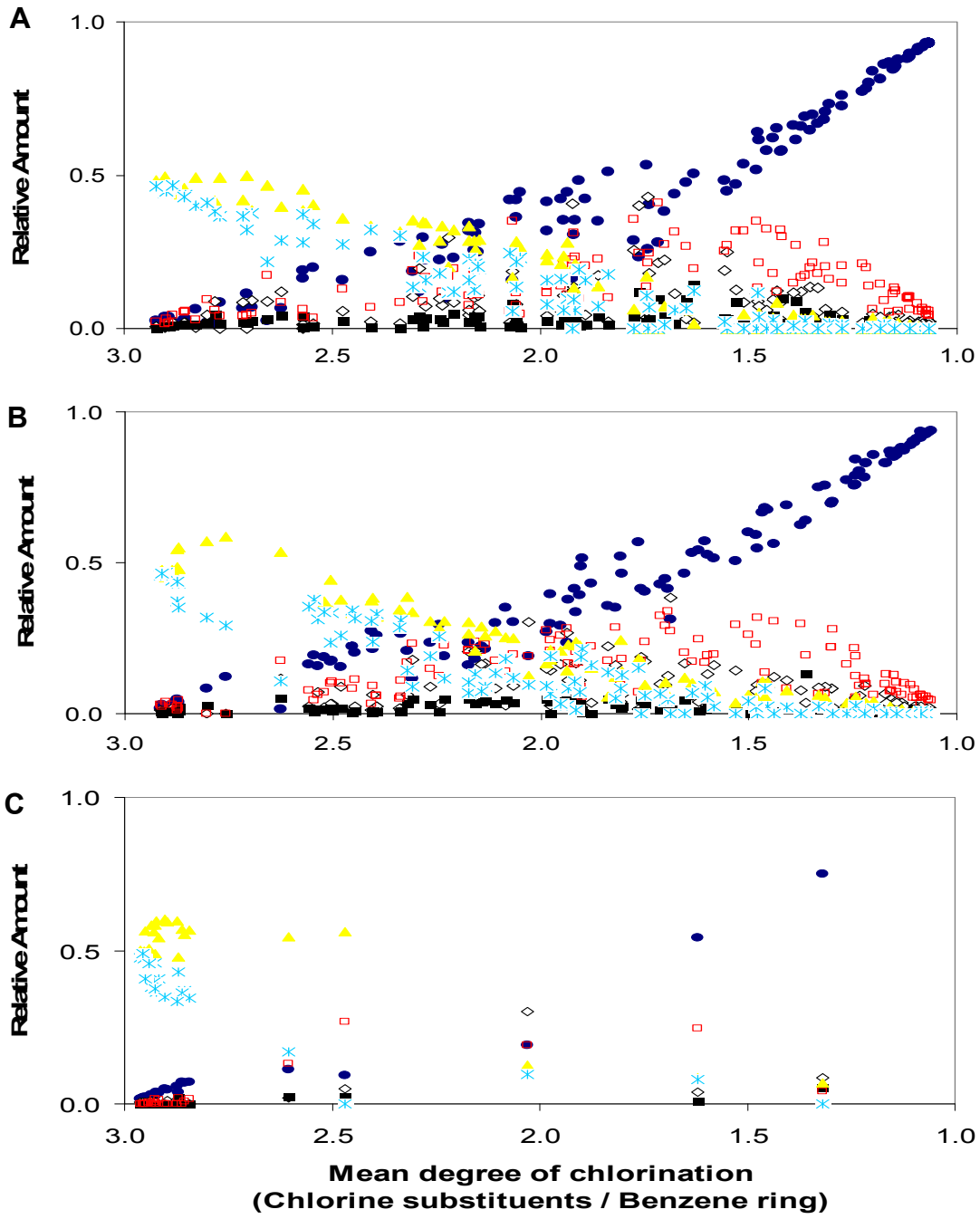

Figure 15: Chlorobenzene dechlorination patterns from a mixture of 1,2,3- and 1,2,4-trichlorobenzene in seven consecutive transfers of the mixed culture enriched from Hue canal's sediment. **Panel A:** Standard cultures; **Panel B:** Cultures in which the inoculum was briefly exposed to oxygen; **Panel C:** Cultures with vancomycin. Symbols: (yellow filled triangle) 1,2,3-trichlorobenzene; (cyan asterisk) 1,2,4-trichlorobenzene; (red open square) 1,4-dichlorobenzene; (black filled square) 1,3-dichlorobenzene; (black open diamond) 1,2-dichlorobenzene; (blue filled circle) monochlorobenzene.

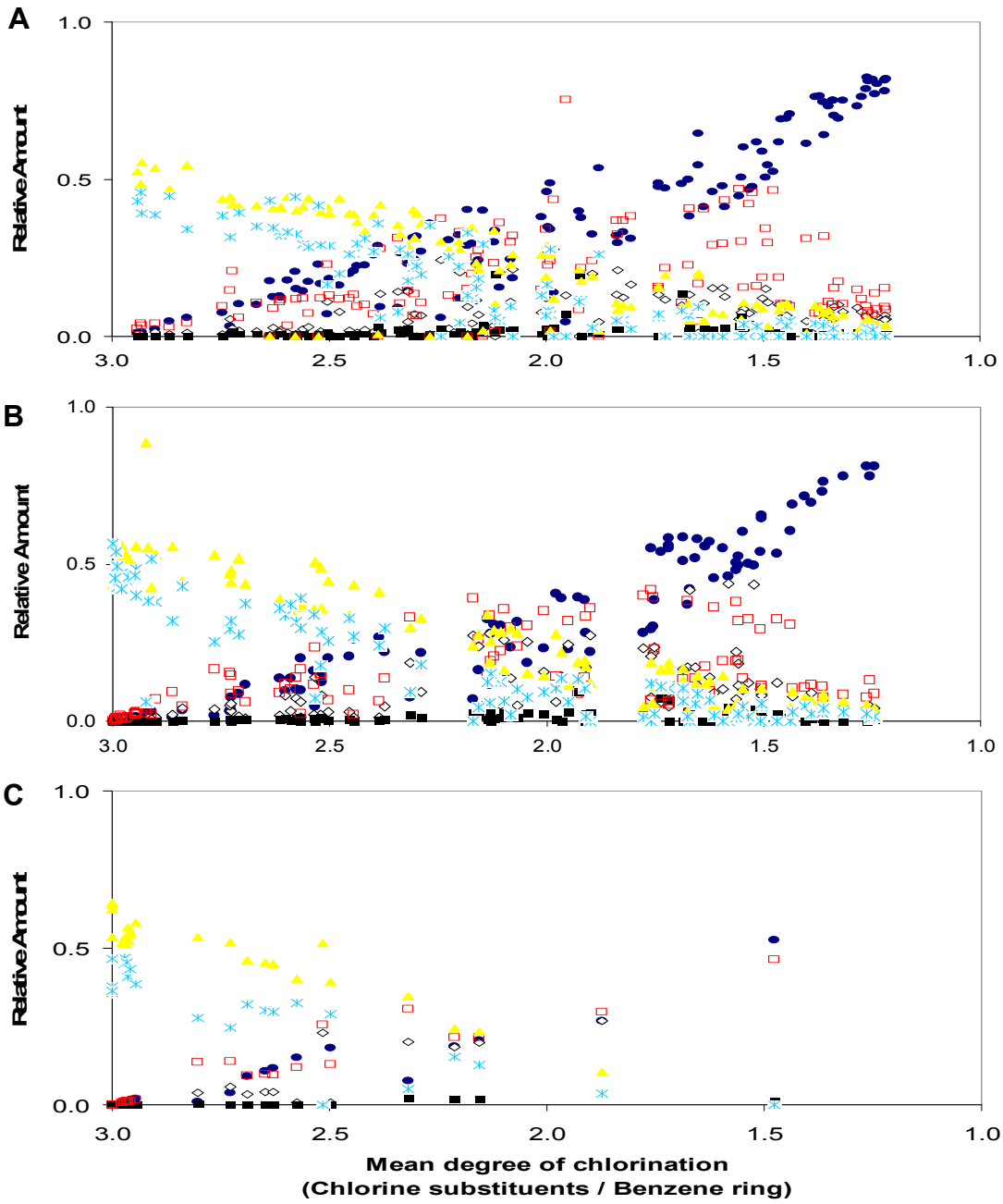

Figure 16: Chlorobenzene dechlorination patterns from a mixture of 1,2,3- and 1,2,4-trichlorobenzene in seven consecutive transfers of the mixed culture enriched from dioxin contaminated-soil in Hue. **Panel A:** Standard cultures; **Panel B:** Cultures in which the inoculum was briefly exposed to oxygen; **Panel C:** Cultures with vancomycin. Symbols: (yellow filled triangle) 1,2,3-trichlorobenzene; (cyan asterisk) 1,2,4-trichlorobenzene; (red open square) 1,4-dichlorobenzene; (black filled square) 1,3-dichlorobenzene; (black open diamond) 1,2-dichlorobenzene; (blue filled circle) monochlorobenzene.

3.2 Dechlorination of hexachlorobenzene by mixed cultures

Culture flasks containing synthetic medium were amended with hexachlorobenzene crystals as electron acceptor and inoculated with sediment samples from Germany or Vietnam or dioxin–contaminated soil. Hexachlorobenzene was added directly as crystals to the medium (approximately 10 mg per 30 mL of liquid medium) before the flasks were sealed and autoclaved. There was no detection of products from hexachlorobenzene transformation in the cultures amended with soil. Only one of ten cultures containing sediments from Leipzig's lake reductively dechlorinated hexachlorobenzene after 1 month of incubation. Products from hexachlorobenzene transformation were monochlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene. Low concentrations of 1,3,5-trichlorobenzene were found after 100 days of incubation but disappeared later after about 200 days of incubation (Figure 17). Interestingly, all the cultures spiked with sediment from Hue's canal bio-transformed hexachlorobenzene after 1 month of incubation and the products of hexachlorobenzene transformation were similar to those of cultures from Leipzig's lake. Monochlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene were found but 1,2-dichlorobenzene did not appear. Traces of 1,3,5-trichlorobenzene were detected after a period of 30 days of incubation but the appearance of this highly persistent organic compound was not stable over time (Figure 18). The products and pathway of transformation of hexachlorobenzene by mixed cultures obtained from different inocula but the same sampling area were similar. Negative controls containing hexachlorobenzene but no inocula and cultures inoculated with autoclaved samples did not show any products from hexachlorobenzene within the period of 200 days of incubation.

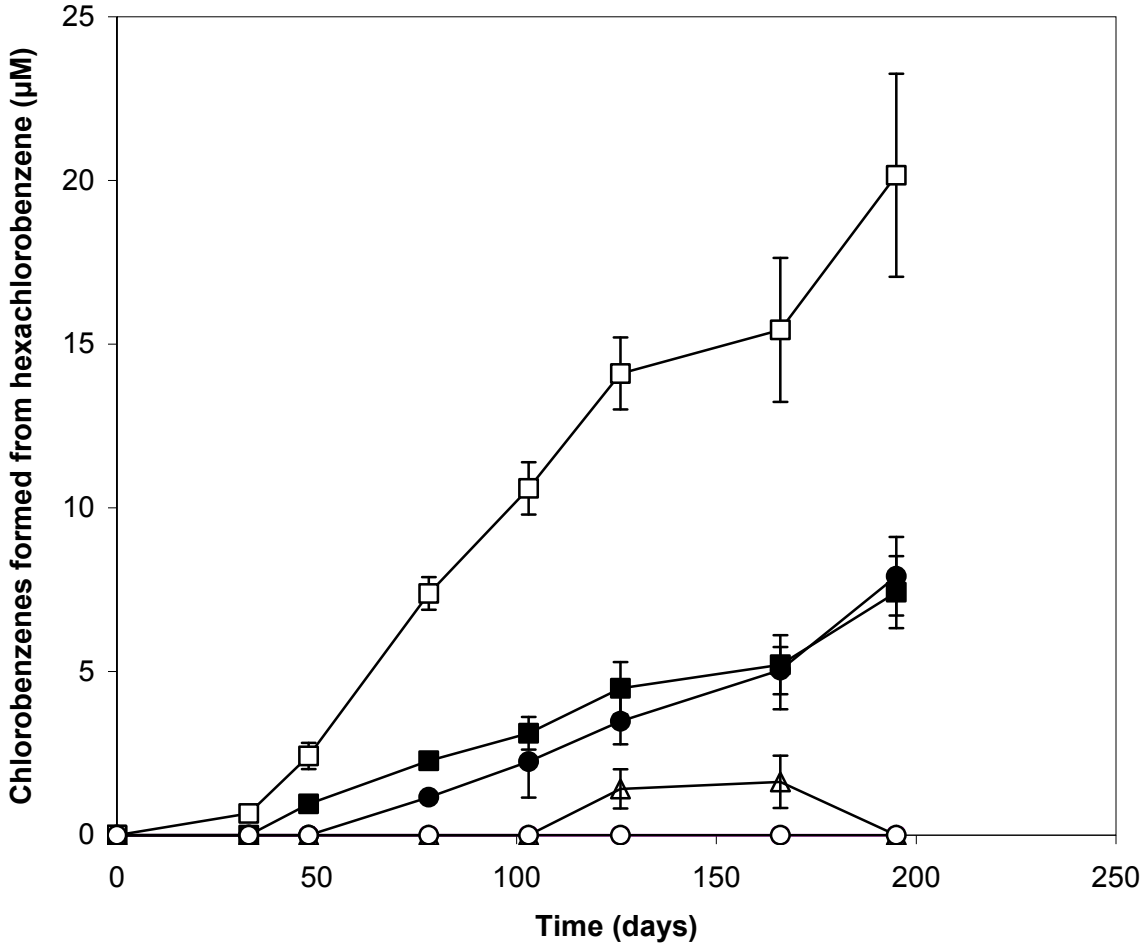

Figure 17: Dechlorination products from hexachlorobenzene by a mixed culture from Germany. Hexachlorobenzene was added in crystalline form and could not be quantified. Penta- and tetrachlorobenzenes were not detected as intermediates. Symbols: (open triangle) 1,3,5-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) all products from hexachlorobenzene transformation of negative control without cells but with hexachlorobenzene and negative control with autoclaved cells and hexachlorobenzene.

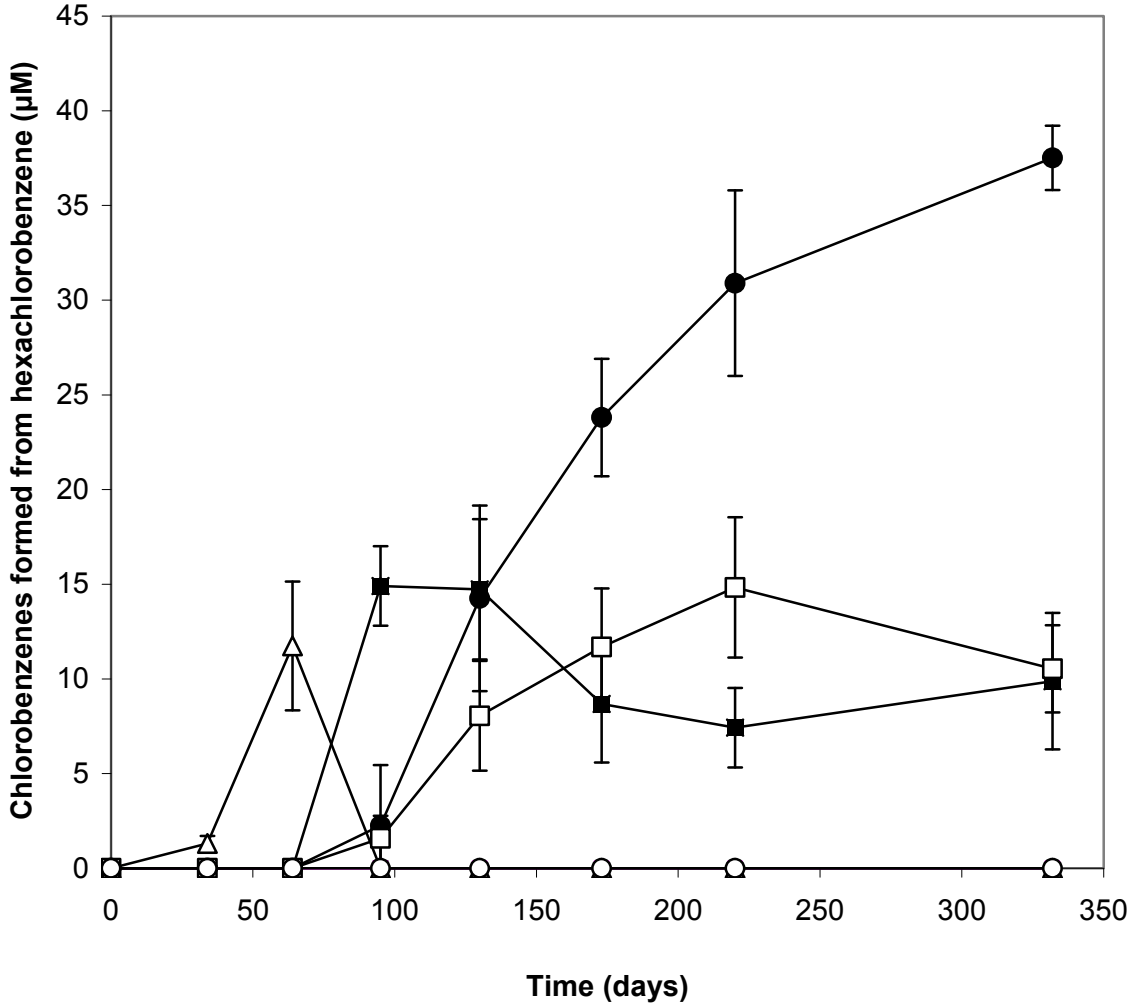

Figure 18: Dechlorination products from hexachlorobenzene by a mixed culture from sediment of Hue canal, Vietnam. Hexachlorobenzene was added in crystalline form and could not be quantified. Penta- and tetrachlorobenzenes were not detected as intermediates. Symbols: (open triangle) 1,3,5-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) all products from hexachlorobenzene transformation of negative control without cells but with hexachlorobenzene and negative control with autoclaved cells and hexachlorobenzene.

The cultures amended with sediments from Leipzig and Hue were transferred seven times each in triplicate with 5% inocula in the same medium. This resulted in cultures that were completely free of sediment and other undefined components. 1,4-dichlorobenzene and monochlorobenzene were produced as the

main final end-products from hexachlorobenzene transformation in all seven transfers. 1,2- and 1,3-dichlorobenzenes were also found in low concentrations as products of hexachlorobenzene dechlorination. Traces of benzene were detected in the subcultures from the third generation onwards and the concentrations of benzene increased very slowly over time but all of them were under 10 μM. However, most importantly, 1,3,5-trichlorobenzene, a persistent organic pollutant that was produced from hexachlorobenzene by the initial enrichment cultures, was not found in any of the subcultures (Figure 19 A and 20 A). The pathway of reductive dechlorination of hexachlorobenzene remained stable over all seven transfers.

