From: Svirsky.Susan@epamail.epa.gov
Sent: Friday, March 30, 2007 5:13 PM
To: Campbell, Scott (MNH); Palmieri, Linda
Subject: Fw: Comments on CMS for Housatonic R

Attachments: bedard 2006.pdf; CMS Comments 3-30-07 FINAL.doc

-----Forwarded by Susan Svirsky/R1/USEPA/US on 03/30/2007 05:12PM -----

To: tim gray <housriverkeeper@verizon.net>, Susan Svirsky/R1/USEPA/US@EPA
From: "Dr. Peter L. deFur" <pldefur@igc.org>
Date: 03/30/2007 07:07AM
cc: self <PLDEFUR@IGC.ORG>
Subject: Comments on CMS for Housatonic R

Dear Susan-
On behalf of the Housatonic River Initiative, I am submitting the attached comments on the Corrective Measures Study for the Housatonic River cleanup.

Please acknowledge receipt of these comments.

Yours truly,

Peter deFur

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Comments on the Corrective Measures Study
Dr. Peter L. deFur
Environmental Stewardship Concepts
Richmond VA
for
Housatonic River Initiative

March 30, 2007

This report on the Corrective Measures Study was prepared by ESC on behalf of the Housatonic River Initiative (HRI). The CMS was distributed February 27th and delivered February 28th. The brief comment period provided only enough time to examine the most significant portions of the CMS in depth. The first few chapters are mostly background and technical information that had been public in early documents. Comments on some of the materials were submitted in reviewing the Ecological and Human Health Risk Assessment. Most of the key information on cleanup is contained in Chapters 4 and 5. Chapter 4 has the descriptions of possible methods and the selection of the range of methods that will be considered and which methods will no longer be considered. Chapter 5 has much of the information on what range of alternatives and combinations will be considered in each stretch of the river.

The CMS is unexceptional for the most part; the document is weak regarding the substantive documentation of the major cleanup methodologies. The document takes a "routine business as usual" approach to cleaning up the PCB contamination from 135 miles of the Housatonic River in two states. The document is well organized and logical, with good reference to the figures and tables that seem to be well presented. There are, however, major substantive problems with the report. The list of these problems follows. Each of these issues or problems is explained below. There are separate sections on Appendices A and B.

1) Connecticut is ignored
2) The data are not sufficient to conclude Connecticut has no problems worth treating
3) There is almost no consideration of innovative methods
4) The major methods are dig up, cover up and let the river cover it up
5) There seems to be no comprehensive source control plan to prevent recontamination once any cleanup has been conducted
6) The decision to set the floodplain boundary based on 1 ppm soil concentration is arbitrary and without any scientific foundation- the waters determine the floodplain.
7) GE's objections to use the scientifically accepted and demonstrated TEQ methods have no place in the document, not even the appendix. Appendix A should be removed.

8) Capping is still a fairly new method in many waters, without clear effectiveness.
9) Natural Recovery is another phrase for let the river cover it up and is also questionable and without substantially demonstrable effectiveness.
10) Neither of the above two approaches (capping and “natural recovery”)is comprehensively documented at other sites.
11) Institutional Controls intended to restrict access to, or use of the river and its resources are just not sufficiently effective and the CMS provides no documentation for the effectiveness of institutional controls.
12) The IMPG’s are not protective and therefore cannot be used as a safe benchmark, and must be combined with protective measures that compensate for the inadequacy of the IMPG’s.

Given the fact that GE has recently announced the closure of the Pittsfield facility, and will no longer have a facility in the boundaries of the contaminated site, EPA should require a performance bond of GE. The cost of fully dredging and treating the 135 miles of river and floodplain is likely to reach many millions of dollars.

The CMS does not address the critical question of the expected life time of PCB’s buried in sediments, soil, or a landfill. The CMS retains the remedy of “natural recovery” for all sites, and considers “capping” for most underwater contaminated sediments. These “remedies” will leave PCB’s in place untreated and rely on some natural process to deal with the problem through covering up or perhaps breakdown. The CMS must provide some estimate of the time that can be reasonably expected before these PCB contaminated sites will no longer have PCB levels that pose a risk to human health or the environment. Research conducted here has not revealed any such estimates in the open literature. The experience of contaminated sites with PCB’s remaining in soils and sediments since the original manufacture more than 40 years ago indicates that the PCB’s will never breakdown in buried sediments, deep soils or landfills.

1) Connecticut is all but ignored. You will not find a statement in the document that says GE does not care about the CT portion of the river or that GE will never clean the CT portion of the river or that EPA does not consider it worth the trouble. But the silence on CT is deafening. There is bare mention of the CT portion of the river in section 3, and the one table that includes CT concludes that only 5% of the PCB mass is in CT, despite the fact that there are not enough data to reach that conclusion. (see our comments on the HHRA and ERA).

There is no mention of Connecticut in the ARAR’s listing except for meeting Connecticut water quality standards. This omission is further evidence (as if it were needed) of the whole document ignoring CT. Tables for section 5 are very illuminating. These tables indicate that the CMS plans to comply with federal laws and regulations, and those of Massachusetts, but only with the Connecticut water quality standards that require water flowing into the state meet state

standards. The CMS proposes no activities in Connecticut, hence there is no need to comply with Connecticut laws and regulations.

2) Data for fish tissue, sediment and floodplain levels in CT are insufficient. We commented on this gross deficiency during the risk assessment phase of the process. So few sediment data exist, especially behind the dams, that it is not possible to accurately depict the extent of contamination in CT. Now the CMS is proposing no further action beyond natural processes to cleanup contamination that has not been adequately characterized.

3) No Innovation. Innovative methods and alternative technologies are all but absent. The only mention of alternatives is use of Thermal Desorption of PCB's from dredged sediments, hardly a new or innovative method. This lack of innovation may in part stem from the federal Superfund act, CERCLA, which seems to have no incentive for new technologies. This feature of Superfund stands in stark contrast to the Clean Air and Water Acts which are intended by design to force new technologies by rewarding companies that use them. Not so with Superfund for either the companies conducting the cleanup or EPA and other agencies responsible for overseeing the efforts.

Innovative methods for cleaning contaminated dredged materials are more numerous than presented in the CMS. The CMS considers thermal desorption, but not sediment washing, sonic removal, deactivation of the contaminants or other such methods.

Two *in situ* methods of note are worth considering in the CMS under the present circumstances. First, researchers at Renselaer Polytechnic Institute have identified the bacterium that can breakdown PCB's (Bedard et al 2006 and 2007). In a paper about to appear in the April issue of Applied and Environmental Microbiology, Bedard et al (2007) describe isolation, and effectiveness of the bacterium that is responsible for anaerobic dechlorination of PCB's. This process removes chlorine (substituting H) from the most accumulative, toxic PCB's, rendering them far les toxic and accumulative and also susceptible to aerobic degradation. Bedard et al (2007) state: "Hence, through the combined action of anaerobic processes N and LP, highly chlorinated PCB congeners can be dechlorinated to PCB congeners with a low degree of chlorination that are susceptible to aerobic degradation and mineralization (i.e., complete detoxification)."

This discovery is a major step in the development of bioremediation processes for such compounds. The two pathways for bacterial breakdown of PCB's are with oxygen (aerobic) or without oxygen (anaerobic), the latter being more effective in removing chlorine from the highly substituted congeners.

The second "in situ" method is known as "Oil- Free Technologies" and has been used to breakdown chlorinated (and non-chlorinated) organic wastes at a number

of European sites. This method uses an enzyme extracted from earthworms to inject into soils and sediments and breakdown the contaminants. The web site, www.oilfreetech.com/index.htm, provides additional details on the effectiveness of removal and loss of PCB's from contaminated sediments and soils.

Both of these methods offer the opportunity to remediate contaminated sediments or soils without dredging or the massive disturbances of soil removal and replacement. Given the fact that this cleanup process will continue to take many years, the fact that these have not yet been used is not sufficient justification for ignoring these innovative opportunities.

4) The major methods are removal, cover with clean sand or watch the river cover it up. These methods are the same ones EPA has approved in other places in recent years, in other places. EPA seems to be comfortable with what they have always done and not with insisting that the solutions here are tailored to the unique or special characteristics of the river. The Housatonic River is a diverse water body with fast flowing sections, small ponds and large reservoirs in CT. The river flows through agricultural lands and woods, supporting warm and cold water fish species as it passes from rural to urban and into rural landscapes. The public (and EPA) should expect a greater array and range of technologies and options.

5) There is no distinct source control plan. Sites where the contamination derives from land-based sources require a comprehensive effort to find all sources and pathways and eliminate these. The CMS discusses a number of actions taken or underway, but these need to be coordinated by some entity and have a responsible person in a regulatory agency with the authority to insure that the source assessment is completed. Failure to have a coordinated and comprehensive source control program will result in the likelihood of recontamination of cleaned areas with new contamination. This problem has occurred in a number of other sites, including the St. Lawrence River, Eagle Harbor, and the Lower Duwamish River (Superfund site in Washington state). A source control program, not simply a list of activities, is needed for this site.

6) The floodplain is not defined by PCB levels. The CMS sets the boundaries of the floodplain considered for cleanup based on the extent of contamination of 1 ppm or greater, rather than on the basis of flooding. Floodplains are set by flooding events (see the US Geological Survey) not by arbitrarily set cleanup goals or standards here or on any other case. If the CMS is going to use the 1 ppm boundary, then the term floodplain has to be qualified so as to get the proper scientific meaning of the term. Floodplain already has a meaning and the CMS should not be inventing new definitions or meanings of scientific terms.