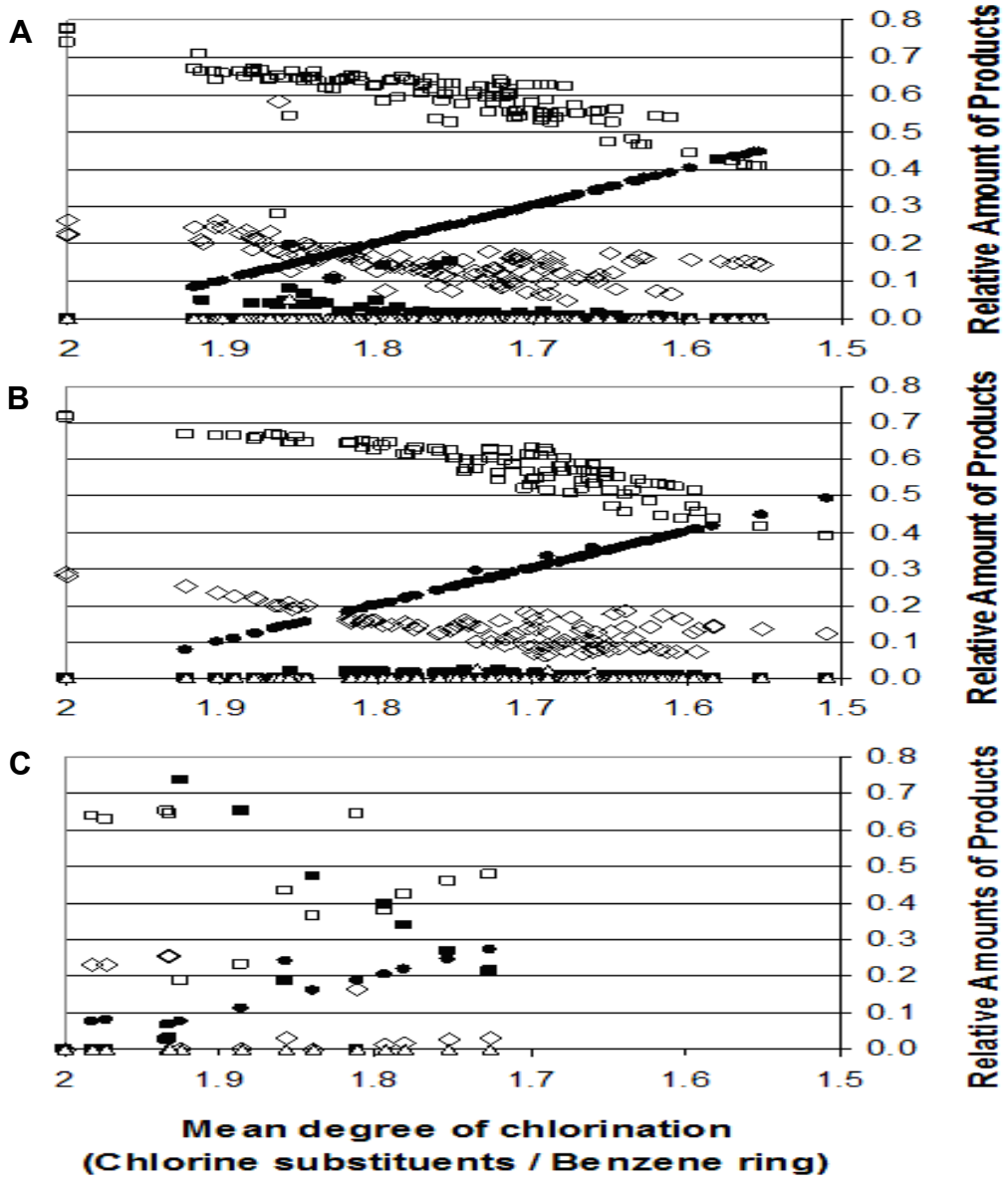

Figure 19: Product formation patterns from hexachlorobenzene in seven consecutive transfers of the mixed culture enriched from Germany. The plot was calculated according to a previously described procedure (Hölscher et al. 2010) and allows comparison of dechlorination pathways independent from the dechlorination rate. Hexachlorobenzene was added in crystalline form and could not be quantified. Penta- and tetrachlorobenzenes were not detected. **Panel A**: Standard cultures; **Panel B**: Cultures in which the inoculum was briefly exposed to oxygen; **Panel C**: Cultures with vancomycin. Symbols: (open triangle) 1,3,5-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene (filled circle) monochlorobenzene.

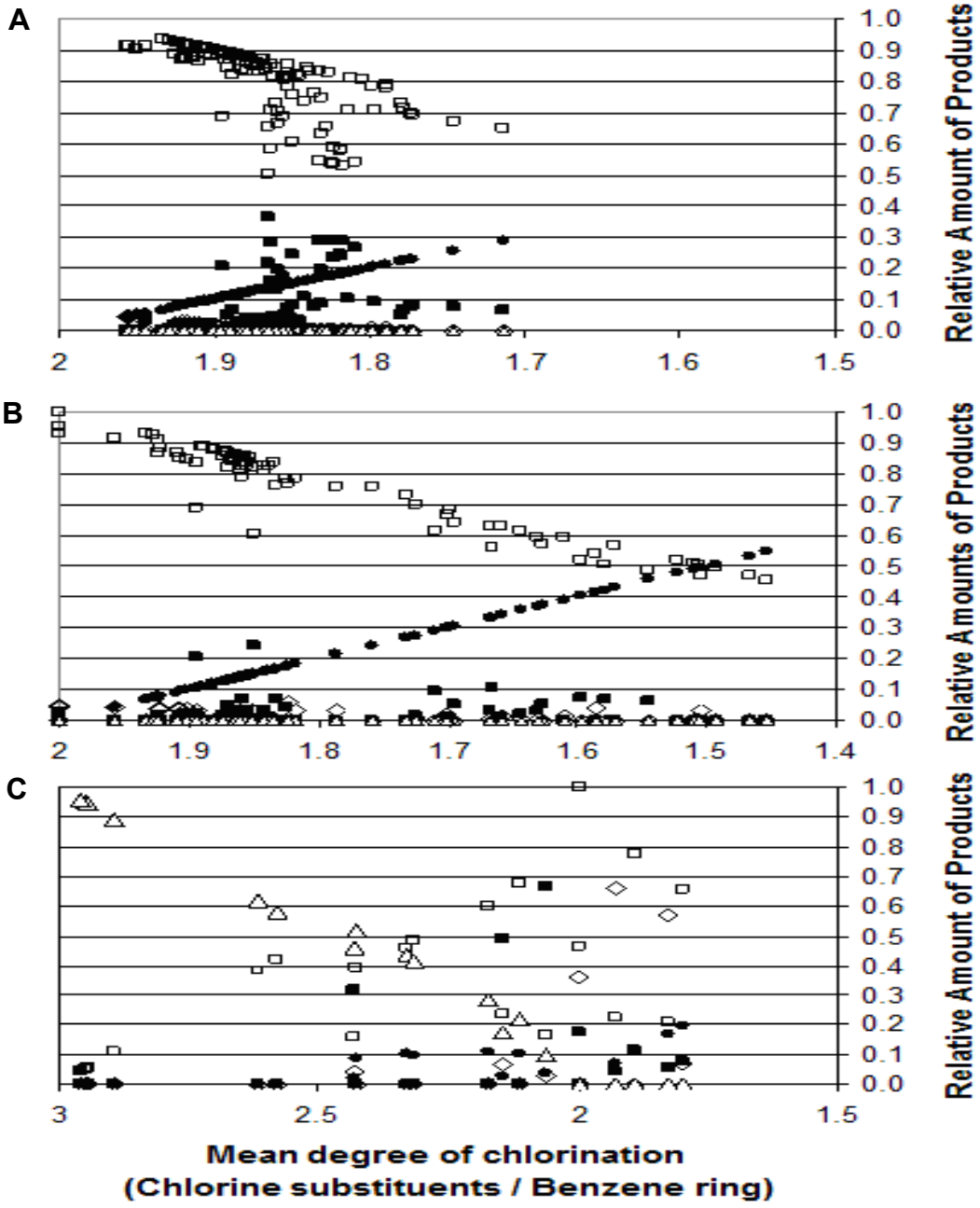

Figure 20: Product formation patterns from hexachlorobenzene in seven consecutive transfers of the mixed culture enriched from Hue sediment. Hexachlorobenzene was added in crystalline form and could not be quantified. Penta- and tetrachlorobenzenes were not detected. **Panel A**: Standard cultures; **Panel B**: Cultures in which the inoculum was briefly exposed to oxygen; **Panel C**: Cultures with vancomycin. Symbols: (open triangle) 1,3,5-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene.

The pathway of biotransformation of hexachlorobenzene was illustrated by testing all isomers of trichlorobenzenes, dichlorobenzenes and monochlorobenzene as single substrate with subcultures from Hue's canal and Leipzig's lake sediments. Results of transformation of all isomers of trichlorobenzene indicated that 1,3,5-trichlorobenzene was transformed to 1,3-dichlorobenzene and monochlorobenzene as intermediate and final end-product, respectively for both subcultures. There was a difference in transformation of 1,2,3-trichlorobenzene and 1,2,4-trichlorobenzene between sub-cultures from Leipzig lake and Hue canal. Sub-cultures from Leipzig lake reductively dechlorinated 1,2,3-trichlorobenzene to 1,2-dichlorobenzene and monochlorobenzene and 1,2,4-trichlorobenzene to 1,4-dichlorobenzene. For the sub-cultures from Hue canal, 1,4-dichlorobenzene and 1,2-dichlorobenzene were the products of 1,2,4-trichlorobenzene and 1,2,3-trichlorobenzene degradation, respectively. Besides, monochlorobenzene was also found in both cultures amended with 1,2,3- and 1,2,4-trichlorobenzene as single electron acceptor. With all dichlorobenzene isomers, monochlorobenzene was the final end-product of bio-degradation of 1,2- and 1,3-dichlorobenzene by subcultures from Leipzig and Hue canal. Moreover, traces of benzene were also found in Hue canal subcultures amended with 1,3-dichlorobenzene (Figure 22). Only benzene but not monochlorobenzene was found in 1,4-dichlorobenzene cultures inoculated with subcultures from Leipzig (Figure 21). In contrast, both benzene and monochlorobenzene were found in 1,4-dichlorobenzene cultures spiked with Hue canal's subcultures. Results of monochlorobenzene testing reveal that none of the two subcultures dechlorinated this substrate when it was added solely.

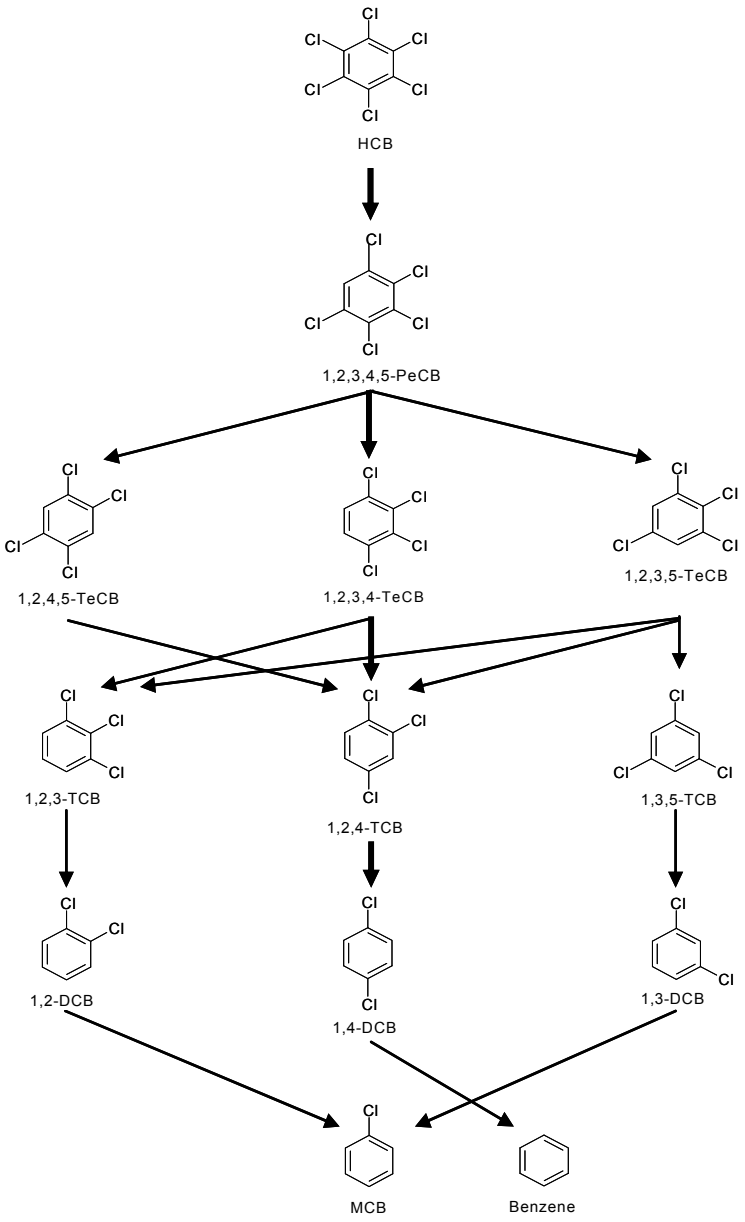

Figure 21: Proposed reductive dechlorination pathway of hexachlorobenzene by mixed cultures from lake's sediments in Leipzig, Germany. The bold black arrow was the main pathway.

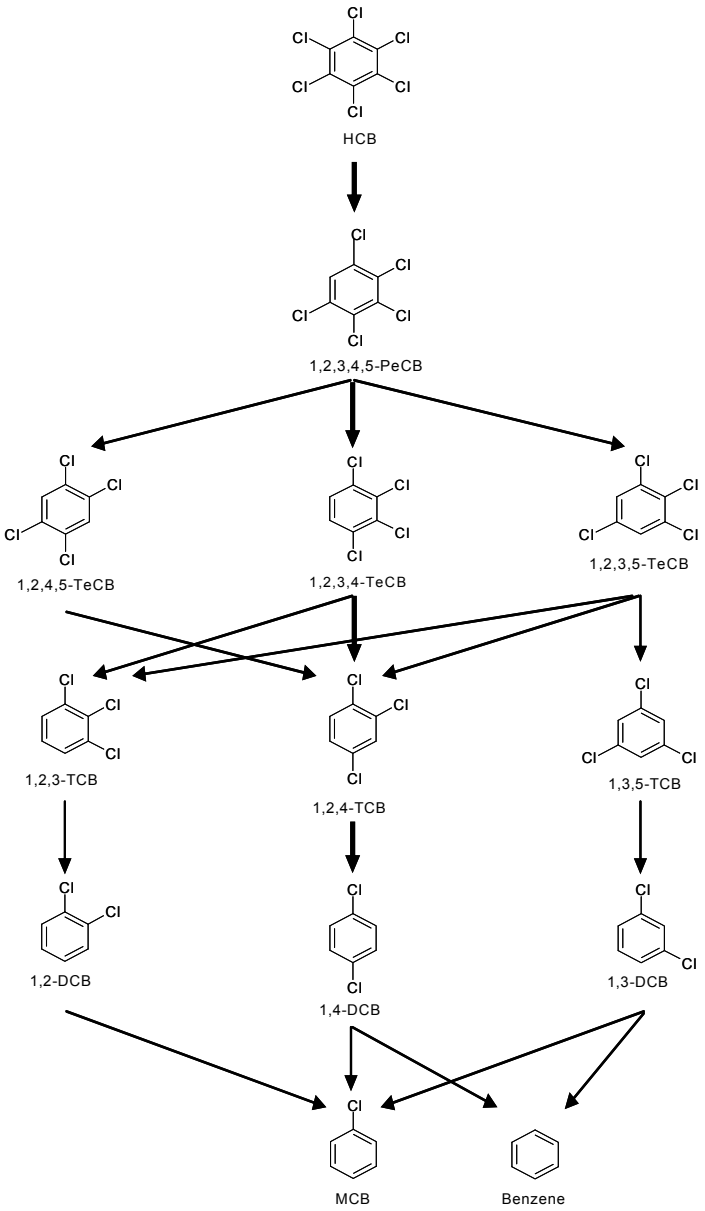

Figure 22: Proposed reductive dechlorination pathway of hexachlorobenzene by mixed cultures from sediments in Vietnam. The bold black arrow was the main pathway.

3.3 Dechlorination of 1,3,5-trichlorobenzene by mixed cultures

The transformation of the most persistent of the three trichlorobenzene isomers, 1,3,5-trichlorobenzene was investigated in cultures spiked with sediments from Leipzig lake, Hue canal or dioxin–contaminated soil. There was no detection of any product of 1,3,5-trichlorobenzene transformation in the cultures containing sediment form Leipzig lake or dioxin–contaminated soil. However, seven out of

nine cultures amended with sediments from Hue canal showed activity against 1,3,5-trichlorobenzene to 1,3-dichlorobenzene and monochlorobenzene as intermediate and final end-product, respectively (Figure 23). No reductive dechlorination products from 1,3,5-trichlorobenzene were found in negative controls without cells or autoclaved inocula but containing 1,3,5-trichlorobenzene for the period of about eleven months of incubation.

The cultures were also transferred seven times each in triplicate with 5% inocula in the same medium. The reductive dechlorination ability of 1,3,5-trichlorobenzene of mixed cultures growing in synthetic medium that was completely free of sediment remained unchanged over the seven generations. Benzene was also found in the third generation of subcultures onwards with a low concentration below 10 µM although its concentration increased with time (Figure 24).

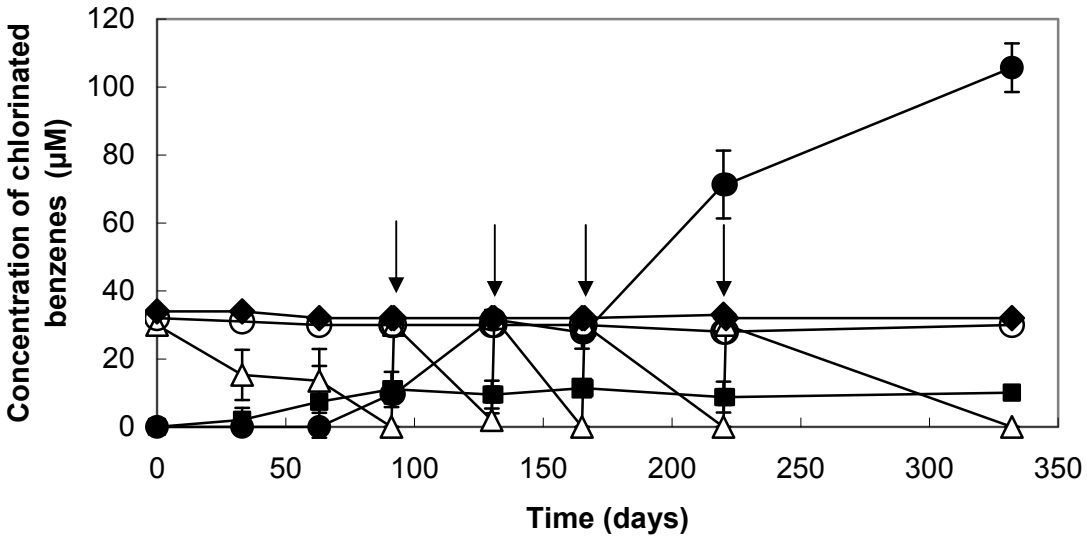

Figure 23: Dechlorination of 1,3,5-trichlorobenzene by a mixed culture enriched from the canal sediment in Hue, Vietnam. Symbols: (open triangle) 1,3,5-trichlorobenzene; (filled square) 1,3-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,3,5-trichlorobenzene of negative control without cells but with 1,3,5-trichlorobenzene; (filled diamond) 1,3,5-trichlorobenzene of negative control with autoclaved cells and 1,3,5-trichlorobenzene. The downwards arrows indicate time points of additional amendment with 1,3,5-trichlorobenzene. These values represent calculated, not measured concentrations.

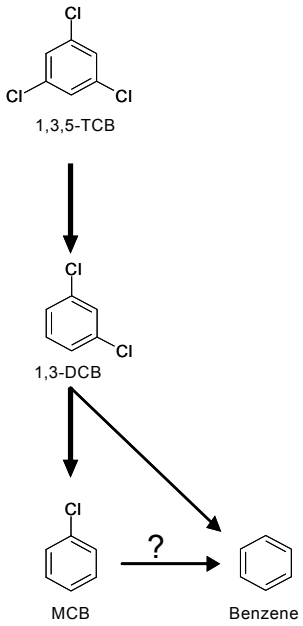

Figure 24: Proposed reductive dechlorination pathway of 1,3,5-trichlorobenzene by mixed cultures from sediments in Vietnam. The bold black arrow was main pathway.

3.4 Effects of vancomycin on the transformation of mixture of 1,2,3- and 1,2,4-trichlororbenzenes, hexachlorobenzene and 1,3,5-trichlorobenzene by mixed cultures

To investigate the influence of cell wall antibiotics on the dechlorination activity of bacteria in mixed cultures originating from all sources of inocula, vancomycin was applied at a concentration of 5 mg L^{-1} to the second most active subcultures in triplicate. The results demonstrate that dechlorination activities towards all compounds were strongly inhibited by the presence of vancomycin and that the dechlorination pathway was changed. Transformation of a mixture of 1,2,3- and 1,2,4-trichlorobenzene in cultures from all different inocula was only found within 6 months of incubation with low concentrations of 1,4-dichlorobenzene and monochlorobenzene as intermediate and final end-product, respectively. However, after six months of incubation monochlorobenzene and all isomers of dichlorobenzene were formed. Transformation of this mixture of chlorinated benzenes was not exposed in further subcultures containing vancomycin which received inocula from previous vancomycin-containing cultures (Figure 14 C; 15 C

and 16 C). The same trend was repeated with the vancomycin-containing cultures amended with hexachlorobenzene or 1,3,5-trichlororbenzene, only trace amounts of 1,3,5-trichlorobenzene and very low concentrations of 1,3-dichlorobenzene were formed from hexachlorobenzene and 1,3,5-trichlorobenzene, respectively within 6 months of incubation. However, stronger dechlorination started after 6 months of incubation. Then, monochlorobenzene and all of the isomers of dichlorobenzene and 1,3,5-trichlorobenzene were produced from hexachlorobenzene (Figure 19 C and 20 C). When further subcultures were set up from vancomycin-containing cultures, again containing vancomycin, only 1,3,5-trichlorobenzene was found with very low concentration. This was true also for 1,3,5-trichlorobenzene cultures containing vancomycin, which first were slow in dechlorination to 1,3-dichlorobenzene but in which transformation rates increased significantly after 6 months of incubation (Figure 25). Further application of vancomycin to a next transfer containing 1,3,5-trichlorobenzene revealed that dechlorination activity was completely inhibited by this antibiotic.

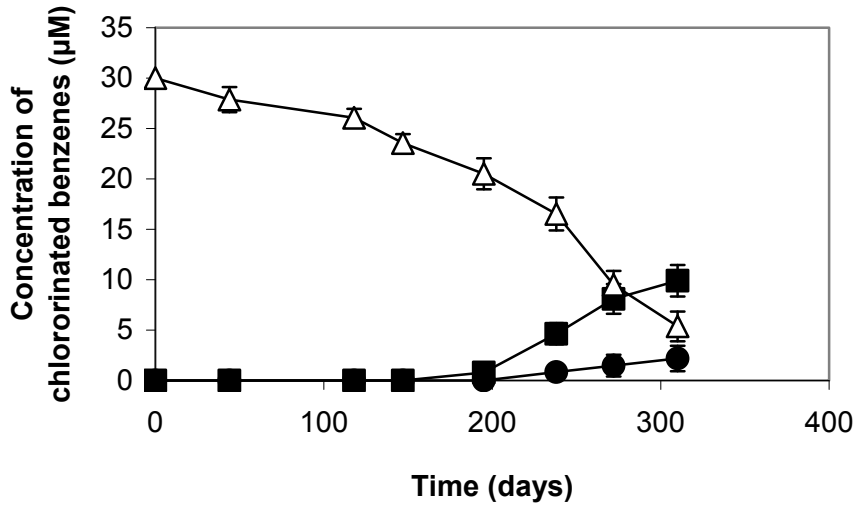

Figure 25: Reductive dechlorination of 1,3,5-trichlorobenzene in the presence of 5 mg L^{-1} vancomycin by the mixed culture enriched from canal sediments in Hue, Vietnam. Symbols: (open triangle) 1,3,5-trichlorobenzene; (filled square) 1,3-dichlorobenzene; (filled circle) monochlorobenzene. 1,3,5-trichlorobenzene loss within the first 150 days represents the rate at which 1,3,5-trichlorobenzene was escaping from the cultures through the injured Teflon liner into the septa and was also seen in negative controls.

3.5 Effects of oxygen exposure of the inoculum on the transformation of a mixture of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene and 1,3,5-trichlorobenzene by mixed cultures

The oxygen tolerance of dechlorinating bacteria was tested by exposing inocula of the second transfer to oxygen till the inocula turned pink before injecting into fresh medium. The pink colour is originating from the redox indicator resazurin and demonstrates a positive redox potential in the sample. In cultures supplied with an oxygen-exposed inoculum, transformation of a mixture of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene and 1,3,5-trichlorobenzene occurred at the same rate as in cultures with inocula without oxygen exposure. The main products of biodegradation of a mixture of 1,2,3- and 1,2,4-trichlorobenzene were monochlorobenzene and 1,4-dichlorobenzene and small concentrations of 1,2- and 1,3-dichlorobenzenes were found in all of subcultures (Figure 14 B; 15 B and 16 B). In addition, benzene was also detected in some subcultures with all concentrations below 10 μM. Hexachlorobenzene was mainly transformed to monochlorobenzene and 1,4-dichlorobenzene with smaller amounts of other dichlorobenzenes being formed. 1,3,5-trichlorobenzene was not produced from hexachlorobenzene in cultures with oxygen-exposed inocula (Figure 19 B and 20 B) as also seen in the positive control cultures without oxygen (Figure 19 A and 20 A). Benzene was produced in some subcultures with trace concentrations although its concentration increased with time. Similarly as in cultures with non-oxygen-exposed inocula, 1,3,5-trichlorobenzene was transformed via 1,3-dichlorobenzene to monochlorobenzene as the final end product (Figure 26). The patterns of reductive dechlorination of the mixture 1,2,3- and 1,2,4-trichlorobenzene and two persistent organic compounds, hexachlorobenzene and 1,3,5-trichlorobenzene remained unchanged over all of the subcultures, each exposed to oxygen. There was no change in the rate of reductive dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene and 1,3,5-trichlorobenzene transformation between cultures exposed to oxygen and those not exposed to oxygen (positive control). However, the rate of the sub-step of 1,3-dichlorobenzene transformation to monochlorobenzene in the 1,3,5-

trichlorobenzene dechlorinating subcultures was slower in most of the oxygen-exposed cultures compared to those without oxygen treatment.

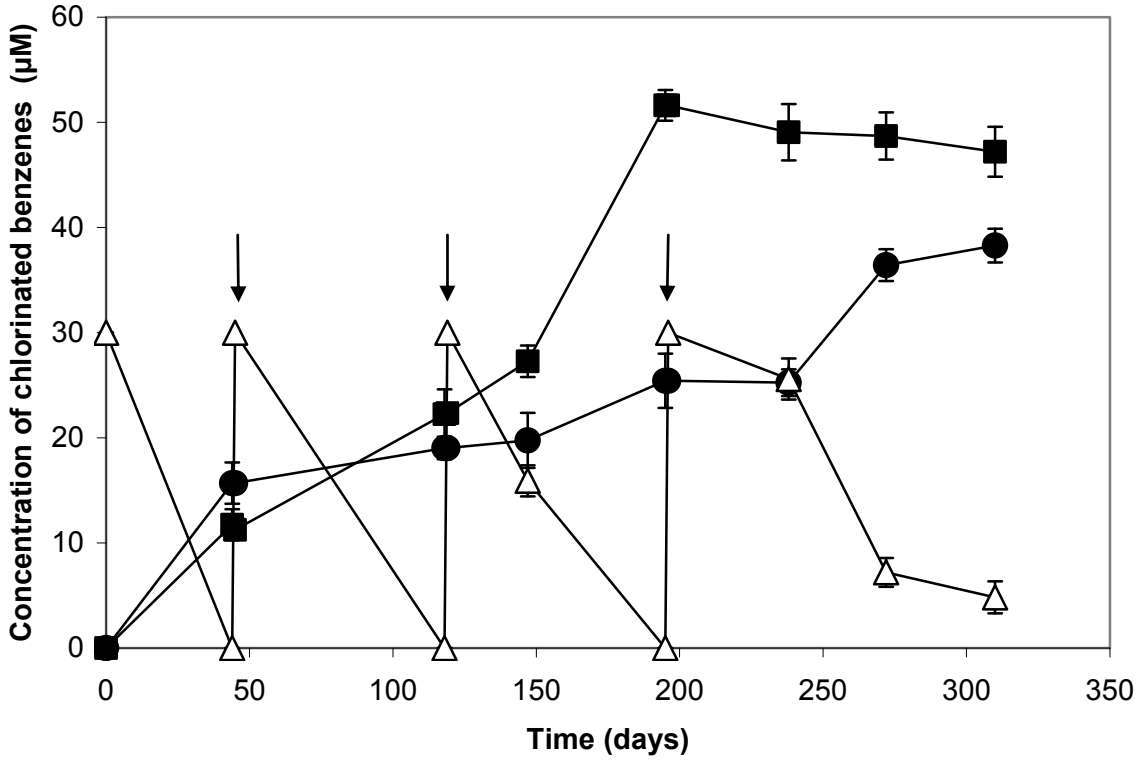

Figure 26: The effect of oxygen on the reductive dechlorination of 1,3,5-trichlorobenzene by a mixed culture enriched from the canal sediment in Hue, Vietnam. Symbols: (open triangle) 1,3,5-trichlorobenzene; (filled square) 1,3-dichlorobenzene; (filled circle) monochlorobenzene; the downwards arrows indicate time points of additional amendment with 1,3,5-trichlorobenzene. These values represent calculated, not measured concentrations.

3.6 Detection of *Dehalococcoides mccartyi* strains in mixed cultures

To screen for the presence of *Dehalococcoides mccartyi* strains in mixed cultures, the *Dehalococcoides*-targeted primers 728F and 1172R (Löffler et al. 2000) were used for PCR amplifications of DNA samples extracted from mixed cultures growing on the medium containing a mixture of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene or 1,3,5-trichlorobenzene. With some of the samples no bands were detectable by agarose gel electrophoresis. Other samples gave bands but

their sizes were smaller than expected and also smaller than PCR products obtained from genomic DNA of *Dehalococcoides mccartyi* strains CBDB1 and 195 used as positive controls. In addition, the results of cultures exposed to oxygen, which has been described to be toxic for *Dehalococcoides* strains, and cultures added with vancomycin, an antibiotic inhibiting the synthesis of a peptidoglycan cell wall which is absent in *Dehalococcoides* (Adrian et al. 2000; He et al. 2003; Maymó-Gatell et al. 1997) confirmed again the absence of *Dehalococcoides* strains in all mixed cultures (Figure 27).

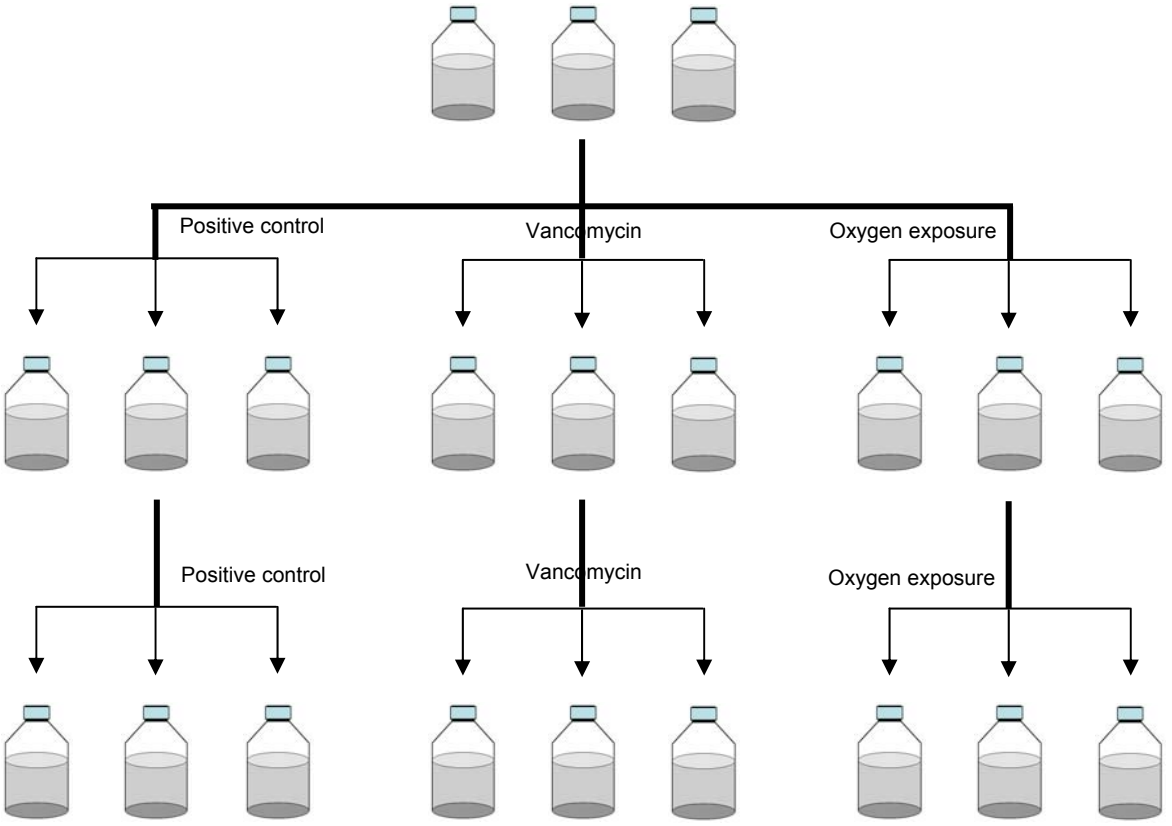

Figure 27: Cultures were tested with vancomycin and oxygen exposure. The antibiotic and oxygen-exposed inocula were applied to the second generation and further applications for a total of seven transfers were carried out if the cultures showed dechlorination activity. The standard medium contained inocula but did not apply vancomycin and without exposing with oxygen as positive control.

3.7 Isolation, morphology and phylogenetic analysis of a pure strain

From active mixed cultures, an attempt to isolate a pure strain was carried out on a series of shakes containing 2 mL of low melting temperature agarose and 10 mL of fresh medium. Colonies with different colour, shape and size from the shakes of the highest dilution at level of 10^{-5} to 10^{-6} were targeted for the next transfer. However, picking up colonies for the next transfer would be stopped if colonies did not show dechlorination activity by measuring GC of 1 mL of the lowest dilution shake extraction. The dechlorination activity of colonies was confirmed by detection of any traces of dechlorination products from all isomers of trichlorobenzene or hexachlorobenzene as electron acceptors after one month of incubation. The active colonies were supposed to be pure after six or seven consecutive transfers if they showed that they belonged to only one kind of colony with the same shape, size and colour. Chlorinated benzenes and hydrogen were employed as electron acceptors and electron donor respectively for the isolation procedure. Then the pure colonies were picked-up and transferred to flasks containing fresh liquid medium and chlorobenzenes as electron acceptor and hydrogen as electron donor. The purity of the isolated strain was confirmed by sequencing the 16S rRNA gene and comparing the sequence with BLASTN to the nr-database of NCBI.

One pure chlorobenzene-dechlorinating strain was obtained from the mixed culture inoculated with dioxin-contaminated soil in Hue, Vietnam. The isolated 16S rRNA gene sequence analysis of this bacterium with 1397 base pairs revealed that its 16S rRNA gene was very similar to that of *Desulfotomaculum guttoideum* strain DSM 4024 with 99% sequence identity (Figure 44). Therefore it was designated as *Desulfotomaculum guttoideum* strain VN1. Cells were observed often in pairs of two joined cells or single cells (Figure 28) and stained gram negative. The cells grew in deep-agarose dilutions forming white, small and very faint colonies.

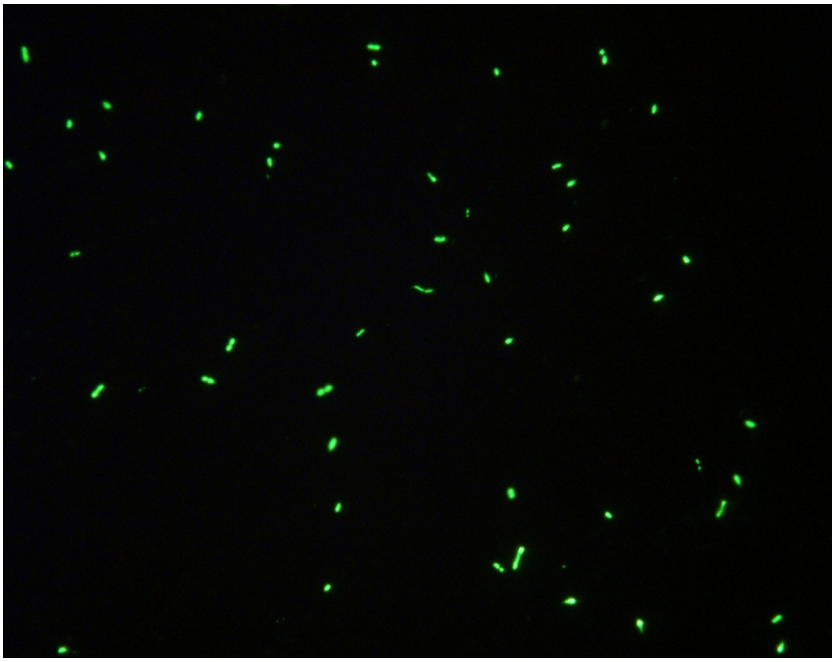

Figure 28: The shape of strain VN1 under the fluorescence microscope after staining with SYBR Green.

3.8 Dehalogenation ability of strain VN1

3.8.1 Dechlorination ability of strain VN1

To investigate the dechlorination ability of strain VN1, hexachlorobenzene, all trichlorobenzene isomers, all dichlorobenzene isomers or monochlorobenzene were added as sole electron acceptor to the cultures containing strain VN1. Hexachlorobenzene was directly added as crystal to the medium with approximately 10 mg per 30 mL of liquid medium before the flasks were sealed and autoclaved and other electron acceptors were applied as solution in acetone. The most active cultures in triplicate were transferred at least three times to confirm the dechlorination activity and stable dechlorination pathway. In the hexachlorobenzene cultures, dechlorination did not occur. The same was repeated with the culture amended with 1,3,5-trichlorobenzene. However, dechlorination activity occurred only in the cultures containing 1,2,3-trichlorobenzene and 1,2,4-trichlorobenzene as sole electron acceptor. 1,2,3-trichlorobenzene was mainly transformed to 1,2-dichlorobenzene and monochlorobenzene as intermediate and

final end-product respectively and a trace concentration of 1,3-dichlorobenzene (Figure 29 A). In the cultures supplemented with 1,2,4-trichlorobenzene, only 1,4-dichlorobenzene was detected (Figure 29 B). A further transformation of 1,4-dichlorobenzene to monochlorobenzene or benzene was not found in the cultures containing 1,2,4-trichlorobenzene and in the cultures spiked with 1,4-dichlorobenzene as original substrate. Similarly, 1,3-dichlorobenzene was not dechlorinated by strain VN1 when this chlorinated organic compound was tested as single initial substrate. However, there was an opposite result with 1,2-dichlorobenzene. This compound was quickly transformed to monochlorobenzene by strain VN1 as final end-product (Figure 29 C). Reductive dechlorination of monochlorobenzene was not found in strain VN1 when cultures received only this compound. When strain VN1 was cultivated with a mixture of 1,2,3- and 1,2,4-trichlorobenzene, the products were similar to those of the parental mixed culture from which strain VN1 was isolated: Monochlorobenzene and all isomers of dichlorobenzene; however, monochlorobenzene and 1,4-dichlorobenzene were the main products (Figure 29 D). Nevertheless, monochlorobenzene, 1,2- and 1,3-dichlorobenzene originated completely from 1,2,3-trichlorobenzene transformation not from 1,2,4-trichlorobenzene, whereas monochlorobenzene and 1,3-dichlorobenzene in the parental mixed culture were produced from both trichlorobenzenes. The growth of cells was monitored by counting cells after staining with SYBR Green I on agarose slides at the same time of measuring dechlorination activity. The results of cell counting indicated that the number of cells increased parallel with the increase of concentrations of dechlorination products. Negative controls containing all tested congeners of chlorobenzene as unique substrate but no strain VN1 did not generate any products.