7) The appendix (A) complaining of the TEQ method should be removed. I have a problem with GE dismissing the use of the TEQ approach for both human health and wildlife. In fact, I expect that USFWS will take this action to court to

enforce the use of TEQ's for wildlife. The hesitations expressed in the NRC 2006 report were not a denunciation of the method, rather NRC endorsed using TEQ's. Doubts about TEQ's are not shared by the international scientific community, as evidenced by the latest publication of TEQ's (Van den Berg 2006). EPA and the international scientific community represented on the World Health Organization (WHO) use and endorse the TEQ method. Further detailed comments on Appendix A are presented below.

Using the TEQ's will make an important difference in the cleanup. At present, ignoring the TEQ contributions of non-PCB active chemicals reduces the boundary for cleanup and the extent of cleanup. The 1 ppm level in soils is not inclusive of all related chemicals because the TEQ chemicals are absent. Adding the other chemicals that contribute to TEQ's will move the 1 ppm boundary beyond present locations and expand the area requiring attention.

8) Capping is not demonstrably effective. Before relying on capping, GE and EPA must produce for the public a comprehensive list of sites where capping has been used for PCB contaminated sediments. This site listing needs to include the site characteristics, hydrology, cap duration, effectiveness and biota tissue levels as a function of time. This information needs to demonstrate the long term effectiveness of the technique in rivers such as the Housatonic. There are so little data presented in the CMS that no reasonable person would accept the capping method until and unless documented thoroughly.

9) Monitored Natural Recovery has a poor track record. Monitored Natural Recovery is not an active treatment; the only process known in this context is burial of old contaminated sediments with cleaner or less contaminated new sediments. The process that the CMS reports to be ongoing in section 4.2.3.1, MNR, in Woods Pond is burial, not recovery in the sense that the contamination is reduced. The contaminated sediments are covered up the way a dog covers up something it wants to hide. The facts on MNR for highly persistent, chlorinated organic chemicals in sediments in the US is not good. Several examples show how natural recovery fails to provide timely cleanup.

Kepone in the tidal freshwater James River was covered by layers of sediment (see Nichols1990) to the point that surface sediments were reduced. But fish still take up Kepone, more than 30 years after the end of manufacture and release.

The Hudson River offers another example of MNR. Now after more than 25 years following the decision to do nothing, the sediments have to be removed from the river because fish tissue PCB levels remain unacceptable with insufficient decline for the foreseeable future.

Newark Bay and the Passaic River are additional places where PCB's, dioxins and pesticides from the 1960's are still present and causing problems. The

buried sediments from decades ago are still presenting risks to human health and the environment.

MNR means burial for future generations to deal with, as in the Hudson River.

10) The CMS has to comprehensively document Capping and MNR as effective methods. There is no comprehensive examination of the long term effectiveness of caps or of monitored (or unmonitored) natural recovery. The few references to these methods include several that are quite recent and in tidal waters with completely different characteristics. The sites to which the CMS referred in reference to either capping or "natural recovery" are listed in Table 1 below, with a comment regarding the applicability of the site. None of the sites provided a good comparison with or reference for the Housatonic River in terms of hydrology, duration of remedy, climate, river characteristics, etc. Two of the sites had no documentation that could be found using Google searches, EPA sites or the peer reviewed literature in a university library. These two sites are not good cases to use as documentation because there is no public information available on them.

Additional information and comments, with references, is presented in Table 1 below. This information documents the inapplicability of many of these sites to the Housatonic River. Other sites have not yet conducted the work, or have recently completed activities, with no long term results possible.

11) Institutional Controls (IC) do not work effectively. The method of IC is not effective where there are subsistence fishers or trappers or access is via multiple avenues- land and water. When the river is used all times of day, and the fence is not monitored, people are either hungry, desperate or fiercely independent, the fences are not likely to keep people from using the floodplain and river.

Consumption advisories for animals in the Housatonic floodplains is marginally practical, and only in a few places. It is not clear how this "control" is going to work in remote and rural areas. This plan does nothing to address the needs and rights of native Americans who live and trap and catch in the river and floodplain. How will anyone determine the effectiveness of such advisories?

12) The IMPG's are not protective and certainly not conservative. Both citizens and state and federal agencies objected to these numbers. The objections were processed-base concerning the effort to derive these numbers that replace the risk assessment outcomes. The cleanup effort went to a great deal of expense and trouble to have human health and ecological risk assessments completed for PCB contamination in the Housatonic River. Now, these goals seem to replace the risk assessments with simplistic calculations unrelated to real conditions in the river. The absolute numbers may well be wrong. The IMPG's use central tendency exposures, rather than maximum

credible events (i.e. osprey are assumed to be present in the Housatonic water shed for 3 days, a weak assumption at best).

One example of the failure of the IMPG's is the effects of PCB's at tissue levels of 5.6 to 14.2 ppm in young fish (Fisher et al., in Rice et al., 2003). The IMPG's for fish is 55 ppm for warm water fish and 14 ppm for cold water fish. Young fish will likely succumb at PCB concentrations less than the IMPG's.

Evidence for the Applicability of Remedies to the Housatonic River

The cleanup sites used in the CMS to substantiate the effectiveness of capping or "monitored natural recovery" turn out to offer a different picture when examined more closely. Many of these sites are not comparable to the Housatonic, others have a poor record with remaining contamination problems in sediment or in fish. In several cases, the cleanup sites have been recontaminated because there was no source control program.

Table 1- Applicability of Cleanups Cited in the CMS. Sites listed in the CMS, documenting or supporting dredging, MNR and capping. The Table comments on the comparability or applicability of each site to the Housatonic River site. Details are given in the text below. No information or documentation could be found for Compressor Station 229 or the North Avenue Dam Site.

Site	Location/date	Issues	cite
Sangamo Weston	Pickens, SC 1994	Site is a lake, not a flowing river; climate is completely different;	1.
Fox River	Green Bay, WI. 2007	Only pilot work done so far, the remedy has not been selected	2
Spokane River	Spokane WA 2005	Recent work; river hydrologically different, effectiveness not confirmed	3
New Bedford Harbor	New Bedford, MA 2005	Coastal, tidal, marine waters; deep waters, incomplete	4
Eagle Harbor	Puget Sound, WA 2002	Different contaminants, calm harbor, problems related to CDF	5
St. Lawrence/ Grasse River	Massena, NY 2006	Fish advisories still in place	6
Manistique River and Harbor	Manistique, MI 2002	Thoroughly dredged prior to capping	7

Sheboygan Harbor and River	Sheboygan, WI 2000	Supports the capping remedy	8
Onondaga Lake	Syracuse, NY 2005	Site is a lake, not a flowing river. Site also dredged	9
Compressor Station 229	NY unknown	No information available	N/A
North Avenue Dam Site	WI unknown	No information available	N/A

1. EPA. 2004. Five-Year Review Report for the Sangamo Weston/Twelve Mile Creek/Lake Hartwell PCB Contamination Superfund Site. US EPA Region IV Atlanta GA
2. WDNR. 2006 Basis of Design Report: Lower Fox River and Green Bay Site. Prepared by Shaw Environmental Inc.
3. WSDE. 2005. Draft Cleanup Action Plan, Spokane River Upriver Dam PCB Site. Toxics Cleanup Program, Eastern Regional Office. Spokane, WA.
4. EPA. 2005. Five-Year Review Report for the New Bedford Harbor Superfund Site. US EPA Region 1 Boston, MA.
5. EPA. 2002. Five-Year Review Report: Wyckoff/Eagle Harbor Superfund Site. US EPA Region X Seattle Washington.
6. NYSDEC and the St. Lawrence River at Massena RAC. 2006. St. Lawrence River at Massena, New York Remedial Action Plan Status Report. Division of Water.
7. Great Lakes Commission and MDEQ. 2002. Remedial Action Plan Update: Manistique River and harbor Area of Concern Manistique, Michigan. Prepared by Triad Engineering Inc and Terrafirma Environmental Inc.
8. EPA. 2000. EPA Superfund Record of Decision: Sheboygan Harbor and River EPA ID: WID980996367, OU1, Sheboygan, WI. EPA/ROD/R05-00/030 2000
9. NYSDEC and EPA. 2005. Record of Decision: Onondaga Lake Bottom Subsite of the Onondaga Lake Superfund Site, Towns of Geddes and Salina, Villages of Solvay and Liverpool, and City of Syracuse, Onondaga County, New York.

Sangamo Weston (Lake Hartwell)

There are a number of reasons why the cleanup of Lake Hartwell is not applicable to the Housatonic. The primary reason is the type of water body. Compared to a river such as the Housatonic, lakes are incredibly static environments. The potential of weather related scouring from ice or floods is significantly reduced, and the environment is highly depositional. In short, it is a completely difference environment that is not applicable to the conditions in the Housatonic River. In addition to large differences in site characteristics, the long-term effectiveness of the cap in Lake Hartwell hasn't been proven. Fish advisories remain in place and are expected to for some time.

Fox River

The inclusion of the Fox River in the document as an example of the effectiveness of capping is curious, as no capping has been done in the river to

date. In fact, until very recently the preferred remedial alternative for the Fox River was dredging until the responsible parties proposed an amendment to the ROD promoting a combination of dredging, capping, and monitored natural recovery. The proposal is still under review, and was almost universally opposed by local citizens.

A number of the comments that have been received on the proposed amendment have noted that capping the Fox River would be incredibly ill-advised. Frazil ice, or the formation of ice crystals in turbulent water, is a frequent occurrence on the river and has resulted in scouring of over three feet in some places. Groundwater inputs that could potentially rupture caps from underneath were also an issue, along with flood related scour. Given the public resistance and the numerous problems that have been noted with capping this particular river, its inclusion gives absolutely no merit to the concepts of capping or monitored natural recovery.

Spokane River

Capping at the Upriver Dam PCB site has only recently been completed. This plan also received a poor reception from the public when it was introduced. Most notably the plan did not address the flux of PCB contaminated groundwater into the river, its effects on overall PCB levels and the integrity of the cap. Since the cap has only recently been put in place, any determinations about its effectiveness in either the short or long term would be premature. The selection of a remedy at one site does not mean that it will be effective at another, or even effective at all. Without long term data, there can be no evidence that these remedies are at all effective.

New Bedford Harbor

Though a capping remedy has been selected for New Bedford Harbor, the installation of those caps has not yet been completed and its expected effectiveness is still in question. Site-specific characteristics play a key role in the suitability of a remedy, and the New Bedford Harbor shares virtually none of the characteristics of the Housatonic. Deep, tidal marine environments are not subject to the physical stresses of flooding, droughts, etc and sediments within them are generally more stable overall. The selection of a particular remedy is no guarantee of success, and it remains to be seen how long the fish advisories at New Bedford Harbor will remain in place after completion of the caps.

Eagle Harbor

Eagle Harbor is in estuarine waters in the Pacific Northwest and a completely different environment than the Housatonic River. Conditions at Eagle Harbor cannot be considered to be evidence of the effectiveness of capping in dynamic environments such as rivers. The caps placed in the harbor range from 3 to 20

feet in thickness, a remedy that would be completely unfeasible in the Housatonic. This is not to say that Eagle Harbor can be considered a capping success story. PAHs are still entering the system through a number of seeps in the intertidal zone. A containment wall has been installed to prevent the groundwater from entering the system but has not been successful. A confined disposal facility (CDF) that was created at the site has also had problems and required repairs in the past. These problems at the site do not indicate that long term success of the capping remedy is likely and will require indefinite monitoring. It should also be noted that monitored natural recovery was considered for some areas of the site with lower contamination but was rejected because bacterial breakdown (which is significantly faster for PAHs than PCBs) would not occur fast enough to meet remediation goals.

St. Lawrence/Grasse Rivers

Capping was completed in the St. Lawrence River over ten years ago, but PCB levels in fish tissue still have not dropped below concentrations required to lift fish advisories meant to protect human health. The reasons cited in the review for the continued presence of unacceptable PCB body burdens included continued loading from unremediated soils from adjacent sites. This emphasizes the need to completely cleanup soils within the floodplain of a contaminated watershed, well beyond what GE is proposing in the Housatonic. Capping in the Grasse River, a tributary of the St. Lawrence has not begun and cannot be considered as evidence for the effectiveness of capping.

Manistique River and Harbor

The Manistique River on the upper peninsula of Michigan flows into Lake Michigan. The contaminated site consists of 1.7 miles and is one of the Great Lakes Area of Concern, not a Superfund site. Sediments were contaminated with PCB's and considered a threat to wildlife and fish in the Great Lakes, thus targeted for cleanup. Most of the 1.7 miles were dredged in places to remove the highest levels of PCB contaminated sediments. Some of the residual sediments were capped to prevent recontamination of the ecosystem. In 2004, sediment samples were taken as part of the ongoing monitoring effort. These results indicated a sharp drop in PCB levels in the surface sediments, undoubtedly a result of burial of the older more highly contaminated sediments with cleaner sediments.

Sheboygan River, WI

The remedy in this particular case was dredging a large part of the river to achieve 0.5 ppm PCB's throughout the full extent of the contaminated area. The contaminated section of the river is the last reach before emptying into Lake Michigan and has few hydrological similarities with the Housatonic. Considering

that the remedy in this case is primarily dredging contaminated sediments, there is little applicability to natural recovery or capping.

Onondoga Lake, NY
This site is in a lake, not a river, and the ROD is just 2 years old, signed in 2005. The work has not been completed and there is no record of effectiveness of the cap that was used after dredging. The remedy here was dredge most of the mercury contamination, cap 154 acres and install a pump system to maintain the cap and prevent contamination. The complexity of this site is greater in some respects than most of the Housatonic River sites, but also demonstrates some problems. One of the biggest problems is that interactions between the aquatic system and the adjoining groundwater and soils has to be carefully evaluated and perhaps controlled.

Compressor Station 229
No report could be found

North Avenue Dam Site
No report could be found

Appendix A: Assessment of Feasibility of Evaluating Dioxin TEQs in CMS

The CMS attempts to reject, and argues against the use of the internationally accepted Toxicity Equivalency Quotients (TEQs) originally developed by the World Health Organization (WHO). GE opposes TEQ's was for a variety of reasons, many of which are spurious. The TEQ methodology is an important tool in determining the true risks presented by mixtures of PCBs and other dioxin-like compounds, and contrary to claims in the CMS, have not been discredited by the recent reevaluation by the National Academy of Sciences (NAS). ESC notes that the CMS is an inappropriate venue for discussions regarding sampling methodologies and cleanup goals, which this appendix clearly intends. Previous documents that have undergone extensive peer review and been subject to public comment had already developed cleanup goals based on detailed risk assessments. This attack on the TEQ method appears to be another attempt by GE to subvert goals that have been agreed on by all stakeholders (much like the IMPGs).

The primary rational given in the CMS to disregard the TEQ approach is a lack of data stemming from the decision to sample primarily for total PCBs rather than by Aroclor. To this point, toxicity of each sample was determined by an EPA approved method (the Method Detection Limit approach). GE's own data shows this approach to be highly accurate, with the exception of samples of non-detectable concentrations of PCBs. TEQ values for such samples are highly variable, and this variability is GE's main argument against the EPA's estimation methodology. What GE fails to acknowledge is that dismissing this approach

would deviate from the accepted goals of conservatism in risk assessment. The reason that the Method Detection Limit is used is because of the very reason that GE dismisses it. Low concentrations of dioxin-like PCBs can have widely varying TEQ values, and therefore to maintain the principle goal of regulatory risk assessment (protecting human health and the environment) these samples are assumed to be more potent than what they may actually be rather than subjecting the public and wildlife to unacceptable risks. The approach developed by the EPA to estimate TEQ values from total PCB concentrations must be kept in place as it is the most protective approach for human health and the environment.

GE has also noted what they believe to be a number of problems with the TEQ methodology itself, based on the recent reassessment performed by the NAS. While GE accurately notes that many of the dioxin-like PCB congeners were not included in the NAS reassessment because of concerns regarding the accuracy of previous studies, nowhere in the report does the NAS recommend removing these congeners from the TEQ approach. While the TEQ values of these congeners are being verified in laboratory studies, GE is required to use the best available data, which in this case are the previously established TEQ values. The EPA has continued to use these values at other cleanups around the country and should not make an exception for the Housatonic River.

The other issue raised by GE regarding the TEQ methodology is the determination of the effective dose from dietary intake. The NAS report noted some of the uncertainty involving the bioavailability of dioxin from different media, associated with dietary exposure and the effect of body burdens on the uptake of dioxin-like compounds. There is a valid basis for some of the reported variability, as a number of studies have found highly variable uptake levels in both humans and laboratory animals. However, the magnitude and source of this variability is not particularly clear, and there is evidence that this variability may be a function of an individual's overall condition at the time of exposure. The evidence remains unequivocal that high levels of uptake do occur in situations found at contaminated sites.

Some of the uncertainty in the NAS report is based on studies by Umbreit et al. (1986a, 1986b).In these studies rats were fed soils from three different TCDD contaminated sites. While finding that rats displayed lower absorption rates of dioxins in soils, these data were highly variable with absorption rates spanning three orders of magnitude (Umbreit et al. 1986b). It should be noted that the absorption values obtained from these studies is quite low in comparison to others. One factor in this may be the use of gum acadia in the mixture the test animals were fed. Gum acadia is incredibly hydrophilic, which may have affected the experiments by changing the behavior of the dioxins within the gut. It is not clear that gum acadia is sufficiently similar to soil (likely not) to use the results without change. Even with these potential problems, the same congener (2,3,7,8-TCDD) was examined in all three soils and the data points to site

specific differences in soil chemistry significantly affecting absorption levels. The literature also points to the potential of other factors to influence the uptake of dioxins within the digestive tract.

Schlummer et al. (1989) examined the absorption of a wide range of dioxin compounds in food among individuals with varying body burdens of dioxin. The results of this experiment showed that uptake of dioxin like compounds were significantly influenced by total body burden of individuals. The degree to which uptake was hindered was congener dependant, and more specifically on the body burden of specific individuals (Schlummer et al. 1998). Higher body burdens also appear to increase excretion of dioxins (Rohde et al. 1998). If these factors were not accounted for in other studies it could drastically affect their outcomes in determining dioxin uptake through gastrointestinal absorption.

Schlummer et al. (1989) hypothesized that uptake and excretion were distinct processes, in part because body burdens were insufficient to explain all of the data. The authors proposed that differences in overall fat uptake influence diffusion gradients within gut, altering lipid concentrations in the gut wall and thereby its concentrations of dioxins. The authors hypothesized that these mechanisms could result in nearly complete absorption of dioxin like compounds when they are mixed with high fat content foods in the gut.