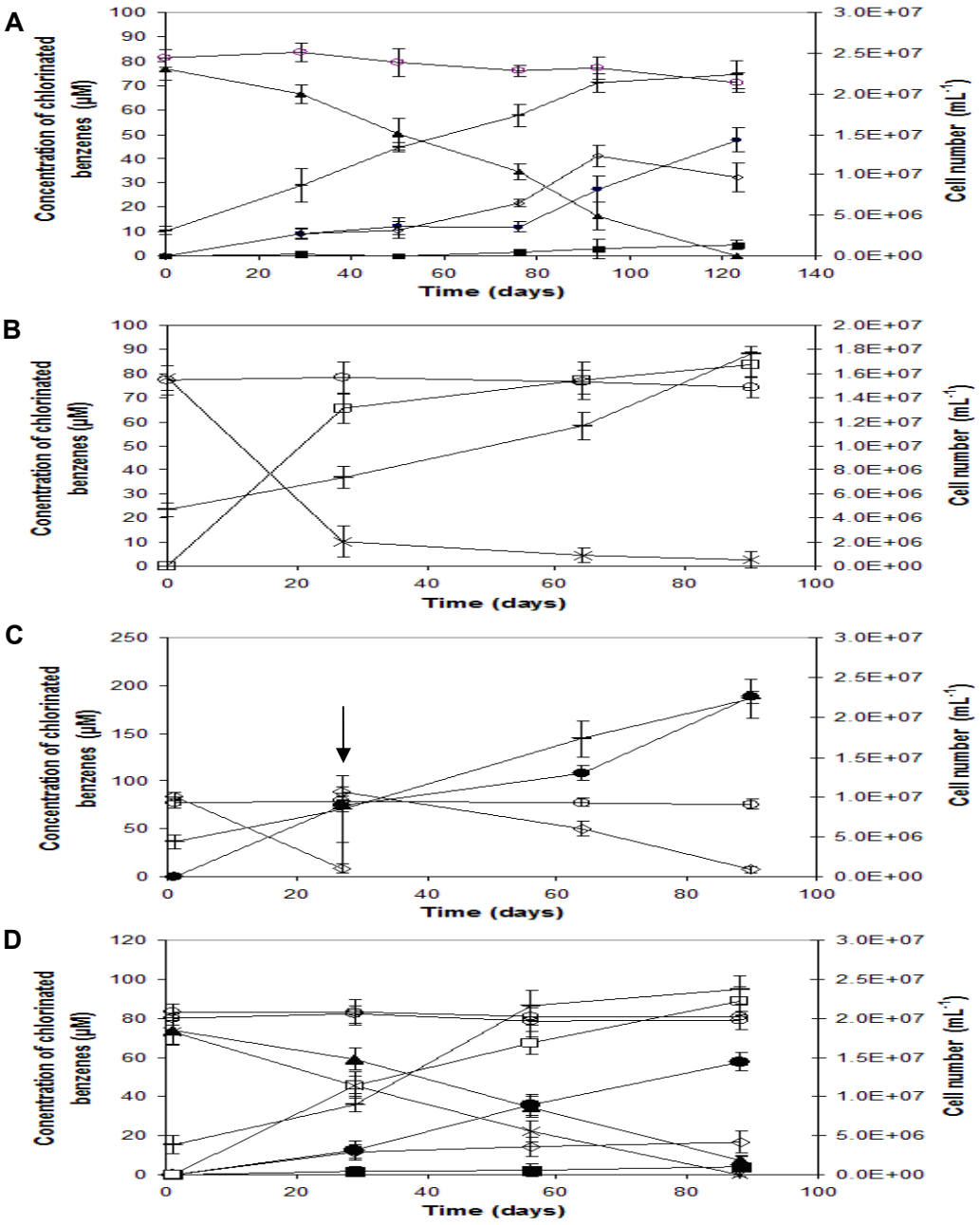

Figure 29: Dechlorination products from 1,2,3-trichlorobenzene **(A)**; 1,2,4-trichlorobenzene **(B)**; 1,2-dichlorobenzene **(C)**; and a mixture of 1,2,3 and 1,2,4-trichlorobenzene **(D)**, by strain VN1. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3- or 1,2,4-trichlorobenzene or 1,2-dichlorobenzene or 1,2,3-trichlorobenzene of a mixture of 1,2,3 and 1,2,4-trichlorobenzene of negative controls without cells; (dash) number of cell mL^{-1}; the downwards arrow indicates time point of additional amendment with 1,2-dichlorobenze. This value represents a calculated, not measured concentration.

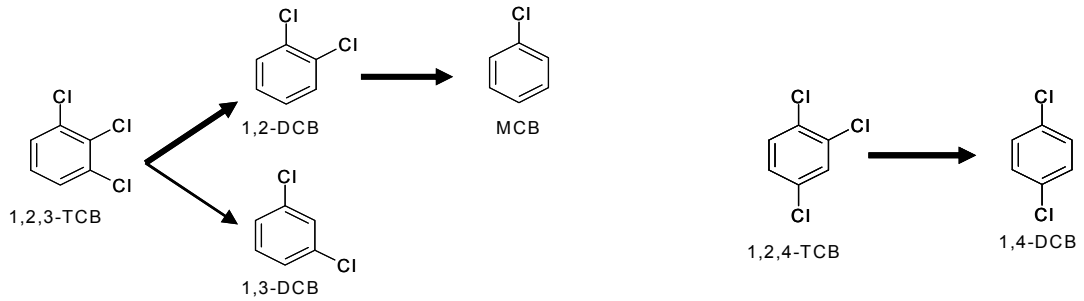

Figure 30: Proposed reductive dechlorination of 1,2,3-trichlororbenzene and 1,2,4-trichlorobenzene by strain VN1. The bold black arrow was the main pathway.

3.8.2 Debromination ability of strain VN1

Strain VN1 was also examined for its debromination ability with different congeners of brominated benzene. 1,2,4-tribromobenzene was completely debrominated by strain VN1 to benzene as main final end-product, monobromobenzene and all isomers of dibromobenzene were detected as intermediates. Similarly, benzene and monobromobenzene were also the final end-product and intermediate, respectively of all isomers of dibromobenzene (1,2-; 1,3- and 1,4-dibromobenzenes) transformation and monobromobenzene was debrominated to benzene when this compound was added to strain VN1 cultures as sole electron acceptor. Interestingly, hexabromobenzene was converted by strain VN1 while this bacterium could not dechlorinate hexachlorobenzene and products of hexabromobenzene transformation were monobromobenzene, all isomers of dibromobenzene and 1,2,4-tribromobenzene. Formation of 1,2,3- and 1,3,5-tribromobenzenes was not confirmed because the used gas chromatographic methods could not differentiate these two tribromobenzene isomers. Debromination activity of strain VN1 remained stable over three consecutive transfers. The results of cell counting illustrate that number of cells increased parallel with debromination during the incubation time (Figure 31 A; 31 B; 31 C and 31 D). No products were found in the flasks containing all tested congeners of bromobenzene as unique substrate but no strain VN1 (negative controls).

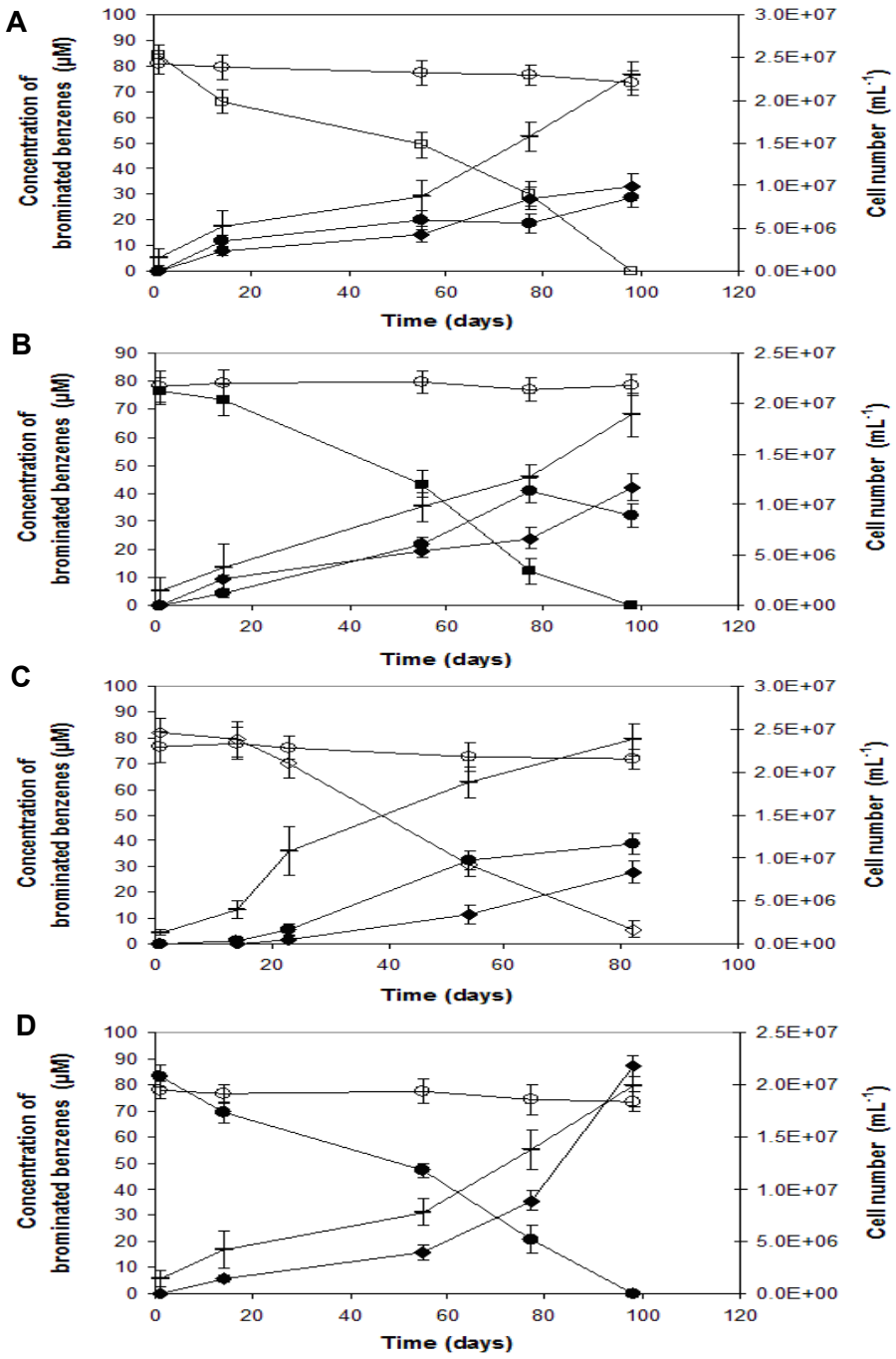

Figure 31 A, B, C, D: Debromination products from 1,4-dibromobenzene; 1,3-dibromobenzene; 1,2-dibromobenzene, monobromobenzene, respectively by strain VN1. Symbols: (open square) 1,4-dibromobenzene; (filled square) 1,3-dibromobenzene; (open diamond) 1,2-dibromobenzene; (filled circle) monobromobenzene; (filled diamond) benzene; (open circle) 1,4-dibromobenzene or 1,3-dibromobenzene or 1,2-dibromobenzene or monobromobenzene of negative control without cells; (dash) number of cells mL^{-1}. The error bars represent the standard deviation of the measurements of triplicate cultures.

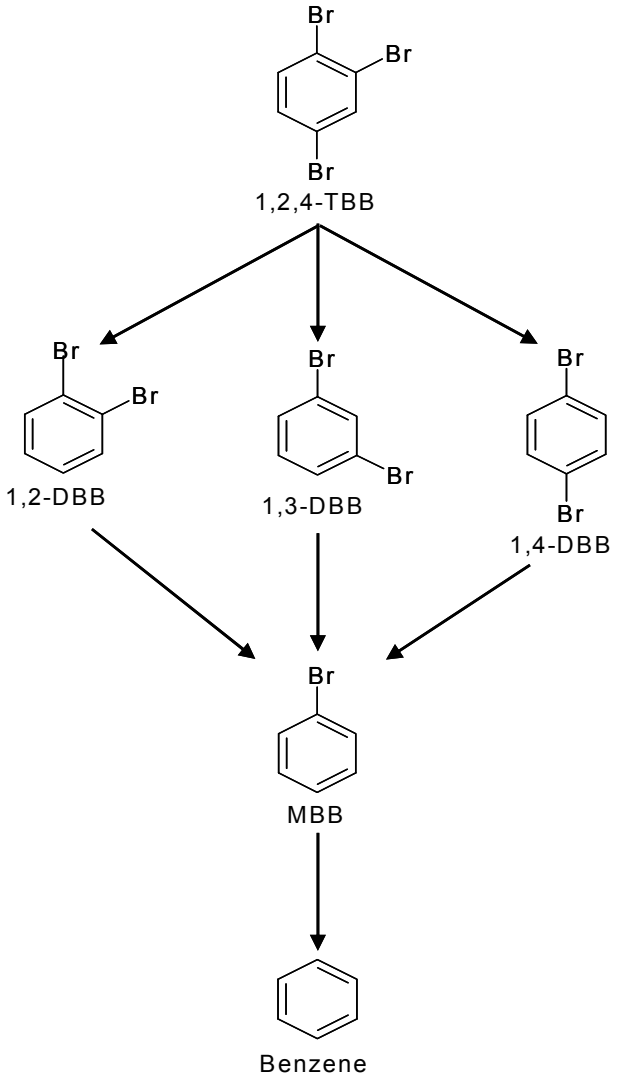

Figure 32: Proposed reductive debromination pathway catalyzed by strain VN1.

3.9 Temperature test

Strain VN1 was tested with a wide range of temperatures from 5^0C to 60^0C, namely the cultures were tested at 5^0C, 10^0C, 20^0C, 30^0C, 40^0C, 50^0C and 60^0C. Cell growth was controlled by counting after defined incubation times. The results of the temperature test show that the optimum growth temperature was 30^0C (Figure 33) and the highest debromination activity was found at this temperature although debromination activity also occurred at 20^0C. The number of cell increased over time of incubation at 30^0C.

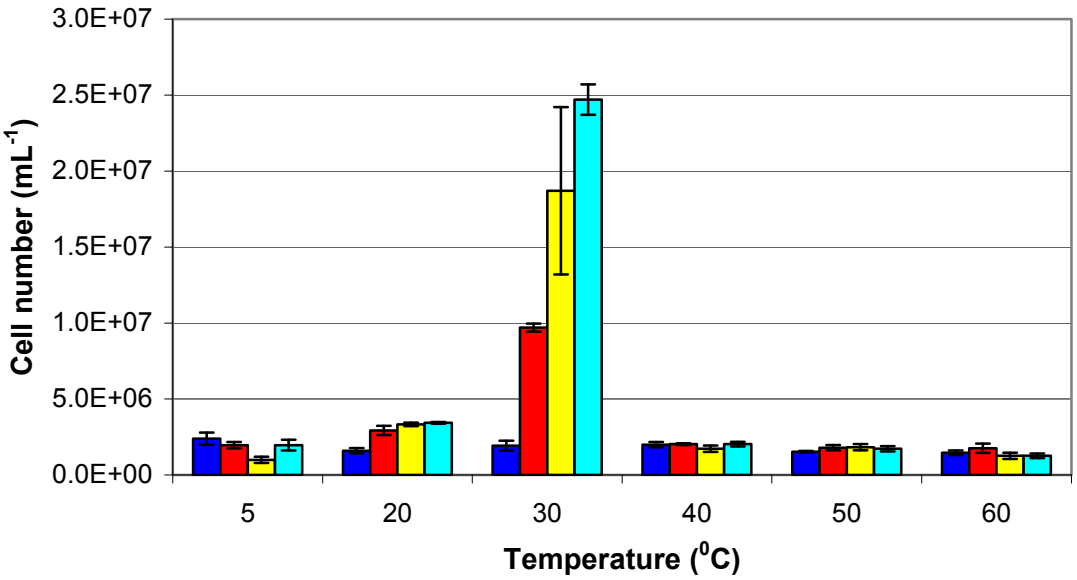

Figure 33: Cell numbers of strain VN1 growing at different temperatures. Symbols: (dark blue colour) 1 day incubation; (red colour) 27 days; (yellow colour) 37 days; (cyan colour) 63 days.

3.10 Oxygen exposure test

Strain VN1 was tested for oxygen tolerance. Inocula containing strain VN1 were exposed to oxygen until the inocula turned pink to ensure all cells were briefly exposed to oxygen. The test showed that strain VN1 is resistant to brief exposure to oxygen. The number of cells increased over time of incubation and the products of the dechlorination activity were the same as culture receiving inocula without oxygen exposure (Figure 34 A and B).

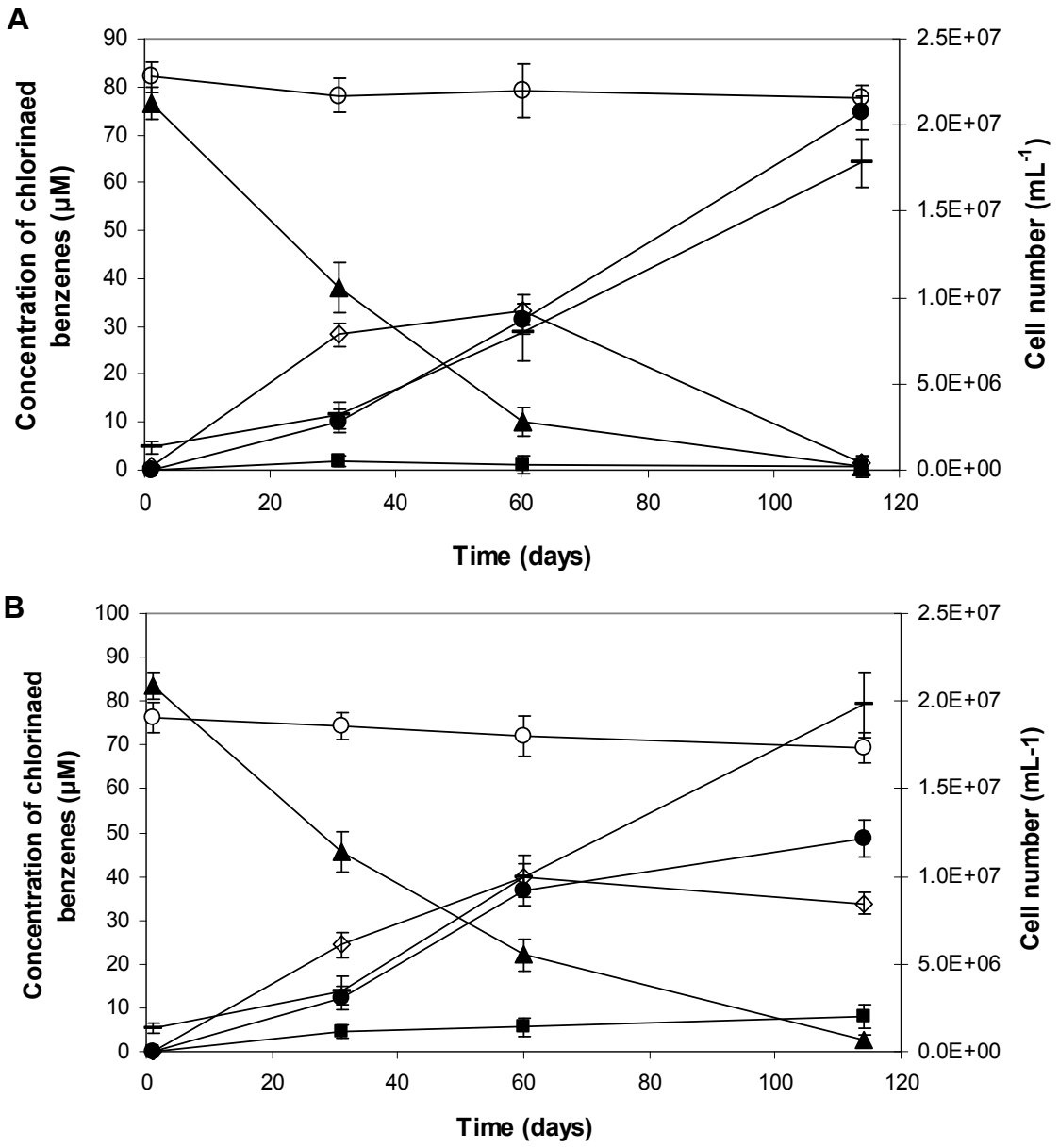

Figure 34 A and B: Dechlorination products from 1,2,3-trichlorobenzene by strain VN1 with and without oxygen exposure, respectively. Dechlorination products from 1,2,3-trichlorobenzene by strain VN1. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with 1,2,3-trichlorobenzene; (dash) number of cells mL^{-1}.