The above literature points to at least three factors playing significant roles in the absorption of dioxins and dioxin like compounds outside of congener type when ingested with soil: soil chemistry and other characteristics, total body burdens of dioxins, and fat content within the gut. These factors can change the absorption of dioxins significantly, possibly by an order of magnitude or more. The degree to which the above variables change overall absorption is still highly uncertain, with relatively few studies performed in the area and significant differences in their results.

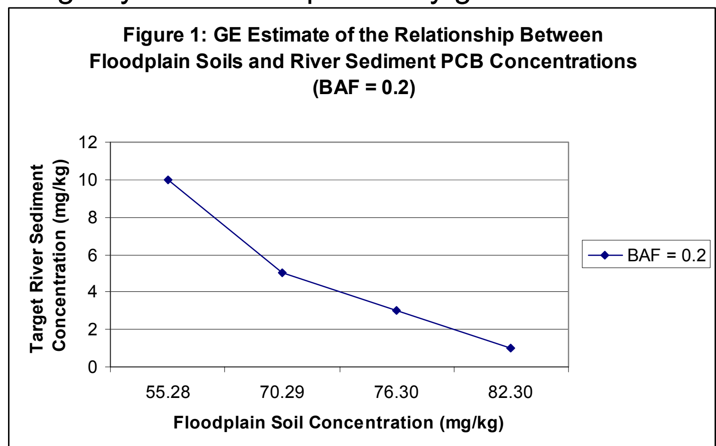
Based on these data, it is clear that dioxin absorption is affected by a number of environmental factors and physiological conditions in the affected people. Under some situations, dioxin is completely absorbed. EPA has addressed this variation by protecting all exposed individuals and assumes 100% absorption, based on the current information that has not been refuted. The current assumptions regarding uptake should stand. It is interesting to note that GE has spent considerable effort in attacking the TEQ methodology based on the uncertainties inherent in its use in the past. Now GE is seeking to disregard the methodology based on data that represent a variation not atypical of the human population and site conditions. NAS and WHO have both recently affirmed the use of the TEQ methodology, noting some of the issues raised by GE while failing to make any firm recommendations and urging more research because of the uncertainties noted above (Van den Berg et al. 2006, NAS 2006).

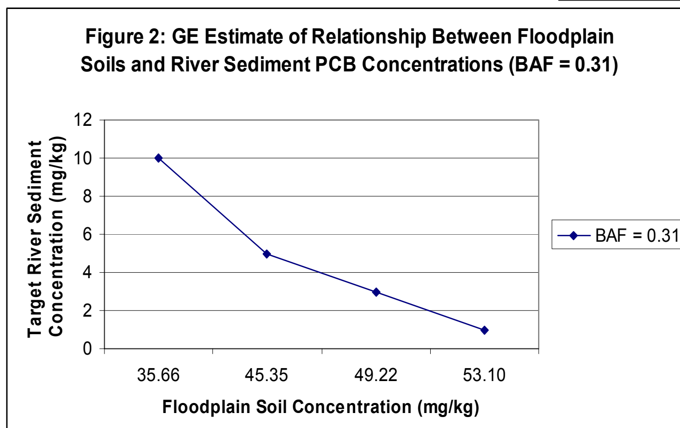
We emphasize that the **EPA, NAS and WHO have reaffirmed the use of the TEQ methodology**. EPA and USFWS recently developed guidelines for using TEQ's for wildlife (EPA, 2003). GE attempts to imply that the NAS did not fully endorse the methodology when in fact they have done the exact opposite. This approach has been examined in a number of reviews and all independent researchers and government entities that have examined it have all concluded that it is the best approach to determining risks from mixtures of dioxins and dioxin-like compounds. Allowing GE to avoid the use of this methodology would fly in the face of the literally decades of research that led to the TEQ development. The EPA must maintain the current requirement that GE use TEQs when determining cleanup levels and risks in the Housatonic River.

Appendix B: Development of Target Floodplain Soil Concentrations Associated with PCB IMPG for Insectivorous Birds

This appendix attempts to calculate floodplain soil concentrations that will result in and maintain a previously Interim Media Protection Goal (IMPG) developed for wood ducks by GE. The IMPG is an estimate of the maximum acceptable average concentration of PCBs in the diet of a target receptor, in this case wood ducks. Significant concerns about the values generated by GE for these goals, as well as the very concept of their creation have been raised by citizens, state environmental agencies, and federal regulatory agencies. If the methodology used in this appendix is any indication, those fears were particularly well founded.

A quick review of Table B-1 on the bottom of page B-3 should give any regulatory agency or neutral observer pause in trusting any values independently generated by GE. The data are plotted in figure form in Figures 1 and 2 below. This judgment can be rendered quickly, almost even without examining the methodology behind those numbers. GE expects the public, scientific community, and regulatory agencies to accept that wood ducks could be protected if *more* PCBs were added to floodplain soils.





The reversal of the logical trend in floodplain PCB

concentrations is the result of significant problems with Equation 6 on page B-2. The appendix claims that this equation was developed from the equation for wood ducks in the document that generated the IMPGs (GE 2006). However, ESC reviewed the cited section and found no such equation or one that Equations 1-6 resembled. Equation 6 was used to generate the values in Table B-1. As the equation is set up, the numerator of the equation decreases with increasing target river sediment values while the denominator stays constant. This results in the calculated Target Soil PCB Concentration declining rapidly as Target Sediment PCB Concentration increases.

GE's approach in effect allows for significantly higher concentrations in floodplain soils in areas with low sediment concentrations. GE focuses on the wood duck IMPG to determine these values, but the wood duck safety is not the proper endpoint for such an investigation. To determine floodplain soil concentration goals, the interaction between the floodplain and river sediments needs to be examined. The appendix does not take this approach.

By focusing on the wood duck IMPG alone, GE is viewing PCB concentrations in wood ducks as a zero-sum game- fewer PCBs in sediments allows for greater concentrations of PCBs in floodplain soils based on the invertebrate diet of wood ducks. Increases in sediment concentrations as a result of erosion of floodplain soils are not considered, nor are alternate routes of exposure that need to be examined if true risks to wood ducks were calculated such as incidental soil ingestion and inhalation. Therefore, GE is poorly implementing a methodology that does not deal with the facts of the situation.

GE should be examining the most sensitive receptors, both aquatic and terrestrial in determining a floodplain soil concentration that would be protective of wildlife. River sediment concentration goals will presumably not be arbitrarily decided, and will be based on data insuring the protection of one or more species. Whatever the selected goal, sediment concentrations cannot be maintained without controlling sources contributing from PCB loading. If high concentrations of PCBs are allowed to remain in the floodplain, these PCBs will eventually find their way back into the river through runoff and erosion related processes. These processes can also work in reverse, potentially depositing PCBs from the sediment into the floodplain. Any remediation goals that do not take these fluxes from river sediment to the floodplain cannot be considered accurate.

If the river sediment remediation goal is set to 1.0 mg/kg, it can be reasonably anticipated that this level cannot be maintained with floodplain soils that contain PCB concentrations in excess of 50 mg/kg. It should be also noted that at these concentrations simply being in close proximity to soils can have adverse effects for organisms outside trophic concerns, and is one reason they are considered hazardous waste under TSCA. The EPA has set the limit of 50 mg/kg because the agency believes that concentrations over those values "present an

unreasonable risk of injury to health within the United States" (EPA 1976). If wood ducks are to be the target receptor for protecting wildlife in the Housatonic, their exposure should be considered in the conceptual model depicted in Figure 3.

The above underscores the flaws in the IMPG creation process, and shows just how inappropriate they are for any sort of regulatory use. Because the values generated by this appendix are meant to be integrated into overall cleanup goals, it is important that all assumptions and equations used be accurate. In this case, neither are and should be a signal to regulatory agencies that GE is either not acting in good faith or simply does not have staff competent enough to be left in charge of such critical calculations.

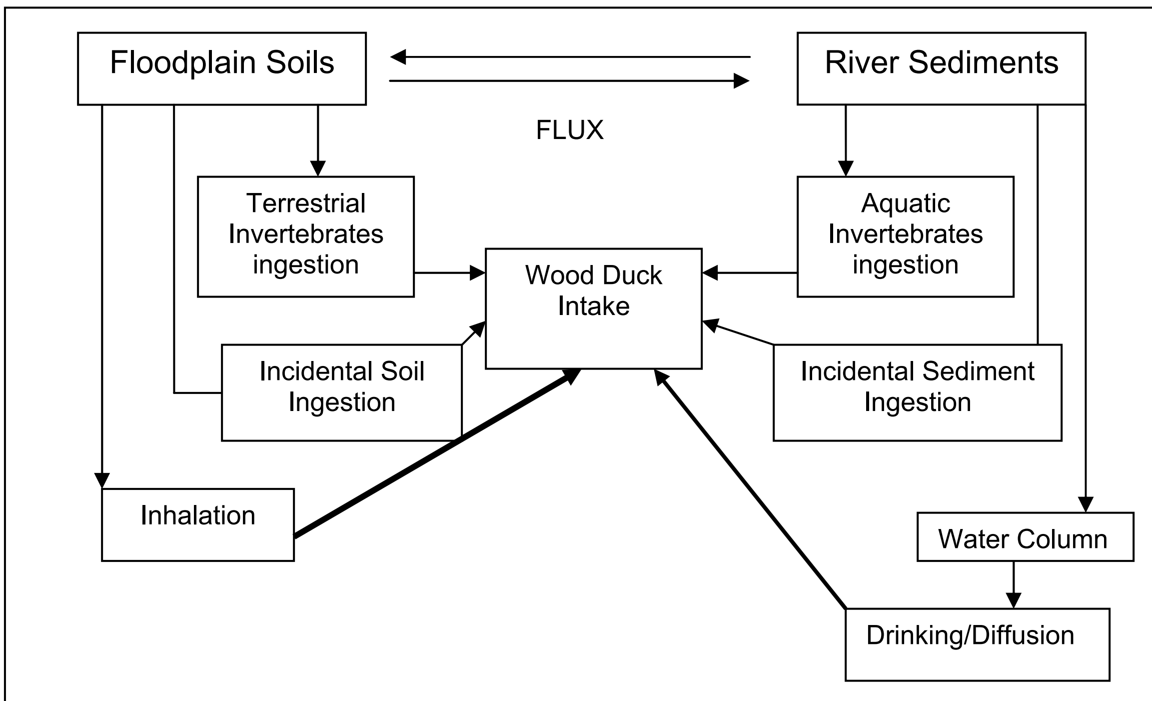


Figure 3 Conceptual model for Wood Duck Uptake of PCB's in the Houstaonic River ecosystem. The resulting equation is: Total intake = Food (terrestrial) + Food (aquatic) + Water + Inhalation + Soil (incidental) + Sediment (incidental)

<u>Section and Page specific Comments:</u>

Section 2.2.2- The description of the hydrology focuses on Reach 5 (abc) to the near exclusion of other reaches. See page 2-9, top.