3.11 NaCl, Na_2S and antibiotics tests

The results of NaCl and Na_2S tests show that dechlorination activity and cell growth were not found in cultures at all concentrations of NaCl and Na_2S after 120 days of incubation whereas dechlorination activity and cell growth were found in normal medium with 0.1% of NaCl and without Na_2S as positive control. This indicates that strain VN1 could not tolerate even low concentration of NaCl or Na_2S. Moreover, two antibiotics, vancomycin and gentamicin were selected to test for the antibiotic resistance of strain VN1 with various concentrations of 5 mg L^{-1}; 10 mg L^{-1} and 20 mg L^{-1}. A combination of the two antibiotics with 5 mg L^{-1} each was also tested (Figure 35). Dechlorination activity and cell growth were only detected in the cultures containing inoculum exposed and without exposed oxygen and gentamicin 5 mg L^{-1} (Figure 36 A and 36 B), but not in the cultures containing both gentamicin and vancomycin 5 mg L^{-1} each. Dechlorination activity and cell growth were not found in cultures at any concentrations of vancomycin.

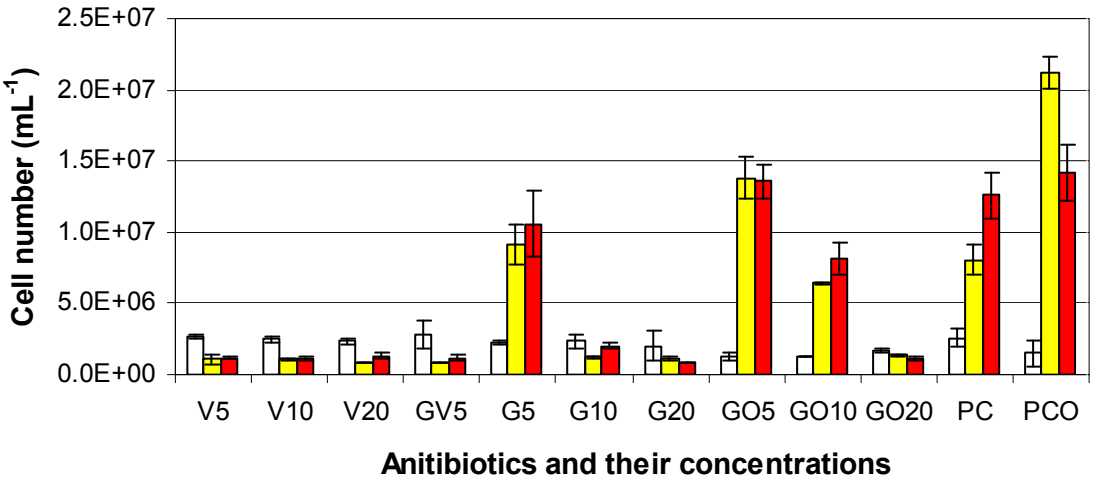

Figure 35: The effects of different concentrations of gentamicin and vancomycin on the growth of strain VN1. Symbols: (V) vancomycin; (G) gentamicin; (GO) gentamicin, with inoculum exposed to oxygen; (GV) combination of gentamicin and vancomycin; (PC) positive control without antibiotics; (PCO) Positive control cultures without antibiotics containing inocula briefly exposed to oxygen; The numbers (5; 10; 20) indicate the concentration of gentamicin or vancomycin at 5 mg L^{-1}; 10 mg L^{-1}; 20 mg L^{-1}; (white bars) number of cells after 1 day of incubation; (yellow bars) number of cells after 37 days; (red bars) number of cells after 127 days.

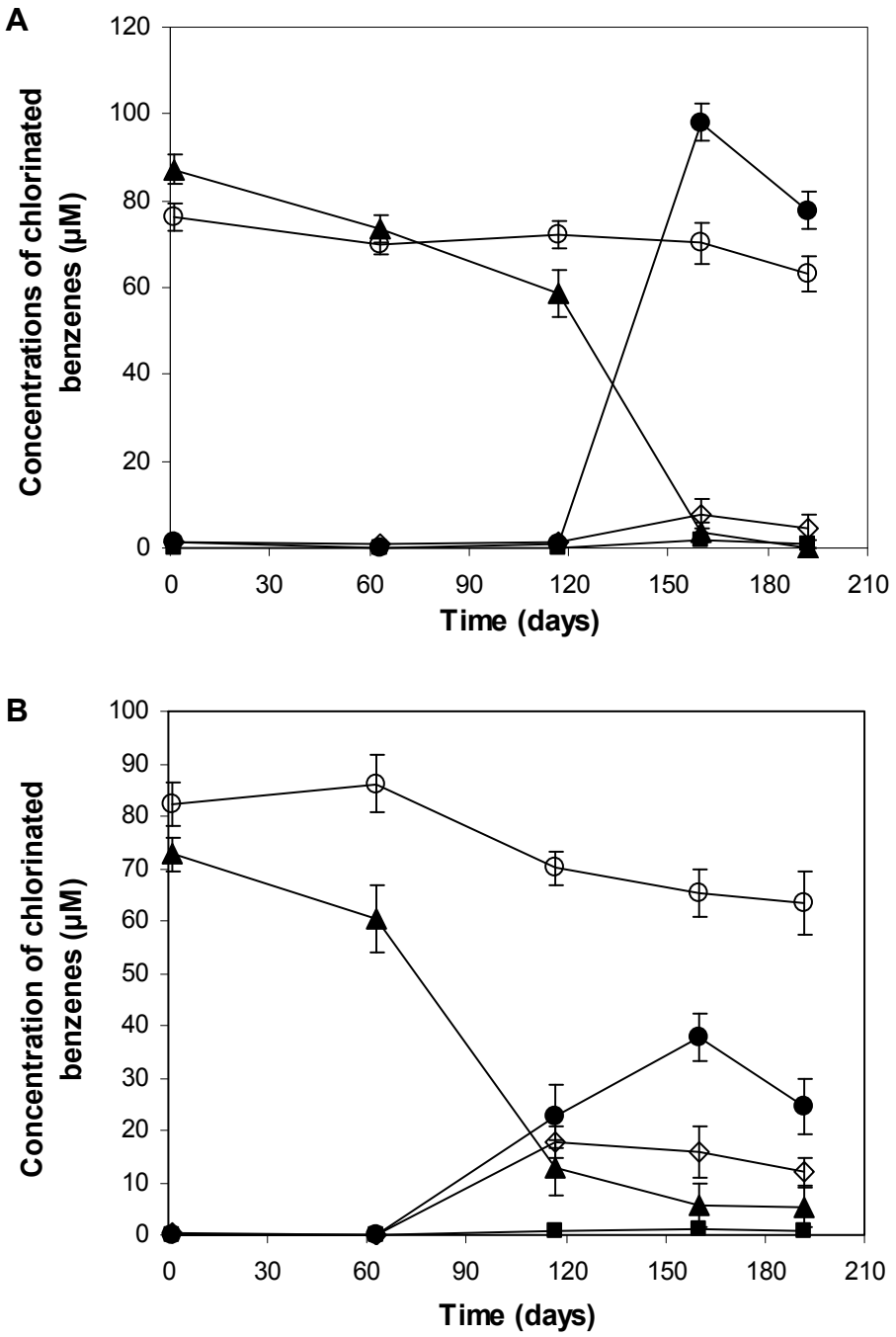

Figure 36 A and B: Dechlorination products from 1,2,3-trichlorobenzene by strain VN1 inmedium with of gentamicin 5 mg L^{-1} and a combination of gentamicin 5 mg L^{-1} with inoculum exposed to oxygen, respectively. Symbols: (filled triangle) 1,2,3-trichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with 1,2,3-trichlorobenzene.

3.12 Changes of pH of the cultures during incubation and its effects on the dechlorination ability of strain VN1

The results demonstrate that the highest cell number and the highest rate of dechlorination were found at initial pH level around 7.3, which the medium was adjusted by $NaHCO_3$ buffer 1 M 1% (v/v). The growth of cell number and dechlorination activity were also detected at the pH levels 7.0 and 7.5, which the media were adjusted by NaOH 5 M (Figure 37).The highest dechlorination activity of strain VN1 was found at pH levels from 7.0 to 7.35. The dechlorination activity increased slightly when the pH of the cultures was in the range of 7.0 and 6.7 but the dechlorination activity decreased when pH of the cultures was below 6.7 (Figure 38). The pH of vials without containing strain VN1 remained stable at around 7.35 over time of incubation. The pH in the medium of dechlorinating cultures descended slowly with time (Figure 38).

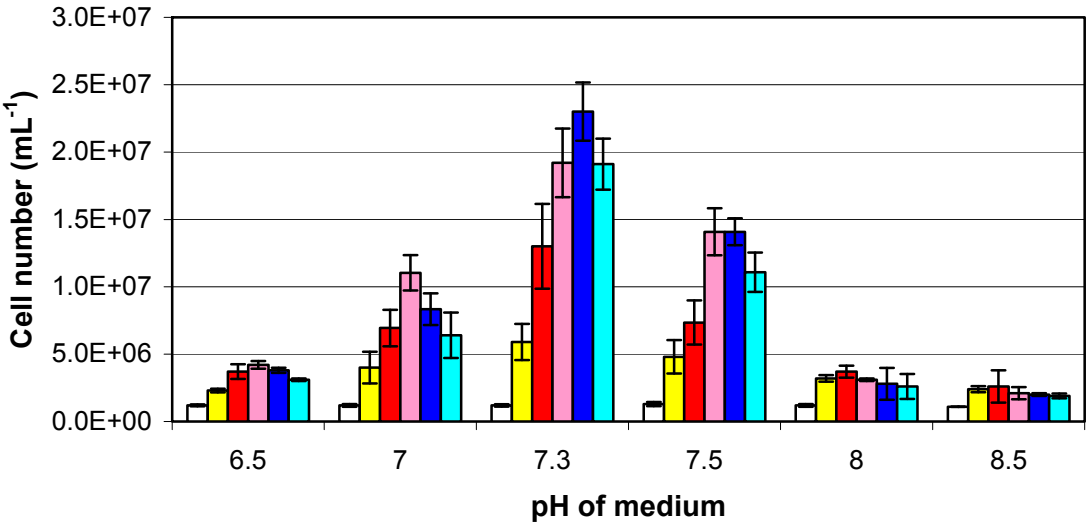

Figure 37: Cell growth depended on the pH of the medium. The levels of pH at 6.5; 7.0; 7.5; 8.0 and 8.5 of cultures were adjusted with NaOH 5 M but without containing $NaHCO_3$ buffer 1 M whereas pH at 7.3 was only adjusted by adding $NaHCO_3$ buffer 1 M with 1% (v/v). Colored bars indicate different incubation times: (white bars) 1 day; (yellow bars) 31 days; (red bars) 60 days; (pink bars) 92 days; (blue bars) 119 days; (cyan bars) 150 days.

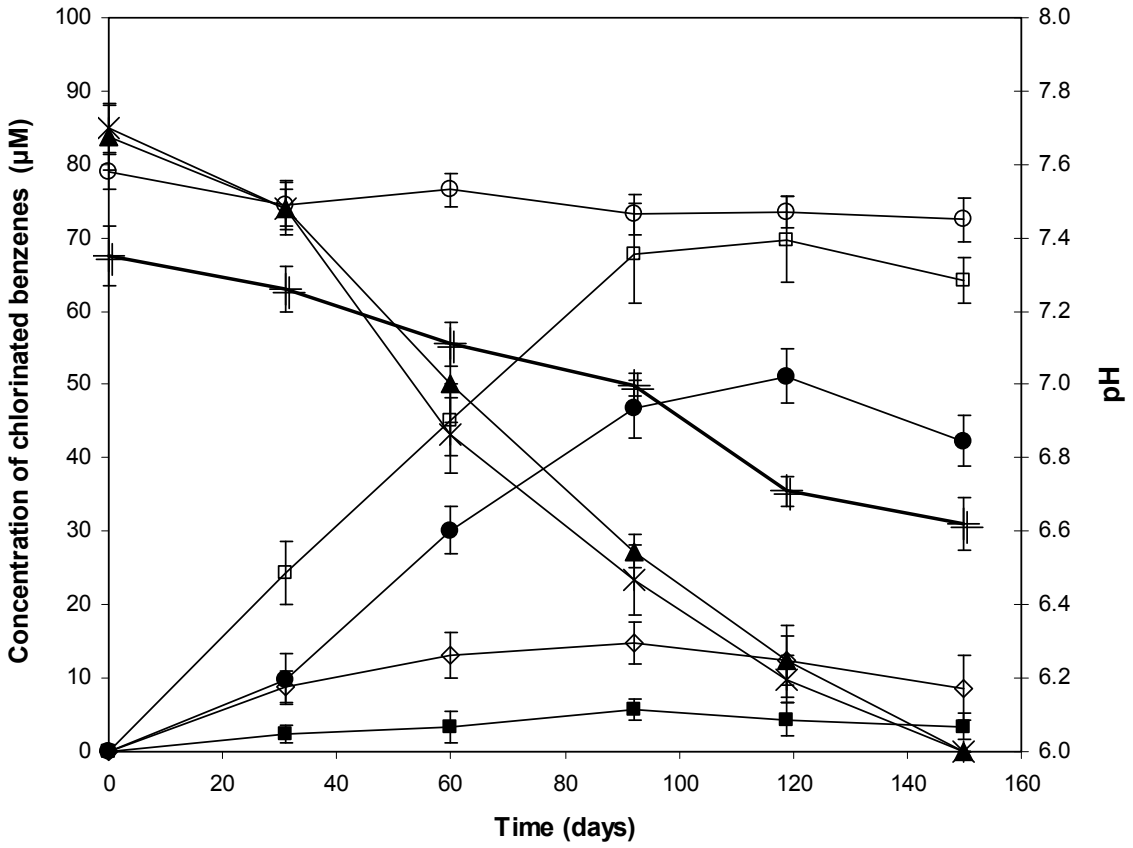

Figure 38: Changes of pH and dechlorination activity of strain VN1 cultures which pH was adjusted by adding $NaHCO_3$ buffer 1 M with 1% (v/v). Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (crosses) pH; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with a mixture of 1,2,3- and 1,2,4-trichlorobenzene.

3.13 Test for sulfate-reducing activity

Strain VN1 was shown to be a sulfate-reducing bacterium by testing H_2S formation from reducing $Na_2S_2O_3$, Na_2SO_4 or Na_2SO_3 with final-concentration of 10 mM as electron acceptors and pyruvate or hydrogen as electron donors. H_2S was only found in the cultures with $Na_2S_2O_3$ as electron acceptor and pyruvate or hydrogen as electron donors (Figure 39), cell number grew overtime of incubation. H_2S was not detected in the cultures with Na_2SO_4 or Na_2SO_3 as electron acceptors and pyruvate or hydrogen as electron donors although the cell number grew overtime of incubation in the medium amended with Na_2SO_4 but not with Na_2SO_3. This

indicates that Na_2SO_3 is an inhibitor for strain VN1 growth but Na_2SO_4 is not. H_2S was not found by abiotic production in the flasks containing Na_2SO_4 or $Na_2S_2O_3$ or Na_2SO_3 as electron acceptors and pyruvate or hydrogen as electron donors without inocula as negative controls. Similarly, H_2S was not detected in the flasks containing the standard medium, hydrogen and inoculum but without $Na_2S_2O_3$, Na_2SO_4 or Na_2SO_3 as positive control. Moreover, strain VN1 did not grow and dechlorinate a mixture of 1,2,3- and 1,2,4-trichlorobenzene in the sodium molybdate-containing medium. However, this strain still grew and dechlorinated a mixture of 1,2,3- and 1,2,4-trichlorobenzene in the cultures containing standard medium without amending the sodium molybdate as positive control. This reveals that strain VN1 is a sulfate-reducing bacterium.

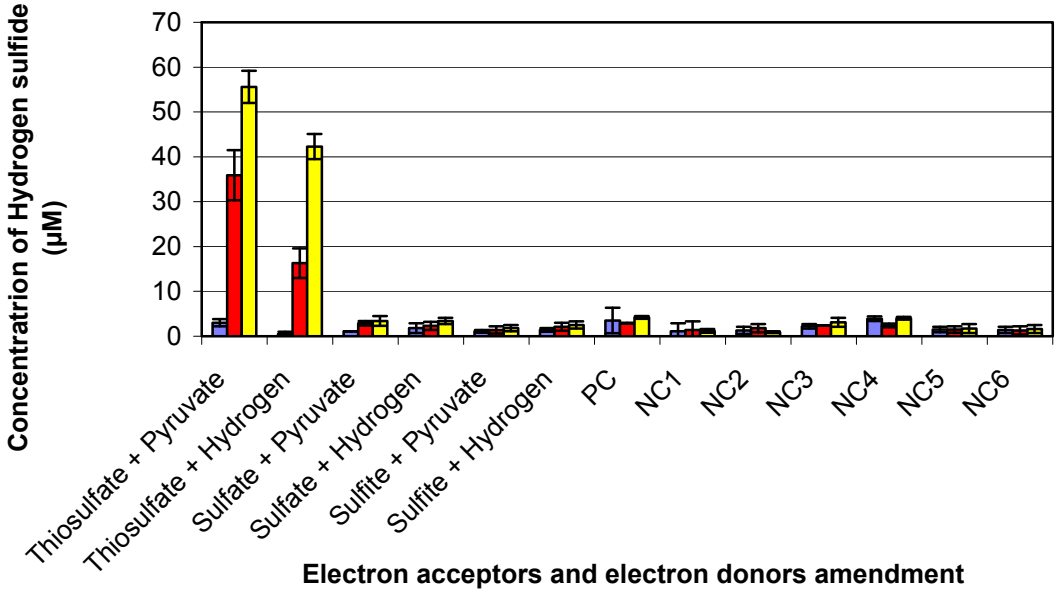

Figure 39: Formation of H_2S in the cultures of strain VN1 containing thiosulfate, sulfate or sulfite as electron acceptor and pyruvate or hydrogen as electron donor. Symbols: (PC) positive control containing the standard medium and inocula but without thiosulfate, sulfate or sulfite; (NC1-6) negative controls did not contain inoculum but the standard medium and electron donor (pyruvate: NC2; NC4 and NC6 or hydrogen: NC1; NC3 and NC5) and electron acceptor (thiosulfate: NC1 and NC2 or sulfate: NC3 and NC4 or sulfite: NC5 and NC6); (blue bars) concentrations of H_2S after 1 day; (red bars) concentrations of H_2S after 18 days; (yellow bars) concentrations of H_2S after 32 days.

3.14 Nutrient and physiological characterization of strain VN1

A complex experiment was set up with many combinations of reducing agents, electron donors, electron acceptors and additional fermentable substrates to physiologically characterize strain VN1 (Table 2). First, the results show that strain VN1 could grow and dechlorinate a mixture of 1,2,3- and 1,2,4-trichlorobenzene in medium without vitamins supplementation (experiments of NV; KM; LM, Table 2). Secondly, strain VN1 grew and dechlorinated in acetate-free medium demonstrating that acetate was not needed as a carbon source (experiments of DH and DK, Table 2). Hydrogen played a very important role in the growth of strain VN1 in the medium without containing TiCi 0.1 M. The cell number of VN1 grew in the basal media without TiCi but with only hydrogen and CO_2 supplied by buffer $NaHCO_3$ and biogon gas and the concentration of acetate increased overtime (Figure 40 A and 40 B) (experiments of NH; DP; DD; GM; DA; DF, Table 2). This confirmed that *Desulfotomaculum guttoideum* strain VN1 is an acetogenic bacterium and CO_2 or bicarbonate is a carbon source. However, hydrogen was not necessary for dechlorination activity and this demonstrates that hydrogen is not an electron donor for dechlorination activity of VN1 (Figure 41 A) (experiments of DH; DL; Table 2). Dechlorination activity always occurred in the medium with presence of TiCi 0.1 M as reducing agent. However, when TiCi was replaced by TiNTA, cells grew but dechlorination activity disappeared (experiments of NH, CM, GT, PT, Table 2). The result reveals that citrate is an electron donor but not a carbon source. In the basal medium without any reducing agents and without hydrogen but with only sodium citrate 4 mM, cell number increased and dechlorination activity occurred during the incubation time of 90 days (experiments of KM; LM, Table 2). This result one again confirmed that citrate is an electron donor and the concentration of acetate went up because acetate was the product of citrate conversion. This suggests that strain VN1 dechlorinated a mixture of 1,2,3- and 1,2,4-trichlorobenzene via co-metabolism. Interestingly, the cell number of strain VN1 increased significantly also in medium without spiking a mixture of 1,2,3- and 1,2,4-trichlorobenzene (experiments of GM; DA; DF; DNT; DG; DM, Table 2).

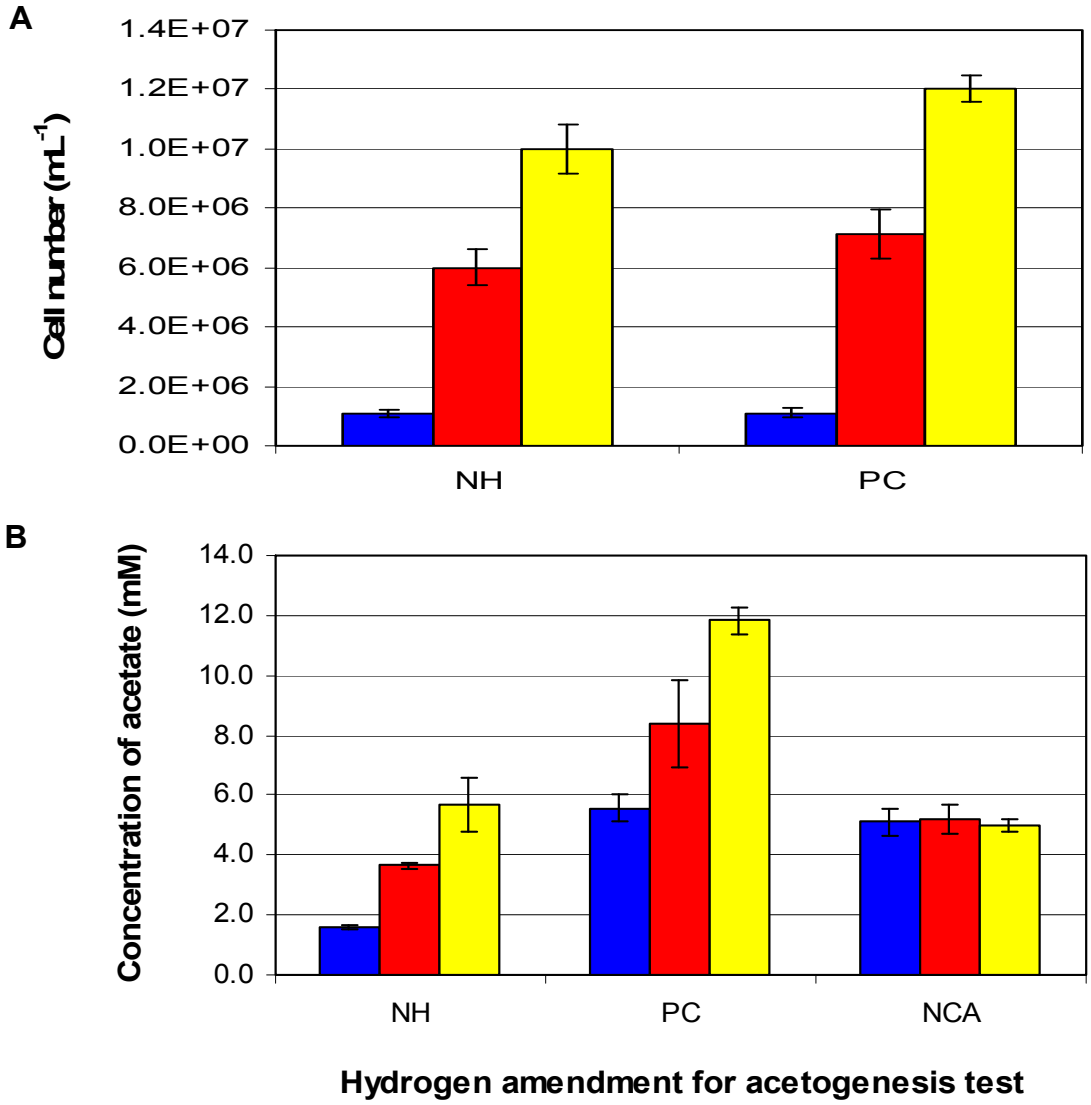

Figure 40: Cell numbers (**A**) and acetate concentration produced (**B**) by strain VN1. Symbols: (NH) triplicate cultures growing in medium with hydrogen as electron donor, trichlorobenzenes as electron acceptor and carbonate buffer as the only carbon source. In these cultures titanium (III) citrate was substituted by titanium (III) NTA to avoid introduction of citrate as fermentable substrate and no acetate was added; (NCA) not inoculated negative control. The medium contained TiCi 0.1 M, hydrogen and 5 mM acetate; (PC) positive control; strain VN1 growing in standard medium containing hydrogen as electron donor, trichlorobenzenes as electron acceptor and acetate as carbon source. In addition the medium contained TiCi 0.1 M as reducing agent; (blue bars) after 1 day of incubation; (red bars) after 31 days of incubation; (yellow bars) after 50 days of incubation.

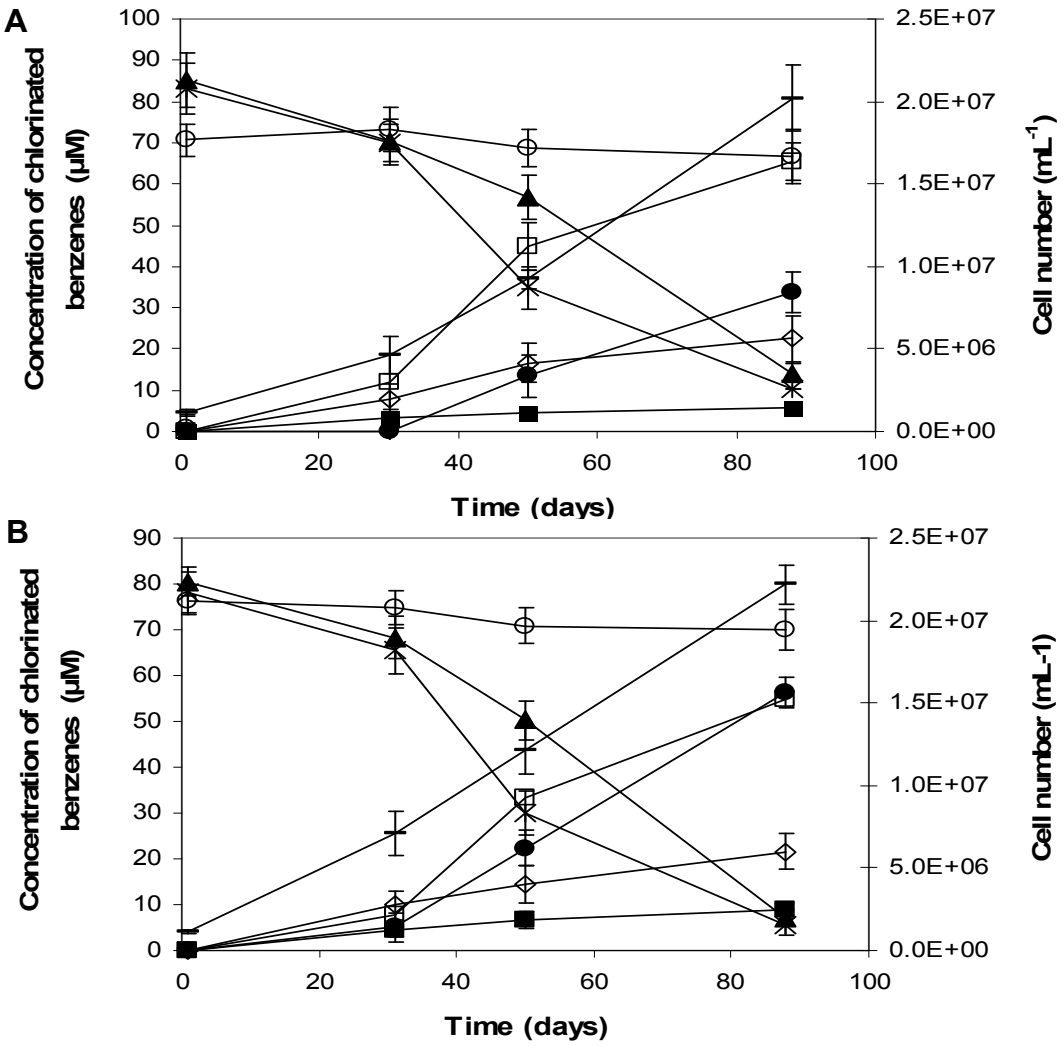

Figure 41 A: Dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by strain VN1 and cell growth on the medium with trichlorobenzenes as electron acceptor, TiCi 0.1 M vitamins, 5 mM acetate and carbonate buffer as the carbon source but without hydrogen supplement (experiment of DL, Table 2). **B:** Dechlorination of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by strain VN1 and cell growth on the same medium as the experiment of DL, Table 2 but with hydrogen as positive control (experiment of PC, Table 2). Symbols: (filled triangle) 1,2,3-trichlorobenzene; (asterisk) 1,2,4-trichlorobenzene; (open square) 1,4-dichlorobenzene; (filled square) 1,3-dichlorobenzene; (open diamond) 1,2-dichlorobenzene; (filled circle) monochlorobenzene; (open circle) 1,2,3-trichlorobenzene of negative control without cells but with 1,2,3- and 1,2,4-trichlorobenzene; (dash) number of cells in mL^{-1}.

In the medium amended with pyruvate and TiCi, the cell number increased very sharply and was higher than those of standard medium as positive control (Figure

42) (experiments of PY, PT, Table 2). Similarly, the concentration of acetate of the cultures of VN1 with the medium containing pyruvate and TiCi increased dramatically from 6.6 to 20.4 mM after 31 days (Figure 43) (experiments of PY, PT, Table 2).

In the medium supplemented with glucose, the cell number was nearly the same as that of standard medium (Figure 42) (experiments of GL; GT, Table 2) and concentrations of acetate of the cultures with medium containing glucose and TiCi went up only slightly after 31 days (Figure 43) (experiments of GL, GT, Table 2). The results demonstrate that strain VN1 can ferment pyruvate but not glucose and that acetate is a product of pyruvate fermentation of strain VN1. Pyruvate stimulated the cells growth very strongly but pyruvate was not an electron donor for dechlorination. The evidence for this is that dechlorination activity was lost in cultures with pyruvate as fermentable substrate and TiNTA as reducing agent instead of TiCi 0.1 M (experiments PT and GT, Table 2).

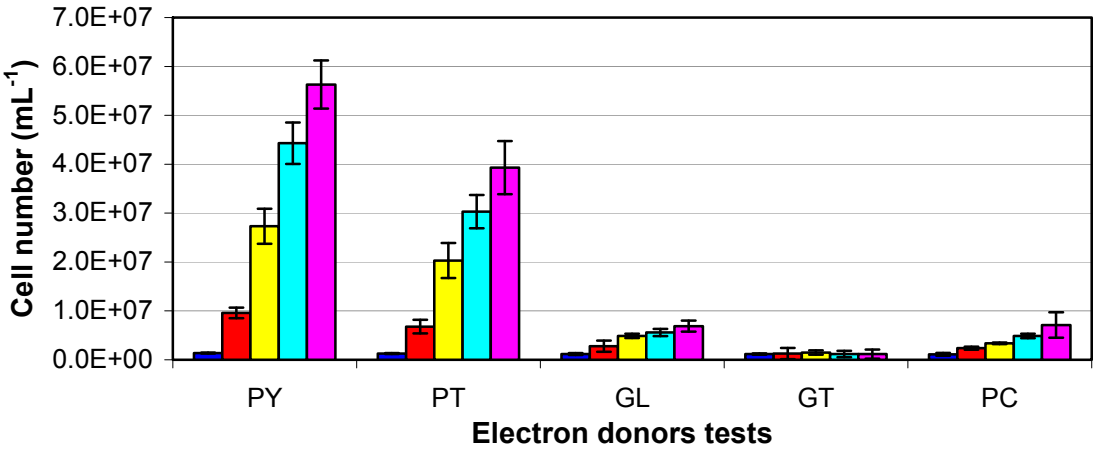

Figure 42: The effects of pyruvate and glucose on the growth of strain VN1. Symbol: (PY) Cultures containing TiCi 0.1 M, acetate 5 mM, pyruvate 4 mM, trichlorobenzenes but without hydrogen; (PT) Cultures with the same medium as PY, but using TiNTA instead of TiCi 0.1 M as reducing agent, therefore, no citrate was present.; (GL) The medium containing TiCi 0.1 M, acetate and glucose but without hydrogen; (GT) The medium containing TiNTA instead of TiCi 0.1 M, acetate, glucose but without hydrogen; (PC) The medium containing TiCi 0.1 M acetate and hydrogen but not pyruvate and glucose as positive control; blue, red, yellow, cyan and pink bars are cell number of strain VN1 after 1, 5, 12, 18 and 31 days of incubation, respectively.

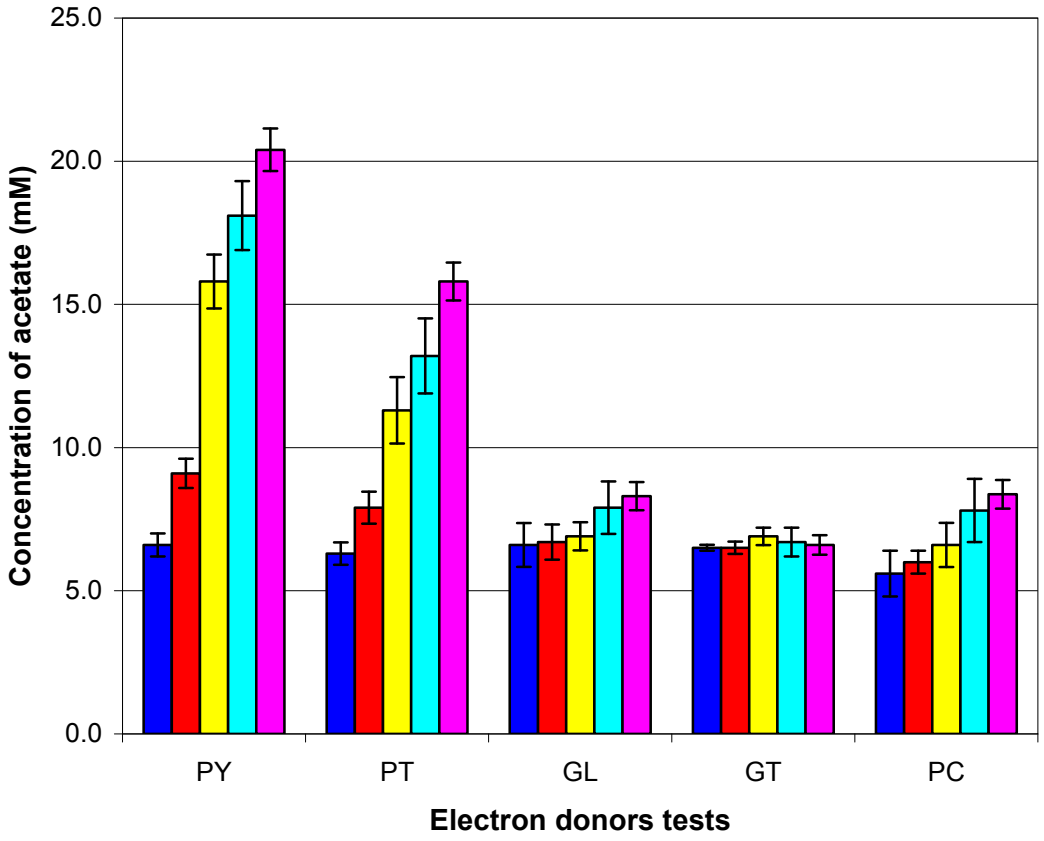

Figure 43: The concentrations of acetate produced by strain VN1 in the medium amended with pyruvate or glucose. Symbol: (PY) The medium containing TiCi 0.1 M, acetate and pyruvate but without hydrogen; (PT) The medium containing acetate, pyruvate and TiNTA instead of TiCi 0.1 M but without hydrogen; (GL) The medium containing TiCi 0.1 M, acetate and glucose but without hydrogen; (GT) The medium containing acetate, glucose and TiNTA instead of TiCi 0.1 M but without hydrogen; The medium containing TiCi 0.1 M, acetate and hydrogen but not pyruvate and glucose as positive control; (blue bars) concentration of acetate after 1 day of incubation; (red bars) concentration of acetate after 5 days of incubation; (yellow bars) concentration of acetate after 12 days of incubation; (cyan bars) concentration of acetate after 18 days of incubation; (pink bars) concentration of acetate after 31 days of incubation.