Section 2.2.3 floodplain. Why is the floodplain defined in terms of PCB [] at all? Why is the floodplain not defined by flooding and waters and use a USGS definition?

See also page 2-11 Table 2-3 for floodplain data- only includes through reach 7- nothing in CT

Page 2-15- What plantings in the ½ mile clean-up?

Page 2-19 to 2-20- GE is now conducting a pilot scale capping in silver Lake. Where are the documents and data?

Page 4-7 CT has been ignored in this CMS. There is no specific statement in the document that says GE does not care about the CT portion of the river or that GE will never clean the CT portion of the river or that EPA does not consider it worth the trouble. But the silence on CT is deafening. There is bare mention of the CT portion of the river in section 3, and the one table that includes CT concludes that only 5% of the PCB mass is in CT, despite the fact that there are not enough data to reach that conclusion. (see our comments on the HHRA and ERA).

Section 4.2.3.2. Enhanced Sedimentation. This approach is to build dams or other flow retarding structures to alter the hydrodynamics to favor sedimentation in the river. This process will create more ponds such as Woods Pond, altering the habitat and the aquatic species assemblages. This process will change the nature of the river and move cold water fish species out of areas of the river and enhance the habitat for species that prefer slow moving waters. This change will also increase the residence of fish in such slow waters with contaminated sediments. As a result to the greater exposure time, the fish will likely suffer increased PCB and other chemical contamination.

We know of no sites where this plan has been done, so this should never have been even suggested, according to the criteria in the CMS. One criterion was the method had not been proven on another site in the US. Besides that fatal flaw, the resources agencies are not going to approve such a radical alteration of habitats that will alter the fish populations so drastically.

Section 4.2.5.3 Rechannelizing the river- the CMS lists a couple of places where this method has been used and certainly the Corps has moved rivers or streams for various reasons, including Pittsfield. GE took the opportunity to fill in the empty oxbows with waste that included PCB's. But this method is an option that has to be considered a "last resort" and still leaves PCB's that have to be contained with structures that may, in due time (100 years), fail.

Section 4.4.2.1 Access limitations under the IC section
This method of IC is not effective where there are subsistence fishers or trappers or access is via multiple avenues- land and water. When river use is all times of

day and the fence is not monitored and the people are either hungry, desperate or fiercely independent, the fences are not likely to hold.

Section 4.4.2.2 Deed restrictions (ERE) this section blatantly ignores CT. The CMS leaves no doubt that no action is planned for CT- no mention is made of how CT or the localities in CT view the matter of PCB contamination in the floodplain, but GE has ignored the CT portion of the river from the start and EPA shows no greater interest than GE.

Section 4.4.2.3- Conditional Use- With GE closing the plant in Pittsfield and leaving town, there will be no GE staff present to monitor or respond to community needs, problems encountered after remediation, etc. EPA should require GE to maintain an office in Pittsfield for the purpose of responding to community inquires and dealing with "use" issues into the future.

Section 4.4.2.4 Consumption advisories for animals in the floodplains. This plan is not going to work for a remote and rural area such as many miles of the Housatonic River and its floodplain. The native Americans who live and trap and catch in the river area will be forever denied use of the natural resources on which they once relied. Just how will anyone determine the effectiveness of such advisories?

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Development and Characterization of Stable Sediment-Free Anaerobic Bacterial Enrichment Cultures That Dechlorinate Aroclor 1260

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We have developed sediment-free anaerobic enrichment cultures that dechlorinate a broad spectrum of highly chlorinated polychlorinated biphenyls (PCBs). The cultures were developed from Aroclor 1260-contaminated sediment from the Housatonic River in Lenox, MA. Sediment slurries were primed with 2,6-dibromobiphenyl to stimulate Process N dechlorination (primarily *meta* dechlorination), and sediment was gradually removed by successive transfers (10%) to minimal medium. The cultures grow on pyruvate, butyrate, or acetate plus H_2. Gas chromatography-electron capture detector analysis demonstrated that the cultures extensively dechlorinate 50 to 500 μg/ml of Aroclor 1260 at 22 to 24°C by Dechlorination Process N. Triplicate cultures of the eighth transfer without sediment dechlorinated 76% of the hexa- through nonachlorobiphenyls in Aroclor 1260 (250 μg/ml) to tri- through pentachlorobiphenyls in 110 days. At least 64 PCB congeners, all of which are chlorinated on both rings and 47 of which have six or more chlorines, were substrates for this dechlorination. To characterize the bacterial diversity in the enrichments, we used eubacterial primers to amplify and clone 16S rRNA genes from DNA extracted from cultures grown on acetate plus H_2. Restriction fragment length polymorphism analysis of 107 clones demonstrated the presence of *Thauera*-like *Betaproteobacteria*, *Geobacter*-like *Deltaproteobacteria*, *Pseudomonas* species, various *Clostridiales*, *Bacteroidetes*, *Dehalococcoides* of the *Chloroflexi* group, and unclassified *Eubacteria*. Our development of highly enriched, robust, stable, sediment-free cultures that extensively dechlorinate a highly chlorinated commercial PCB mixture is a major and unprecedented breakthrough in the field. It will enable intensive study of the organisms and genes responsible for a major PCB dechlorination process that occurs in the environment and could also lead to effective remediation applications.

Polychlorinated biphenyls (PCBs) are priority pollutants that were used worldwide for a variety of applications for more than 50 years. PCB molecules are composed of a biphenyl backbone substituted with 1 to 10 chlorines. They were manufactured by catalytic chlorination of biphenyl to obtain specified weight percentages of chlorine for different applications. Consequently, they are complex mixtures, each composed of 60 to 90 PCB congeners. In the United States PCBs were produced under the trade name Aroclor. Several hundred million pounds of PCBs were released into the environment and pollute many rivers, lakes, and harbors worldwide. These PCBs persist in the sediments, accumulate in biota, and biomagnify in the food chain. Multiple adverse health effects have been attributed to them, and they are suspected human carcinogens (62). Microbial reductive PCB dechlorination provides a natural means of detoxifying PCBs in aquatic sediments because it reduces their persistence and increases their biodegradability and metabolism by other prokaryotes and by higher organisms (14, 16, 50, 51, 55).

There have been numerous reports of microbial dechlorination of Aroclors observed in situ and in laboratory experiments with sediment slurries (3, 9, 17, 18, 24, 34, 35, 47, 48, 53, 54, 66). Furthermore, eight microbial PCB dechlorination processes have been described (8, 9). Each of these dechlorination processes dechlorinates multiple PCB congeners in commercial PCB mixtures such as Aroclors, but they differ in the PCB congeners that are substrates, the PCB congeners that are products, and the chlorine positions targeted. However, until now no microbial PCB dechlorination process has ever been obtained in a sediment-free culture. Our objective was to develop and characterize a stable sediment-free culture that retained the ability to dechlorinate the same broad spectrum of PCBs that is dechlorinated in situ. The source of the sediment used to develop our cultures was one of the most highly PCB-contaminated rivers in the country, the upper Housatonic River in western Massachusetts. This river is contaminated with up to 668 mg/kg of Aroclor 1260 (15), a PCB mixture that is 60% chlorine by weight and is composed of congeners with 5 to 9 chlorines and an average chlorine number of 6.3.

Here we report the development and characterization of sediment-free enrichment cultures that extensively dechlorinate Aroclor 1260. The pattern of dechlorination is essentially identical to that of Process N, the major dechlorination activity observed in the Housatonic River. Hexa- through nonachlorobiphenyls were dechlorinated to less toxic and less persistent tri- through pentachlorobiphenyls. Our phylogenetic characterization of one sediment-free culture provides the best evidence to date of the bacteria that are actually involved in PCB dechlorination in the environment and suggests that *Dehalococcoides* spp. are involved.

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MATERIALS AND METHODS

Sediment collection and storage. Sediment samples were collected by repeated core sampling near the western shore of Woods Pond and transferred to 1-gallon glass jars which were then filled to the top with site water, sealed, and stored at 4°C until use.

Reagents. PCB congeners (purity, 99.9%) were purchased from AccuStandard (New Haven, CT). 2,6-Dibromobiphenyl (26-BB) (purity, 99.9%) was a gift from GE Corporate Research and Development (Niskayuna, NY).