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VN1     1    ACACATGCA-GTCGAGCGAAGCGATTCAAATGAAGTTTTCGGATGGATTTTGAATTGACT   59
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
4024    37   ACACATGCAAGTCGAGCGAAGCGATTCAAATGAAGTTTTCGGATGGATTTTGAATTGACT   96

VN1     60   TAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACAGGGGGATAACAGTCG  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    97   TAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACAGGGGGATAACAGTCG  156

VN1     120  GAAACGACTGCTAATACCGCATAAGCACACAGTGCCGCATGGYACGGTGTGAAAAACTCC  179
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
4024    157  GAAACGACTGCTAATACCGCATAAGCACACAGTGCCGCATGGTACGGTGTGAAAAACTCC  216

VN1     180  GGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTTGGTGAGGTAACGGCCCACCAAGC  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    217  GGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTTGGTGAGGTAACGGCCCACCAAGC  276

VN1     240  CGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCCA  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    277  CGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCCA  336

VN1     300  AACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCG  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    337  AACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCG  396

VN1     360  ACGCCGCGTGAGTGAAGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATG  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    397  ACGCCGCGTGAGTGAAGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATG  456

VN1     420  ACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    457  ACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  516

VN1     480  GGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCACTGCAAGTCTG  539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    517  GGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCACTGCAAGTCTG  576

VN1     540  GAGTGAAAGCCCGGGGCTCAACCCCGGGACTGCTTTGGAAACTGTGGTGCTAGAGTGCAG  599
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    577  GAGTGAAAGCCCGGGGCTCAACCCCGGGACTGCTTTGGAAACTGTGGTGCTAGAGTGCAG  636

VN1     600  GAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCA  659
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    637  GAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCA  696

VN1     660  GTGGCGAAGGCGGCTTACTGGACTGTAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAA  719
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    697  GTGGCGAAGGCGGCTTACTGGACTGTAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAA  756

VN1     720  ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGC  779
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    757  ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGC  816

VN1     780  AAAGCTCTTCGGTGCCGCCGCTAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAA  839
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    817  AAAGCTCTTCGGTGCCGCCGCTAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAA  876

VN1     840  GAATGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATT  899
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    877  GAATGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATT  936

VN1     900  CGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCGGAATGACCGGGATGTAACGATC  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    937  CGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCGGAATGACCGGGATGTAACGATC  996
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VN1     960   CCTTCCCTACGGGGCATTCCAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG  1019
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024    997   CCTTCCCTACGGGGCATTCCAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG  1056

VN1    1020   ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTAGTAGCCAGCAAGTGAAGT  1079
              |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
4024   1057   ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTAGTAGCCAGCAAGTCAAGT  1116

VN1    1080   TGGGCACTCTGGGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATC  1139
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024   1117   TGGGCACTCTGGGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATC  1176

VN1    1140   ATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAAGCA  1199
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024   1177   ATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAAGCA  1236

VN1    1200   AAGGAGCGATCTGGAGCAAACCCCAAAAATAACGTCTCAGTTCGGATTGTAGTCTGCAAC  1259
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024   1237   AAGGAGCGATCTGGAGCAAACCCCAAAAATAACGTCTCAGTTCGGATTGTAGTCTGCAAC  1296

VN1    1260   TCGACTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGT  1319
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024   1297   TCGACTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGT  1356

VN1    1320   TCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTAACGCCCGAAGTCAGT  1379
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4024   1357   TCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTAACGCCCGAAGTCAGT  1416

VN1    1380   GACCCAACCGTAAGGAGG  1397
              ||||||||||||||||||
4024   1417   GACCCAACCGTAAGGAGG  1434
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Figure 44: Comparison of the 16S rRNA gene of strain VN1 with that of *Desulfotomaculum guttoideum* strain DSM 4024 over 1397 base pairs. The total identity is 99.9% (1396 of 1397 base pairs identical).

Table 2: Experimental set-up to elucidate the requirements of energy and carbon sources of strain VN1. All cultures were set up in triplicate and with the exception of NCA and NCW inoculated with 5% (vol/vol) of strain VN1.

Name	Acetate	TiCi	H_2	CO_2	Vitamin	$NaHCO_3$	TCB	TiNTA	NaCi	GLU	PYR	CG	De	T50	SA50
DH	-	+	-	+	+	+	+	-	-	-	-	**+**	**+**	**10.7**	**4.6**
NH	-	-	+	+	+	+	+	+	-	-	-	**+**	**-**	**9.1**	**4.1**
CM	-	-	-	+	+	+	+	+	-	-	-	**-**	**-**	**2.5**	**0.3**
DL	+	+	-	+	+	+	+	-	-	-	-	**+**	**+**	**9.0**	**3.2**
PC	+	+	+	+	+	+	+	-	-	-	-	**+**	**+**	**10.9**	**6.2**
GM	+	-	+	+	+	+	-	+	-	-	-	**+**	**NI**	**8.3**	**3.3**
GL	+	+	-	+	+	+	+	-	-	+	-	**+**	**+**	**9.9**	**3.6**
GT	+	-	-	+	+	+	+	+	-	+	-	**-**	**-**	**1.0**	**0.1**
PY	+	+	-	+	+	+	+	-	-	-	+	**+**	**+**	**40.0**	**13.8**
PT	+	-	-	+	+	+	+	+	-	-	+	**+**	**-**	**30.0**	**9.5**
DP	+	-	+	+	+	+	+	-	-	-	-	**+**	**-**	**7.0**	**3.1**
DN	-	-	-	+	+	+	-	-	-	-	-	**-**	**NI**	**1.9**	**0.2**
DA	-	-	+	+	+	+	-	-	-	-	-	**+**	**NI**	**11.8**	**2.0**
DB	-	-	-	+	+	+	+	-	-	-	-	**-**	**-**	**1.4**	**0.1**
DC	+	-	-	+	+	+	-	-	-	-	-	**-**	**NI**	**1.6**	**0.3**
DD	-	-	+	+	+	+	+	-	-	-	-	**+**	**-**	**7.0**	**2.1**
DE	+	-	-	+	+	+	+	-	-	-	-	**-**	**-**	**1.8**	**0.3**
DF	+	-	+	+	+	+	-	-	-	-	-	**+**	**NI**	**10**	**2.5**
DNT	-	+	-	+	+	+	-	-	-	-	-	**+**	**NI**	**10.9**	**4.6**
DG	-	+	+	+	+	+	-	-	-	-	-	**+**	**NI**	**10.0**	**6.3**
DJ	+	+	-	+	+	+	-	-	-	-	-	**-**	**NI**	**12.7**	**3.4**
DK	-	+	+	+	+	+	+	-	-	-	-	**+**	**+**	**9.9**	**5.1**
DM	+	+	+	+	+	+	-	-	-	-	-	**+**	**NI**	**10.0**	**5.1**
LM	-	-	-	+	-	+	+	-	+	-	-	**+**	**+**	**8.8**	**2.9**
KM	-	-	-	+	-	+	+	+	+	-	-	**+**	**+**	**9.2**	**3.3**
NCA	+	+	+	+	+	+	-	-	-	-	-	**NIN**	**NIN**	**NIN**	**0**
NCW	-	+	+	+	+	+	-	-	-	-	-	**NIN**	**NIN**	**NIN**	**0**
NV	+	+	+	+	-	+	+	-	-	-	-	**+**	**+**	**9.2**	**4.2**

Abbreviations: NaCi - sodium citrate with concentration of 4 mM; GLU – glucose with concentration of 4 mM; PYR – pyruvate with concentration of 4 mM; CG - cell growth; De - dechlorination; T50 - relative growth. Calculated as the cell number after 50 days divided by the cell number after 1 day of incubation; SA50 - Increase of acetate concentration after 50 days compared to that after 1 day of incubation; NI - No information; NIN - No inoculum; **+**: indicating presence, cell growth or dechlorination; **-** : indicating absence, no cell growth or no dechlorination.

4. Discussion

4.1 Transformation of a mixture of 1,2,3- and 1,2,4-trichlorobenzene by mixed cultures

Dechlorination activity together with various pathways of reductive dechlorination was found in soil and sediments from both Germany and Vietnam revealing that reductively dechlorinating bacteria are diverse and widely distributed. Reductive dechlorination activity was not detected in the flasks with fresh medium and electron acceptors without inocula or in the medium with chlorobenzenes and autoclaved inocula indicating that transformation of chlorobenzenes was carried out by microorganisms in soil and sediments samples.

Production of 1,2-dichlorobenzene in all microcosms from soil and sediments amended with a mixture of 1,2,3- and 1,2,4-trichlorobenzene demonstrates that this product stemmed either from dechlorination of the singly flanked chlorine of 1,2,3-trichlorobenzene or from dechlorination of isolated chlorine of 1,2,4-trichlorobenzene. However, evidence of 1,2-dichlorobenzene in all subcultures tested with 1,2,3-trichlorobenzene and 1,2,4-trichlorobenzene as sole electron acceptor except soil cultures supplemented with 1,2,4-trichlorobenene confirmed that dechlorination of singly flanked and isolated chlorines occurred in all cultures inoculated with sediments and the removal of singly flanked chlorine did not happen in cultures with soil. Similarly, in a previous study we also found dechlorination of singly flanked and isolated chlorines during the transformation of a mixture of 1,2,3- and 1,2,4-trichlororbenzenes by mixed cultures of sludge samples from Southern and Eastern Germany (Hölscher et al. 2010). However, there is a difference between the two studies. For the cultures of sludge samples from Germany, transformation of 1,2,3- and 1,2,4-trichlorobenzene occurred simultaneously or 1,2,3-trichlorobenzene was dechlorinated first and then 1,2,4-trichlorobenzene was transformed. In contrast, transformation of 1,2,4-trichlororbenzene was faster than that of 1,2,3-trichlorobenzene though both trichlorobenzenes were transformed simultaneously in all cultures of soil and

sediments from Vietnam and Germany amended with a mixture of 1,2,3- and 1,2,4-trichlorobenzene.

Earlier reports revealed that dechlorination activity did not occur in mixed cultures containing 1,2,3-trichlorobenzene when its concentration was above 40 μM or 1,3-dichlorobenzene above approximate 70 μM (Holliger et al. 1992) or a mixture of 1,2,3- or 1,2,4-trichlrorobenzene above 30 μM each (Adrian et al. 1998). This illustrates that water-solved chlorobenzenes can inhibit dechlorination activity of chlorobenzene-dechlorinating bacteria due to their toxicity. In our experiments, dechlorination activity still occurred in most microcosms enriched with a mixture of 1,2,3- and 1,2,4-trichlorobenzene at approximately 80 μM each as electron acceptors. The transformation activity of 1,2,3- and 1,2,4-trichlorobenzene even kept continuing in the culture containing monochlorobenzene and 1,3-dichlorobenzene, products of 1,2,3- and 1,2,4-trichlorobenzene degradation, exceeded 1300 μM and 70 μM respectively (Table 1).

There were so far many reports on mixed cultures preferentially or uniquely removing doubly flanked chlorine substituents from 1,2,3-trichlorobenzene (Adrian et al. 2000; Chang et al. 1997; Chang et al. 1998; Chen et al. 2010; Fathepure et al. 1988; Holliger et al. 1992; Middeldorp et al. 1997; Wu et al. 2002). Moreover, some pure strains which preferentially or uniquely removed doubly flanked chlorine substituents were isolated and intensively studied such as the bacterium DF-1 (Wu et al. 2002) and two strictly anaerobic strains belonging to *Dehalococcoides*, strain CBDB1 (Adrian et al. 2000) and strain 195 (Fennell et al. 2004). In contrast, the information about microorganisms preferentially or exclusively dechlorinating singly-flanked or isolated chlorines from 1,2,3- or 1,2,4-trichlorobenzene is limited. To our knowledge, up to now there was only one report comprehensively discussed on mixed cultures preferentially dechlorinating singly flanked chlorines from trichlorobenzenes (Hölscher et al. 2010), especially there was no publication on any pure strain having the ability to dechlorinate chlorines at singly-flanked or isolated positions of chlorobenzenes. Therefore, our findings of preferential singly-flanked or isolated dechlorination patterns by mixed cultures not

only gave a valuable proof of variety of dechlorination pathways of chlorinated benzene compounds but also supplied valuable bacterial sources to isolate pure strains which are responsibility to preferentially dechlorinate singly-flanked or isolated chlorines for further studies on their physico-biochemical characteristics. The dominance of the dechlorination pathway preferentially removing singly-flanked chlorines in the cultures amended with Leipzig sediment and the stability of the dechlorination pattern over seven transfers revealed that bacteria preferentially dechlorinating singly flanked chlorine might be in charge of dechlorination activity in mixed cultures. In contrast, it seems that there was a co-existence of bacteria preferentially removing doubled flanked chlorines and bacteria preferentially removing isolated chlorines in cultures containing soil and sediment from Hue.

Monochlorobenzene was produced as a main final end-product in all cultures. Accumulation of monochlorobenzene to high concentration over a long time of incubation after repeated addition of two isomers of trichlorobenzene indicated that chlorobenzene-dechlorinating bacteria could maintain the dechlorination ability in a long time and could withstand the high toxicity of chlorobenzenes. Therefore, the cultures in our experiments had a great potential for bioremediation of trichlorobenzene- and dichlorobenzene-contaminated sites. Evidence of a further anaerobic biotransformation of monochlorobenzene to benzene were described in the literature (Nijenhuis et al. 2007) (Fung et al. 2009). However, the presence of benzene in enrichment cultures with Hue sediment in our work might derive directly from biodegradation of isomers of dichlorobenzene but not via monochlorobenzene. This can be explained by increasing accumulation of the concentration of benzene coupled with the increase of concentration monochlorobenzene and transformation of monochlorobenzene to benzene did not occur in subcultures spiked with monochlorobenzene as single electron acceptor over a period of six months. Moreover, for the formation of benzene in the cultures from monochlorobenzene two explanations should be taken into account. Firstly, the rate of transformation of monochlorobenzene to benzene was

much slower than that of transformation of isomers of dichlorobenzene to monochlorobenzene, so the concentrations of benzene and monochlorobenzene increased simultaneously. Secondly, bio-transformation of monochlorobenzene to benzene might occur concurrently with dichlorobenzene dechlorination to monochlorobenzene and this detection was confirmed by studies of Nowak et al. 1996 and Quistorff 1999. Therefore, the transformation of monochlorobenzene to benzene in the experiments testing monochlorobenzene as a sole substrate did not occur due to the absence of dechlorination of isomers of dichlorobenzene to monochlorobenzene. A further transformation of benzene to nontoxic products was not investigated although the anaerobic biodegradation of benzene to CO_2 and CH_4 had been illustrated (Liang et al. 2013). The concentrations of benzene in all subcultures were below 10 µM although its concentrations increased over time because of two reasons. The first reason is that benzene concentration measurements stopped while its concentration continued going up this hypothesis was supported by the increase of benzene concentration of parental mixed culture kept going up to 130 µM when this culture was incubated for over 2 years (Table1). The second reason is that benzene is highly volatile so that it could be lost easily through the Teflon-lined rubber septa which were repeatedly pierced for addition of reducing agent, buffer, vitamins and gases as well as for sampling.

4.2 Transformation of hexachlorobenzene and 1,3,5-trichlorobenzene by mixed cultures

Small amounts of benzene, monochlorobenzene and all dichlorobenzene isomers were formed from hexachlorobenzene but without accumulation of 1,3,5-trichlorobenzene in most of the cultures enriched here from sediments in Germany and Vietnam. This reveals a diversity of hexachlorobenzene transformation pathway. Products of hexachlorobenzene degradation by mixed cultures and pure strain in most of previous reports were all three isomers of dichlorobenzene and trichlorobenzene as final products. Especially 1,3,5-trichlorobenzene was often formed as the dominant end product (Adrian and Görisch 2002; Chang et al. 1997; Chang et al. 1998; Fathepure et al. 1988; Fennell et al. 2004; Wu et al. 2002). It is

clear that there was a priority in removing of chlorines at positions with two other flanked substituents in these cultures. However, another previous research was carried out by Ramanand et al. who found singly-flanked chlorine substituents were preferred over doubly-flanked chlorine substituents in soil slurry cultures that were able to reductively dechlorinate hexachlorobenzene without the formation of 1,3,5-trichlorobenzene (Ramanand et al. 1993). In this dissertation, we already described mixed cultures dechlorinating mixtures of 1,2,3- and 1,2,4-trichlorobenzene which catalyzed a different pattern by preferentially dechlorinating singly-flanked chlorine substituents, for instance 1,2,3-trichlorobenzene transformation to mainly 1,2-dichlorobenzene in the cultures enriched with soil and sediments in Vietnam and Germany. In the hexachlorobenzene transforming cultures, the bacteria preferentially dechlorinating singly-flanked chlorine substituents were dominant with main formation of monochlorobenzene, a pattern that was maintained over seven times of transferring in purely synthetic medium. The accumulation of 1,3,5-trichlorobenzene was stably avoided . Detection of 1,3,5-trichlorobenzene in parent cultures inoculated with the original sediments in Germany and Vietnam and the disappearance of this persistent chlorinated benzene in all subcultures over seven times of consecutive transfer illustrate the removal of hexachlorobenzene-to-1,3,5-trichlorobenzene-dechlorinating microbial populations during the enrichment of the mixed cultures . In addition, the fact that both 1,3,5-trichlorobenzene and 1,2-dichlorobenzene were products of hexachlorobenzene transformation in the parent cultures prove that there was co-existence of two types of chlorobenzene-dechlorinating bacteria in the cultures, namely bacteria preferentially removing doubly-flanked chlorines and those preferentially removing singly-flanked chlorines. The instability of 1,3,5-trichlorobenzene formation in parent cultures and subcultures can be explained: in the parent cultures, both types of organisms were active because undefined compounds in the sediments might stimulated the growth of organisms that preferentially dechlorinating doubly flanked chlorines. However, undefined compounds seem to be eliminated in the free-sediment subcultures and preferentially removing double-flanked chorine

bacteria got an intensively competition from other bacteria, especially from preferentially singly-flanked chlorine bacteria. The more prominent formation of monochlorobenzene in the subcultures supports this hypothesis. The co-existence of two types of chlorobenzene-dechlorinating bacteria were also confirmed by appearance of 1,2- and 1,4-dichlorobenzene as sole intermediates of transformation 1,2,3- and 1,2,4-trichlorobenzene respectively when two isomers of trichlorobenzene were spiked to subcultures of Leipzig and Hue as unique electron acceptor. By applying oxygen to inocula or by adding vancomycin to the cultures, this competition was influenced in favor of one or the other organism, respectively. Moreover, the transformation of 1,3,5-trichlorobenzene to 1,3-dichlorobenzene and monochlorobenzene in subcultures tested with only 1,3,5-trichlorobenzene reveals chlorobenzene-dechlorinating bacteria preferentially removing isolated chlorines also existed in the mixed cultures from two different sources of inocula.

Accumulation of benzene was found in most of the subcultures from sediments in Germany and Vietnam. This shows that a complete dechlorination of the highest chlorinated benzene to benzene can occur and this may be the first report on complete transformation of hexachlorobenzene to benzene. Nevertheless, formation of benzene could derive from removing simultaneously two chlorines of 1,3- and 1,4-dichlorobenzene not via monochlorobenzene. This hypothesis could be proved by benzene formation from 1,3- and 1,4-dichlorobenzene in subcultures amended with 1,2-; 1,3- and 1,4-dichlorobenzene as single substrate and absence of benzene formation in subcultures enriched with monochlorobenzene as sole electron acceptor. This result was different from that of Fung et al. 2009, who found formation of benzene from monochlorobenzene but similar to the same phenomenon described by Nowak et al 1996.

Besides a description of the cultures transforming hexachlorobenzene but avoiding to produce 1,3,5-trichlorobenzene during hexachlorobenzene dechlorination also the biotransformation of 1,3,5-trichlorobenzene in cultures amended with only 1,3,5-trichlorobenzene as electron acceptor was investigated. To our knowledge, there are no reports on anaerobic microbial degradation of 1,3,5-trichlorobenzene.

Reductive dechlorination of 1,3,5-trichlorobenzene requires the removal of isolated substituents (not flanked by other chlorine substituents) which are also present in other anaerobically persistent chlorobenzene isomers such as 1,4-dichlorobenzene or monochlorobenzene. Reductive dechlorination of chlorobenzenes did not occur in negative controls containing substrates without inoculum or in flasks containing fresh medium, substrates and autoclaved inoculum indicating that viable microorganisms were the key factor for reductive dechlorination of 1,3,5-trichlorobenzene in the cultures.

Anaerobic reductive dechlorination of 1,3,5-trichlorobenzene to monochlorobenzene or even benzene and the stability of this dechlorination pattern over several transfers show that 1,3,5-trichlorobenzene dechlorinating cultures have the great potential for bioaugmentation in the sites contaminated with 1,3,5-trichlorobenzene which is resistant to mostly aerobic and anaerobic microbial attacks. In addition, the cultures also have significant application to the sites contaminated with hexachlorobenzene which can be biologically converted to 1,3,5-trichlorobenzene as one of the main final end-products.