Microcosm preparation. Microcosms were prepared in an anaerobic chamber (Coy Laboratories) in an atmosphere consisting of 95 to 97% N_2 and up to 5% H_2. Sediment was sieved to remove debris and then combined with anoxic sterile ultrapure H_2O to form a slurry (60% wet sediment, 40% water). Thirty-milliliter aliquots of the slurry were dispensed into 60-ml serum bottles. We added sterile disodium malate (pH 7.0) to a final concentration of 10 mM and 26-BB to a final concentration of 350 μM from a 70 mM stock solution in gas chromatography (GC)-grade acetone (OmniSolv; EM Science). Triplicate microcosms were sealed with Teflon-lined butyl rubber septa (West Company) and aluminum crimp caps and were then incubated in the dark at 22 to 24°C. Sterile slurries for initial transfers were prepared by pasteurizing the microcosms at 75°C for 10 min, incubating them at 22 to 24°C for 24 h, and finally autoclaving them at 121°C for 3 h. The enrichments were transferred onto sterile sediment slurries three times and incubated under a nitrogen headspace. These subcultures were supplemented so that they contained each of the following vitamins at a final concentration of 50 μg/liter: *p*-aminobenzoic acid, D-biotin, folic acid, niacinamide, D-pantothenic acid, pyridoxal, pyridoxamine, pyridoxidine, riboflavin, thiamine, DL-6,8-thioctic acid, and vitamin B_{12}.

Transfer onto medium without sediment. We transferred an inoculum (final volume, 10%) from triplicate sediment slurries to triplicate bottles containing defined medium and incubated them under a headspace consisting of 20% CO_2 and 80% N_2. We used a sulfide-free bicarbonate-buffered minimal medium amended with a selenite-tungstate solution, vitamins (including vitamin B_{12}), and a trace element solution (SL9) as described previously (2) and reduced with a solution of titanium(III) chloride (0.1 M)–citrate (0.2 M), pH 7, as described previously (2). However, after two transfers without sediment, we were unable to transfer a third time until we supplemented the medium with yeast extract. We eventually determined that 0.01% yeast extract maintained high dechlorination activity (see below). The enrichments were supplemented with pyruvate, butyrate, acetate plus formate, or acetate plus H_2. Hydrogen was added by injecting 5 ml with a sterile syringe into a 30-ml culture to obtain a nominal concentration of 7.5 mM. Initially, each carbon source was added at a final concentration of 5 mM. After this, enrichments were fed every 3 to 4 weeks. Acetate or acetate-plus-formate enrichments were fed by adding each compound at a concentration of 5 mM, and pyruvate and butyrate enrichments were fed by adding the compound at a concentration of 3 mM. The medium was amended with Aroclor 1260 (lot 023-150B; AccuStandard, New Haven, CT) at the concentrations indicated below. The Aroclor 1260 (and 26-BB in some cases) was added as a concentrated acetone solution to 300 mg of sterile silica (~240 mesh; Fisher Scientific). The bottoms and walls of the serum bottles were coated with the silica-PCB-acetone mixture by rotating the bottles on a vortex mixer, and then the acetone was gently evaporated with a stream of N_2 gas. After the silica was totally dry, the medium was added, and the silica-PCB mixture was resuspended by vortexing. We believe that this procedure greatly increases the surface area for deposition of PCBs and thus makes these compounds more readily available to the bacteria. Furthermore, the fine silica-PCB mixture is easily suspended and allows highly reproducible samples to be taken. The first few transfers on sediment-free medium were also amended with 26-BB (350 μM).

Extraction of PCBs and bromobiphenyls. We sampled the microcosms at intervals of 7 to 10 days for PCB and bromobiphenyl extraction and analysis during the incubation periods (up to 152 days). For cultures containing significant amounts of sediment, the samples used for analysis (0.2 to 1 ml) were aseptically collected under a stream of sterile, O_2-free N_2 gas by using a micropipette with the end of the tip cut off. After the second transfer without sediment, samples were taken by syringe after the syringe was rinsed with 5 mM sulfide to remove O_2. Samples were transferred to 8-ml glass vials fitted with Teflon-lined screw caps. Halogenated biphenyls were extracted with 4 to 5 ml of anhydrous diethyl ether (Mallinckrodt) by vigorous horizontal shaking on a platform shaker for a minimum of 16 h. Quantitative comparisons of samples extracted by this simple procedure and by a rigorous Soxhlet procedure (EPA 3540) (63) revealed no difference.

GC analysis and quantitation of halogenated biphenyls. Dehalogenation of 26-BB was monitored by GC-mass spectrometry as previously described (12). Congener-specific PCB dechlorination was monitored by high-resolution capillary GC analysis with a ^{63}Ni electron capture detector (ECD) as previously described (11). In brief, we used a Hewlett-Packard model 5890 GC-ECD operated in splitless mode and equipped with a DB-1 capillary column (length, 30 m; inside diameter, 0.25 mm; phase thickness, 0.25 μm; J & W Scientific, Inc., Folsom, CA). We used specially designed calibration standards prepared from Aroclor 1260 supplemented with all 43 of the PCB congeners previously identified as products of Dechlorination Process N (11, 58). The concentrations of the individual components of Aroclor 1260 in our standard were calculated from previously determined weight percent distributions of the congeners in Aroclor 1260 (25, 58). Our customized standard permits quantitation of 84 PCB peaks, including all significant peaks detected in our samples. The GC-ECD data were collected with Dionex AI-450 chromatography software (Dionex Corp., Sunnyvale, CA). PCBs were quantified with a five-point external calibration for the customized PCB standard (542 to 8,668 ng/ml) with a quadratic fit forced through zero. We calculated the mole percent value for each individual peak, the distribution of *ortho*, *meta*, and *para* chlorines per biphenyl, the total number of chlorines per biphenyl, and the PCB homolog distribution.

DNA extraction, cloning, and RFLP analysis. DNA was extracted from triplicate cultures that had been transferred eight times on sediment-free medium. We used a BIO 101 Fast DNA Spin Kit for Soil (Q-Biogene), which worked equally well for DNA extraction from sediment slurries and from sediment-free cultures, with slight modifications of the manufacturer's protocol. A total of 1.5 ml of culture was transferred to an empty 2-ml screw-cap Fast Prep tube and centrifuged at 16,000 × *g* in a microcentrifuge for 15 min, and the supernatant was discarded. This procedure was repeated with a second 1.5 ml of culture. Lysing matrix E was added to the pellet. The second modification of the protocol was to process the preparation twice for 45 s at speed 5.5 with a Fast Prep (Savant). The DNA was diluted 1:10 with sterile PCR-quality water, and 1 μl was used as a template for PCR amplification in a 25-μl reaction mixture with eubacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTA CCTTGTTACGACTT-3′) (42, 43) and Eppendorf MasterTaq according to the manufacturer's instructions. We used the following hot start PCR program. PCR mixtures containing all reagents except *Taq* polymerase were incubated for 2 min at 94°C, and then 1 μl of *Taq* polymerase diluted 1:10 with 10× *Taq* buffer was added. Twenty-five cycles of 45 s at 94°C, 45 s at 45°C, and 45 s at 72°C were carried out, followed by final extension at 72°C for 7 min and a final hold at 4°C. Agarose gel electrophoresis revealed a single band that was the correct size (data not shown). We used the Topo TA Cloning Kit (Invitrogen) to clone 1.5 μl of the PCR product. Ligation was performed for 30 min. Cells were plated on Luria-Bertani (LB) plates with ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (50 μg/ml). White colonies were picked onto fresh LB plates containing ampicillin and X-Gal, and 118 clones were checked for inserts by colony PCR with M13 primers as follows. PCR mixtures (25 μl) were prepared with no template, and then a sterile toothpick was used to gently touch a clone colony. The toothpick was inserted into the PCR mixture and stirred to transfer some cells to the reaction mixture. The amplification program was 7 min at 94°C and then 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension at 72°C for 12 min and a final hold at 4°C. PCR products were analyzed on 1% agarose gels. All but two clones had inserts that were the proper length. The clones were then subjected to restriction fragment length polymorphism (RFLP) analysis as follows. Ten microliters of each PCR product was incubated overnight at 37°C with 2 U of HhaI and 2 U of MspI (TaKaRa) in 20 μl (total volume) of a solution containing (final concentrations) 33 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, and 50 ng/μl bovine serum albumin. Following incubation, 2 μl of 10× loading dye was added to each reaction mixture, and the digests were electrophoresed on a 2% low-melting-point agarose gel (SFR; Amresco, Solon, Ohio) in 1× Tris-borate-EDTA at 170 V. RFLP patterns were analyzed visually.

Plasmid isolation and sequencing. Clones that were representative of each RFLP group were grown, and the plasmids were isolated with a QIAGEN Spin Miniprep Kit used according to the manufacturer's instructions. Cloned 16S rRNA genes that were representative of all unique RFLP groups were sequenced from both ends using the M13F and M13R primers. The sequences were checked for chimeras by CHIMERA_CHECK (44) and then analyzed by the Basic Local Alignment Search Tool (BLAST) (4) and by the Sequence Match tool of Ribosomal Database Project II (RDP II), release 9.33 (updated 10 November 2005; http://rdp.cme.msu.edu) (20). The closest matching sequences and type strains were identified using the RDP II Sequence Match tool. When possible, the new sequences were classified according to Bergey's Taxonomic Outline of Prokaryotes (26), using the RDP II-release 9 Classification tool (20). The sequences were aligned by the Ribosomal Database Project staff by taking rRNA secondary structure into account. Phylogenetic trees were constructed using PAUP* 4.0b

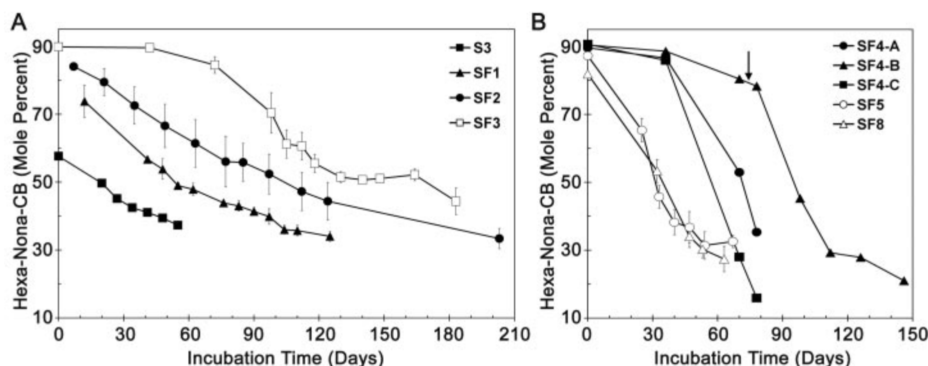

FIG. 1. Time course of dechlorination of Aroclor 1260 at various stages of the enrichment process. S3 is the third transfer on autoclaved sediment; SF1, SF2, SF3, SF4, SF5, and SF8 are the first, second, third, fourth, fifth, and eighth transfers without sediment, respectively. The Aroclor 1260 concentration was 5 μg/ml for SF1 to SF4 and 50 μg/ml for SF5 and SF8. For the S3 cultures, the sediment-associated Aroclor 1260 had been partially dechlorinated in situ. The concentration was approximately 45 μg/g (dry weight). Most data are averages for three replicates; for SF4 the data for three individual samples (SF4-A, SF4-B, and SF4-C) are shown. SF4-B showed minimal dechlorination by day 70 and was reinoculated from SF4-A and SF4-C on day 75 (indicated by an arrow). The error bars indicate the standard deviations of the means.

and were rooted using *Aquifex pyrophilus* as an outgroup. Maximum-likelihood trees were constructed using a heuristic search. Bootstrapped (1,000 bootstraps) neighbor-joining trees (using the Jukes-Cantor model) and maximum-parsimony trees were also constructed. Negative branches were not allowed, and zero-length branches were collapsed.

Nucleotide sequence accession numbers. The nucleotide sequences of cloned 16S rRNA genes have been deposited in the GenBank database under accession numbers DQ168641 to DQ168658.

RESULTS

Establishment of sediment-free cultures. We previously determined that it is necessary to stimulate PCB dechlorination in the sediments studied by the addition of a high concentration (350 μM) of a single PCB congener or brominated biphenyl, a procedure that we called priming (6, 11, 13, 64). Hence, we established active PCB-dechlorinating cultures in sediment slurries carrying out Process N dechlorination by priming with 26-BB as previously reported (11). These cultures were transferred three times onto sterile sediment slurries when extensive dechlorination had occurred but before dechlorination had stopped. Malate, 26-BB, and vitamins were added at each transfer. In each case the 26-BB was completely dehalogenated to biphenyl, and the PCBs were dechlorinated without delay, as shown in Fig. 1, culture S3. We expressed the data as the decrease in hexa- through nonachlorobiphenyls over time because hexa- through nonachlorobiphenyls comprise ~90% of Aroclor 1260. We then transferred the cultures onto sulfide-free bicarbonate-buffered minimal medium reduced with a Ti(III)-citrate solution; the final concentrations of Ti(III) and citrate were 0.8 mM and 1.6 mM, respectively. (We used sulfide-free medium because some organisms, including some sulfate reducers, are sensitive to sulfide.) Acetate and formate (5 mM each) were added as a carbon source and electron donor. PCB dechlorination was slow but steady for the first two transfers onto medium without sediment (Fig. 1, cultures SF1 and SF2), but three attempts to transfer a third time under the same conditions failed. After supplementing the medium with 0.1% yeast extract, we were able to transfer a third time without sediment, but there was a lag of about 2 months before PCB dechlorination began (Fig. 1, culture SF3). Since less than 5% of the 26-BB was dehalogenated after the third transfer without sediment, subsequent transfers were made without 26-BB. We subsequently decreased the yeast extract concentration to 0.01% and added 5 ml H_2 per 30-ml culture as a potential electron donor. These changes reduced the lag time for the fourth transfer to about 35 days in two of the three triplicates (Fig. 1B). However, the third triplicate still showed little dechlorination after 70 days until we reinoculated the culture. Slow dechlorination and samples that showed little or no dechlorination were fairly common. Therefore, beginning with the fifth transfer without sediment, we increased the concentration of Aroclor 1260 from 5 μg/ml to 50 μg/ml (135 μM). This eliminated the lag time, greatly increased the rate of dechlorination (Fig. 1B, cultures SF5 and SF8), and resulted in far more reproducible dechlorination. We also eliminated the formate from subsequent transfers because cultures amended with formate, acetate, and H_2 were no faster, and perhaps slightly slower, than cultures with acetate and H_2 alone. Parallel transfers with pyruvate and butyrate were also carried out, and for several transfers PCB dechlorination was more rapid on these substrates (data not shown). However, by the eighth transfer without sediment, dechlorination was faster in the

TABLE 1. Maximum observed rates of dechlorination at different PCB concentrations

No. of transfers without sediment	Expt	Aroclor 1260 concn		Maximum observed rate of dechlorination (nmol Cl/culture/day) (mean ± SD)[a]
		μg/ml	μM	
1	JN2	5	13.5	4.71 ± 1.10
2	JN3	5	13.5	3.53 ± 1.03
3	JN7	5	13.5	4.88 ± 0.83
5	JN12	50	135.0	169 ± 15
6	JN13	50	135.0	99 ± 6
		250	675.0	290 ± 51
		500	1,350.0	594 ± 93
8	JN18	50	135.0	100 ± 9
		250	675.0	544 ± 44

[a] The concentrations of Cl were calculated from the PCB analyses. Data were calculated per 30-ml culture.

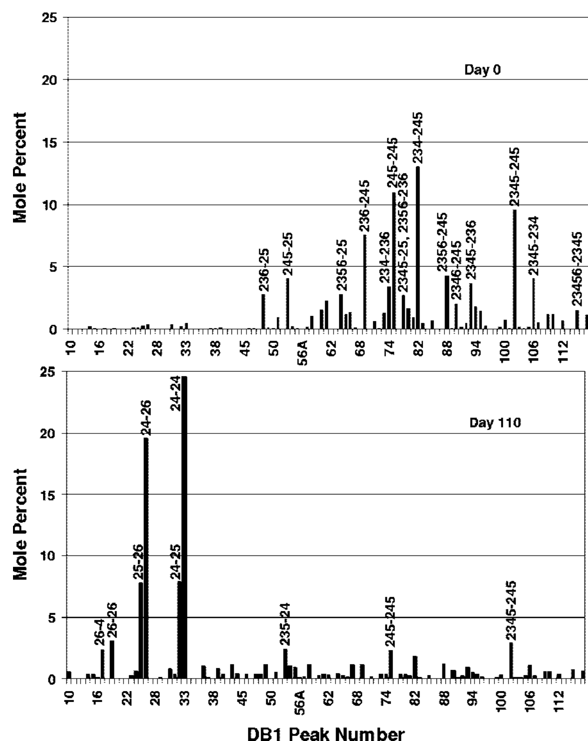

FIG. 2. Change in PCB congener distribution as a result of dechlorination in enrichment cultures transferred eight times without sediment. The data are the averages for three replicates. The concentration of Aroclor 1260 was 250 μg/ml (675 μM). A complete list of the congener assignments for all DB1 peaks is given in references 25 and 64.

cultures transferred with acetate plus H_2 (Fig. 1B, culture SF8); hence, the cultures grown on other substrates (data not shown) were discontinued.

Effect of PCB concentration on dechlorination. Increasing the concentration of Aroclor 1260 from 5 to 50 μg/ml had a profound impact on both the rate of dechlorination and the success of transfer. The maximum observed rate of dechlorination increased by a factor of more than 20 (Fig. 1 and Table 1). Also, transfers with 50 μg/ml had no lag time and were highly reproducible. Increasing the PCB concentration to 250 or 500 μg/ml further increased the rate of dechlorination (Table 1). These concentrations are 4 to 5 orders of magnitude greater than the solubility of Aroclor 1260, which is only 2.7 μg/liter (7.3 nM) (33, 61).

Sediment-free cultures carry out extensive dechlorination of Aroclor 1260. Figure 2 compares the PCB congener distribution for the eighth transfer without sediment at time zero and that after 110 days of incubation at 22 to 24°C. The data are the means for triplicate cultures incubated with 250 μg/ml of Aroclor 1260. Every peak for a congener with six or more chlorines was dramatically decreased. This analysis reveals an unprecedented breadth of substrate range in this sediment-free culture. Four major products were formed: 2,2′,4,4′-(tetra)chlorobiphenyl (24-24-CB), 24-26-CB, 24-25-CB, and 25-26-CB. (In this paper, we shall refer to PCB congeners by listing the substituted positions on each ring separated by a hyphen. Thus, 24-24-CB is the congener substituted at positions 2, 2′, 4, and 4′.) Significant amounts of 26-4-CB and 26-26-CB were also formed. This pattern of dechlorination is typical of Process N dechlorination, the dominant dechlorination activity in the Housatonic River (7, 10, 64). However, none of our previous investigations with sediment slurries have ever shown such extensive dechlorination.