In the cultures enriched with sediment from Hue, it seems that different type chlorobenzene-dechlorinating bacteria could be enriched by adding different chlorinated benzenes. Bacteria that preferentially remove doubly flanked and isolated chlorines were dominant in the cultures spiked with a mixture of 1,2,3- and 1,2,4-trichlorobenzene, while those preferentially removing singly flanked and isolated chlorines were predominant in the cultures amended with hexachlorobenzene and 1,3,5-trichlorobenzene respectively. Yet the cultures enriched with sediment from Leipzig, the chemical properties of different chlorinated benzenes did not decide the dominance of any chlorobenzene-dechlorinating bacteria, singly flanked dechlorinators overwhelmed other chlorobenzene dechlorinating bacteria in cultures supplemented with hexachlorobenzene or a mixture of 1,2,3- and 1,2,4-trichlorobenzene.

4.3 The effects of vancomycin on the growth and dechlorination activities of bacteria

The absence of most products of hexachlorobenzene transformation in all vancomycin applied cultures indicates that vancomycin had a strong inhibitory effect on the chlorobenzene-dechlorinating activity in our cultures. Williams and Bardsley 1999 proved that vancomycin inhibits the synthesis of a peptidoglycan cell wall in bacteria. When working on some pure strains of *Dehalococcoides*, Maymó-Gatell et al. 1997, Adrian et al. 2000 and He et al. 2003 found that *Dehalococcoides* strains grew and possessed reductive dechlorination activity in synthetic medium containing vancomycin because a peptidoglycan cell wall does not exist in *Dehalococcoides* strains. Inhibition by vancomycin therefore reveals that *Dehalococcoides* species were not responsible for chlorobenzenes transformation observed in our enrichment cultures. Gram-positive bacteria are generally highly sensitive to vancomycin. With this we obtained evidence that chlorobenzene-dechlorinating bacteria in our cultures belong to the *Firmicutes*. However, recovery of reductive dechlorination activity was found in most of the vancomycin-containing cultures after six months of incubation. 1,4-dichlorobenzene and monochlorobenzene were then found in the cultures amended with 1,2,3- and 1,2,4-trichlorobenzene. Monochlorobenzene, dichlorobenzenes and 1,3,5-trichlorobenzene were detected in cultures incubated with hexachlorobenzene and 1,3-dichlorobenzene was shown in cultures enriched with 1,3,5-trichlorobenzene after six months of incubation. Reductive dechlorination recovery of chlorobenzene-dechlorinating organisms can be explained by an inactivation, destruction or depletion of the antibiotic after this incubation time or by development of antibiotic resistance allowing bacteria to grow and to dechlorinate. A complete reductive dechlorination inactivity of bacteria when they were treated again with vancomycin in the second application indicates that the concentration of antibiotic applied to the first treatment was not enough to kill or completely inhibit bacteria. The detection of 1,4-dichlorobenzene and monochlorobenzene with low concentration from a mixture of 1,2,3- and 1,2,4-trichlorobenzene in vancomycin-containing cultures and the finding of only 1,3,5-

trichlorobenzene in vancomycin-containing cultures amended with hexachlorobenzene within 6 months indicate that bacteria preferentially removing singly-flanked or isolated chlorines were more vulnerable to this antibiotic than bacteria that preferentially remove doubly-flanked chlorines such as *Dehalococcoides* species. It is shown that there was a selective inhibition by vancomycin on different bacteria in the cultures and formation of 1,3,5-trichlorobenzene from hexachlorobenzene catalyzed by bacteria that preferentially dechlorinate double-flanked substituents was favored by vancomycin while this antibiotic inhibited other bacteria. The results of vancomycin application supplied good evidence to support our hypothesis of a competitive co-existence of different types of chlorobenzene-dechlorinating bacteria in the cultures.

4.4 The effects of oxygen on the growth and dechlorination activities of bacteria

The similarly in dechlorination products between the cultures containing oxygen-exposed inocula and the cultures containing inocula that were not exposed to oxygen indicates that the bacteria preferentially removing singly-flanked or isolated substituents in our cultures can withstand oxygen exposure. The insensitivity to oxygen treatment provided another evidence to support our hypothesis that bacteria preferentially dechlorinating singly-flanked or isolated chlorines in our cultures did not belong to *Dehalococcoides* species, which are described to be extremely sensitive to the exposure of oxygen (Adrian et al. 2000; Maymó-Gatell et al. 1997; Wagner et al. 2012). One again, the absence of *Dehalococcoides* species in all cultures spiked with inocula from different sources could be confirmed by the lack of correct expected sizes after PCR with *Dehalococcoides*-specific primers when compared to genomic DNA of *Dehalococcoides mccartyi* strains CBDB1 and 195 used as positive controls. However, the decrease in the rate of dechlorination in 1,3,5-trichlororbenzene dechlorinating cultures with oxygen-exposed inoculum indicated a change in the populations, possibly affecting bacteria that were important for syntrophic interactions with the dechlorinating bacteria.

4.5 Isolation of a chlorobenzene-dechlorinating strain

Several efforts have been described in the literature to isolate pure dehalogenating anaerobes and several pure strains have been obtained belonging to different phylogenetic groups. However, most of the pure isolated strains so far belong to two important groups of reductive dehalogenating bacteria *Dehalococcoides* and *Desulfitobacteria* which have been intensively studied. The evidence from this study for reductive dechlorination ability of *Desulfotomaculum guttoideum* strain VN1 demonstrates that the diversity of chlorobenzene-dechlorinating bacteria in nature is even larger and that there are more uncultured dechlorinating bacteria waiting for discovering. Moreover, this result gives a hint of testing other pure bacteria with halogenated benzenes which were isolated for other purposes such as sulfate reduction or nitrate reduction. To our understanding, this is the first report on the dechlorination ability of *Desulfotomaculum spp.*. *Desulfotomaculum guttoideum* was first described by Gogotova and Vainstein 1983. However, there was a misclassification of this species when it was first classified and the misclassification was found by a study carried out by Stackebrandt et al. 1997, who detected that the 16S rRNA gene sequence of strain *Desulfotomaculum guttoideum* is closer to the *Clostridium* cluster than to *Desulfotomaculum*. In our study, the result of a phylogenetic tree when comparing the 16S rRNA gene of *Desulfotomaculum guttoideum* strain VN1 to those of other *Desulfotomaculum* strains and to the 16S rRNA gene of some *Clostridium* species one again confirmed of the previous conclusion of Stackebrandt (Figure 45) that *Desulfotomaculum guttoideum* would be better classified as "*Clostridium guttoideum*".

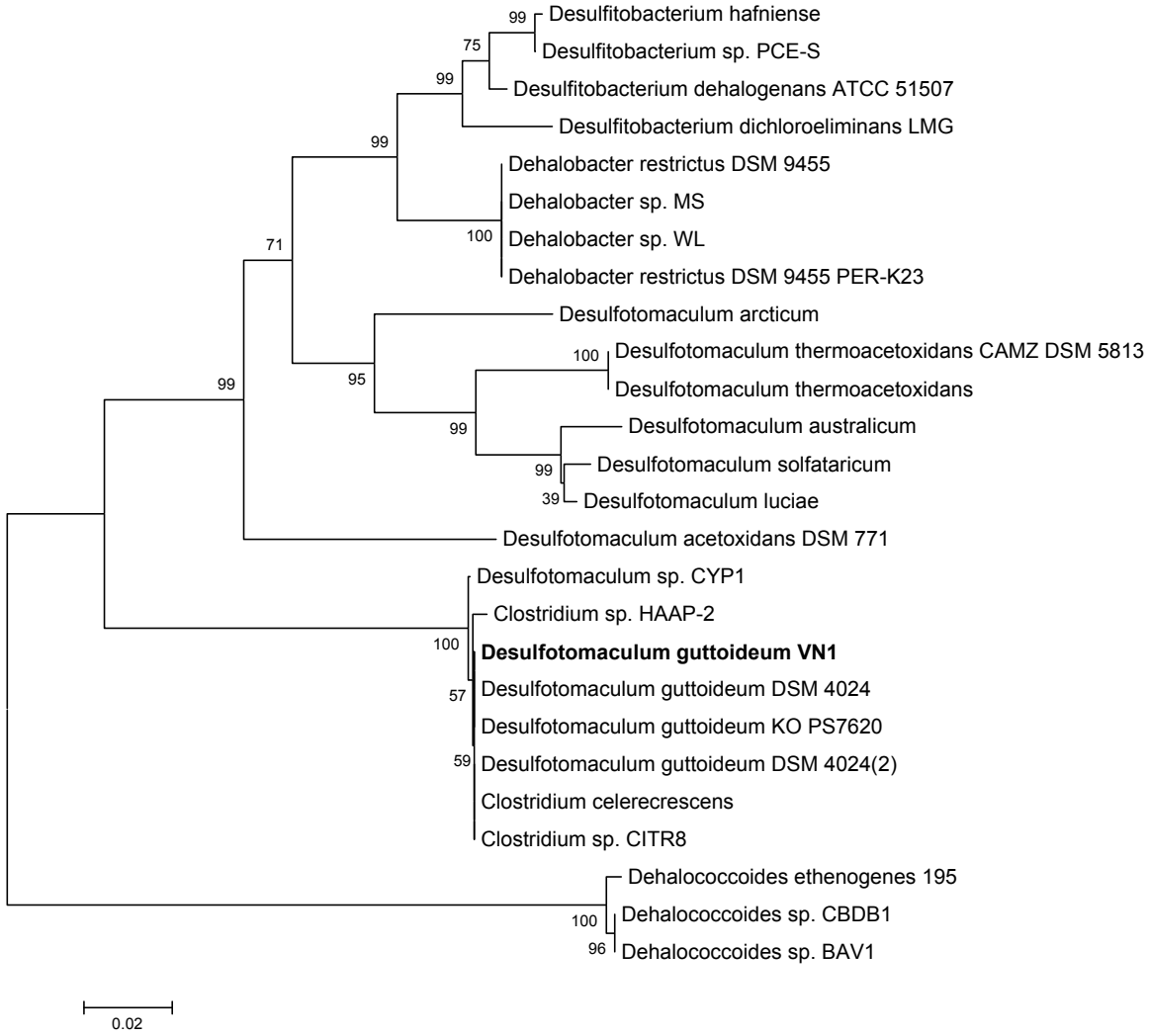

Figure 45: Phylogenetic tree based on 16S rRNA gene sequence comparisons of some described species of the genus *Desulfotomaculum* and some other additional strains focusing on bacteria with described reductive dehalogenating potential. The neighbor-joining tree was constructed from a distance matrix; Bootstrap values of 1000 replications are expressed at the branch points. Bar = 2 nucleotide substitutions per 100 nucleotides. Phylogenetic analyses were carried out with MEGA 5.2.

4.6 Dechlorination ability of strain VN1

1,2,3-trichlorobenzene transformation to mainly 1,2-dichlorobenzene and monochlorobenzene and formation of monochlorobenzene from 1,2-dichlorobenzene in the cultures of strain VN1 containing only 1,2-dichlorobenzene indicate that strain VN1 preferentially dechlorinates singly-flanked chlorines.

Moreover, a trace amount of 1,3-dichlorobenzene was found in 1,2,3-trichlorobenzene cultures and 1,4-dichlorobenzene was the only product of 1,2,4-trichlorobenzene transformation showing evidence of slow double-flanked chlorines dechlorination of VN1. However, 1,3-; 1,4-dichlorobenzene and 1,3,5-trichlorobenzene were not reductive dechlorinated by strain VN1. This shows the pure strain cannot dechlorinate isolated chlorines. To our knowledge, this is the first pure strain that can dechlorinate both singly-flanked and double-flanked chlorines, but preferentially dechlorinating singly-flanked chlorines.

A similarity in products of 1,2,3- and 1,2,4-trichlorobenzene mixture transformation between VN1 cultures and its parental mixed cultures with predominance of 1,4-dichlorobenzene and monochlorobenzene as products reveals that VN1 was the main chlorobenzene-dechlorinating bacterium in its parental mixed cultures. Yet, the mixed cultures reductively dechlorinated 1,2,4-trichlorobenzene as single substrate to predominantly 1,4-dichlorobenzene and monochlorobenzene and small amount of 1,3-dichlorobenzene while strain VN1 transformed this chlorinated compound to only 1,4-dichlorobenzene so it is apparent that other chlorobenzene-dechlorinating bacteria existed together strain VN1 in the mixed cultures.

4.7 Debromination ability of VN1

There was a significant difference between reductive dechlorination and reductive debromination pathways catalyzed by strain VN1 on chlorinated and brominated benzenes, respectively, having the same position of chlorines and bromines on the benzene ring. For instance, strain VN1 dechlorinated 1,2,4-trichlorobenzene to only 1,4-dichlorobenzene while this bacterium debrominated 1,2,4-tribromobenzene to benzene, monobromobenzene and all isomers of dibromobenzene. The same was true for all isomers of dichlorobenzene and monochlorobenzene compared to the isomers of dibromobenzene and monobromobenzene. Strain VN1 transformed 1,2-dichlorobenzene to monochlorobenzene but did not dechlorinate 1,3-, 1,4-dichlorobenzene or monochlorobenzene whereas all isomers of dibromobenzene and monobromobenzene were completely transformed to the non-brominated

benzene. This illustrates that chlorinated benzenes are more persistent than brominated benzenes when compared on the same number of chlorines and bromines as well as the same their position on benzene ring.

In the reductive debromination pathways of tested bromine compounds, strain VN1 preferred to remove bromines at isolated positions with formation of 1,2-dibromobenzene from 1,2,4-tribromobenzene and a quick reductive debromination of 1,3-, 1,4-dibromobenzene and monobromobenzene which did not occur with the respective chlorinated benzenes. Besides, removing doubly-flanked and singly-flanked bromine substituents still existed together with removing isolated bromine substituents in these bromination patterns. Interestingly, a considerable change in reductive dechlorination patterns and reductive debromination patterns on only pure strain was also found in a previous study with a pure strain belonging to *Dehalococcoides mccartyi*, strain CBDB1. In the cultures of CBDB1 supplemented with chlorobenzenes, reductive dechlorination occurred with only removing double-flanked chlorine substituents (Adrian et al. 2000) but with the cultures applied with bromobenzenes, strain CBDB1 also removed singly-flanked bromine substituents. Moreover, bromines at isolated positions of 1,3-; 1,4-dibromobenzene and monobromobenzene were also completely reductively debrominated by CBDB1 (Wagner et al. 2012).

A complete transformation of bromobenzenes leading to formation of a non-brominated product (benzene) which can be further mineralized by aerobic and anaerobic bacterial attacks to form non-toxic products such as CO_2 and CH_4 (Galic and Vogel 1987; Jindrová et al. 2002; Liang et al. 2013; Vogt et al. 2011) showed a great potential in application of strain VN1 to cleaning up brominated benzenes–contaminated sites.

4.8 The effect of pH on dechlorination activity and cell growth of strain VN1

The highest dechlorination activity and highest cell harvest were found at pH around 7.3, which was adjusted by adding $NaHCO_3$ buffer 1 M. This indicates that the pH adjustment and maintenance of fresh medium for strain VN1's growth and dechlorination by sodium bicarbonate buffer is better than NaOH. This can be

explained that *Desulfotomaculum guttoideum* strain VN1 is an acetogenic bacterium so CO_2 is very important for them to synthesize acetate. Therefore, buffer $NaHCO_3$ plays two roles in the cultures of VN1: pH stability and CO_2 supplement.

5. Conclusion

This research investigated the diversity and wide distribution of chlorobenzene-dechlorinating bacteria. Mixed cultures obtained from dioxin-contaminated soil and sediments in Vietnam and sediments in Germany dechlorinated a mixture of 1,2,3- and 1,2,4-trichlorobenzene preferentially removing singly-flanked or isolated chlorines. Hexachlorobenzene, one of the most persistent organic pollutants, was also dechlorinated by mixed cultures originating from sediments in Vietnam and Germany preferentially removing chlorines at isolated and singly-flanked positions. This recalcitrant chlorinated benzene was transformed to avoid accumulating 1,3,5-trichlorobenzene, which was a main final end-product of hexachlorobenzene dechlorination by mixed cultures and some pure chlorobenzene-dechlorinating bacteria in most previous reports. In contrast, only mixed cultures from sediments in Vietnam could dechlorinate 1,3,5-trichlorobenzene, another very recalcitrant organic pollutant and also the product of hexachlorobenzene transformation in many published cultures. Transformation of 1,3,5-trichlorobenzene also occurred demonstrating the unique ability of the cultures to remove isolated chlorine substituents. The dechlorination pathways of 1,2,3- and 1,2,4-trichlorobenzene, hexachlorobenzene and 1,3,5-trichlorobenzene remained stable over seven transfers.

Dehalogenating bacteria which preferentially remove singly-flanked and/or isolated halogen substituents as shown in this study have a great potential for bioremediation of hexachlorobenzene-contaminated sites, because the accumulation of chlorinated and persistent intermediates such as 1,3,5-trichlorobenzene can be avoided. Moreover, the removal of isolated chlorine substituents from 1,3,5-trichlorobenzene or 1,3-dichlorobenzene also has a significant practical value for *in situ* application at 1,3,5-trichlorobenzene-contaminated sites.

Sensitivity to vancomycin and insensitivity to brief exposure to oxygen of chlorobenzene-dechlorinating bacteria in all mixed cultures revealed that the

dechlorinating did not belong to genus *Dehalococcoides,* a group of bacteria that are very sensitive to oxygen but insensitive to vancomycin. An attempt to isolate pure cultures from the active mixed cultures with different sources of inocula was carried out and one pure chlorobenzene-dechlorinating strain was obtained from the mixed cultures amended with dioxin–contaminated soil. The result of 16s rRNA gene sequencing demonstrated that the isolate belongs to *Desulfotomaculum guttoideum* species and was designated as *Desulfotomaculum guttoideum* strain VN1. This study might be the first report on dehalogenation ability by *Desulfotomaculum guttoideum* species and also the first pure strain preferentially eliminating singly-flanked chlorine substituents. This strain could dechlorinate two isomers of trichlorobenzene (1,2,3- and 1,2,4-trichlorobenzene) but not 1,3,5-trichlorobenzene. For all isomers of dichlorobenzene, only 1,2-dichlorobenzene was transformed and hexachlorobenzene was not transformed by this bacterium. Of the brominated benzenes, the strain transformed all investigated brominated benzenes, namely hexabromobenzene, 1,2,4-tribromobenzene, all isomers of dibromobenzenes and monobromobenzene, and the main final end-product of all transformations was the non-brominated benzene compound.

The optimal temperature and pH for dehalogenation activity and growth of strain VN1 strain were 30^0C and 7.3, respectively. Dechlorination activity and cell growth of this bacterium remained when the strain was briefly exposed to oxygen. However, this strain could not grow in medium supplemented with 0.5% to 4.0% of NaCl or 0.2 mM to 1 mM of Na_2S. Similarly, in the presence of vancomycin, an antibiotic inhibiting cell wall peptidoglycan synthesis, dechlorination activity and growth were lost. The same was true for gentamicin at concentrations of 10 mg L^{-1} or more but dechlorination activity and growth of strain VN1 were found at a concentration of 5 mg L^{-1}. Strain VN1 produced H_2S from thiosulfate but not from sulfate or sulfite.

Strain VN1 could grow and dechlorinate in media without addition of vitamins or acetate. Hydrogen was not an electron donor for reductive dehalogenation but it was an electron donor for CO_2 reduction to synthesize acetate. Pyruvate

supported the growth of the bacterium but it did not support dechlorination activity whereas glucose could not be fermented by strain VN1 and was not an electron donor for dechlorination. In contrast, citrate played a determined factor in dechlorination activity as electron donor. Strain VN1 could grow in the medium without chlorobenzene and the reductive dechlorination process was via co-metabolism.

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