Analysis of the PCB dechlorination. Analysis of the individual congener peaks over the course of the incubation showed that the congeners fell into three classes: substrates, intermediates, and terminal products. The results for several examples of each are shown in Fig. 3. All data are the averages for triplicate cultures incubated with 250 μg/ml of Aroclor 1260. Virtually all of the components of Aroclor 1260 were substrates. Hexachlorobiphenyls were dechlorinated at a higher rate than heptachlorobiphenyls (Fig. 3A), and heptachlorobi-

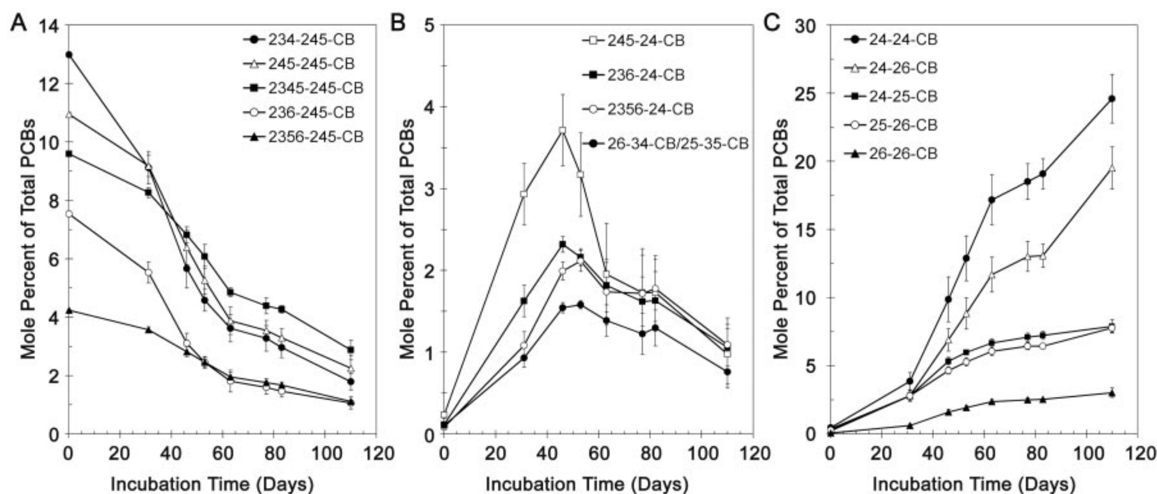

FIG. 3. Time course of changes in specific congeners during dechlorination of Aroclor 1260. (A) Dechlorination of five major components of Aroclor 1260. (B) Formation and subsequent dechlorination of intermediates. (C) Accumulation of major dechlorination products of Aroclor 1260. All data are averages for three replicates. The error bars indicate the standard deviations of the means. The concentration of Aroclor 1260 was 250 μg/ml (675 μM).

TABLE 2. Effect of dechlorination on PCB homolog distribution

PCB homolog[a]	Amt (mol%) (avg ± SD)		% Decrease
	Day 0	Day 110	
Trichlorobiphenyl	0.41 ± 0.02	4.10 ± 0.47	
Tetrachlorobiphenyl	2.19 ± 0.08	65.23 ± 3.52	
Pentachlorobiphenyl	12.53 ± 0.06	10.46 ± 1.30	16.5
Hexachlorobiphenyl	47.33 ± 0.12	8.62 ± 1.49	81.8
Heptachlorobiphenyl	31.17 ± 0.10	8.43 ± 1.06	73.0
Octachlorobiphenyl	5.08 ± 0.08	2.38 ± 0.60	53.2
Nonachlorobiphenyl	1.09 ± 0.20	0.58 ± 0.03	46.5

[a] No mono- or dichlorobiphenyls were detected.

TABLE 3. Effect of dechlorination on chlorine position

Chlorine position	No. of Cl/biphenyl (avg ± SD)		% Dechlorination
	Day 0	Day 110	
ortho	2.44 ± 0.00	2.49 ± 0.01	0
meta	2.47 ± 0.01	0.89 ± 0.09	63.9
para	1.37 ± 0.00	1.24 ± 0.00	9.4
Total	6.27 ± 0.01	4.61 ± 0.10	26.4

phenyls were dechlorinated more rapidly than octa- and nonachlorobiphenyls (data not shown).

The time course of appearance of the five most abundant terminal products is shown in Fig. 3C. All products were formed throughout the entire incubation, although at different rates. To a large extent, the rate of formation of each product was apparently determined by the amount of available substrate that could be dechlorinated to that product. As shown clearly in Fig. 3C, the initial rates of formation of 24-25-CB, 24-26-CB, and 25-26-CB were identical and were only slightly lower than the initial rate of formation of 24-24-CB. This was confirmed by the nearly equal heights of the peaks on chromatograms at the earliest stages of dechlorination. Subsequently, 24-24-CB and 24-26-CB were formed at much higher rates. A likely explanation for this is that the pentachlorobiphenyls which were the immediate precursors of these dechlorination products are present at very low concentrations in Aroclor 1260. For example, Fig. 3B shows that 245-24-CB and 236-24-CB, which are precursors of 24-24-CB and 24-26-CB, respectively, comprise less than 0.25 mol% of the total PCBs in Aroclor 1260. However, the levels of these intermediates subsequently increased to 3.71 and 2.32 mol%, respectively, as they were formed through the dechlorination of hexa- and heptachlorobiphenyls.

A close examination of Fig. 2 shows that there are several additional peaks which were higher at day 110 than at zero time. These are the peaks for 26-34-CB, 236-24-CB, 245-24-CB, and 2356-24-CB (peaks 39, 49, 54, and 67). However, an analysis of these congeners over the entire time course (Fig. 3B) revealed that these congeners are all intermediates whose levels initially increased and which were subsequently further dechlorinated. Other congeners in this class are 246-34-CB/236-246-CB and 234-25-CB (peaks 55 and 58A, respectively).

Effect of dechlorination on PCB homolog distribution. The dechlorination resulted in a shift in the homolog distribution from primarily hexa- and heptachlorobiphenyls to predominantly tetrachlorobiphenyls (Table 2). About one-half of the octa- and nonachlorobiphenyls were converted to less chlorinated congeners, but the experiment was terminated before dechlorination was complete and it is likely that the levels of these homologs would have been further decreased if the experiment had been continued. The level of pentachlorobiphenyls decreased only slightly, not because these compounds were not dechlorinated but because dechlorination was still in progress (Fig. 3B) and because new pentachlorobiphenyls, such as 246-24-CB, 246-25-CB, and 246-26-CB, were formed as terminal dechlorination products. Overall, the level of the hexa- through nonachlorobiphenyls decreased by 76%.

The dechlorination was almost exclusively at the *meta* position, but 9.4% of the *para* chlorines were also removed (Table 3). The *para* dechlorination was most likely from 2,3,4,5- (2345-) and 23456-chlorophenyl rings, where the *para* chlorine is flanked by two *meta* chlorines. This hypothesis was supported by the fact that intermediates with 2356-chlorophenyl rings were formed (Fig. 3B). No *ortho* chlorines were removed.

Specificity of dechlorination. Figure 4 shows the proposed routes of dechlorination of six heptachlorobiphenyl components of Aroclor 1260 to the four major tetrachlorobiphenyl products. On the basis of the quantitative results, we propose that 2345-chlorophenyl rings may be dechlorinated by either of two pathways. It appears that the dominant mode of dechlorination, occurring approximately 60% of the time, is via loss of the *para* chlorine, followed by loss of the *meta* chlorine in position 3 to generate a 25-chlorophenyl ring. The second mode of dechlorination is via sequential loss of both *meta* chlorines, first from position 3 and then from position 5. Hence, we propose that 2345-234-CB is dechlorinated by two different pathways that lead to two different products. The dominant pathway is 2345-234-CB → 235-234-CB → 235-24-CB → 25-24-CB. The second pathway is 2345-234-CB → 245-234-CB → 245-24-CB → 24-24-CB. For each of the six heptachorobiphenyls we indicate the proposed order of chlorine removal in Fig. 4. Note that the chlorination pattern of both rings affects the order in which chlorines are removed. In general, it appears that the substrate preference for chlorophenyl rings is as follows: 23456 ≈ 2346 ≈ 2345 > 234 > 245 > 236 > 2356 > 235 > 34.

Table 4 lists the major congeners of Aroclor 1260 that are dechlorinated to each of the major tetrachlorobiphenyl products and shows the mole percent decrease of each in triplicate cultures incubated with 250 μg/ml of Aroclor 1260 for 110 days. The list of substrates includes some hexa- and pentachlorobiphenyls which not only are present in Aroclor 1260 but also are transient dechlorination products of higher congeners, such as 245-245-CB and 245-234-CB. The latter two congeners are major components of Aroclor 1260 and are also transient intermediates of 2345-245-CB and 2345-234-CB, respectively. Congeners which are proposed to be dechlorinated by two different pathways (initial *para* dechlorination or initial *meta* dechlorination) are present twice in Table 4, and the observed decreases for these congeners are apportioned as proposed above for each of the two pathways and terminal products. Table 4 also lists the observed mole percent increases for intermediates which accumulate (as shown in Fig. 3B) and for the terminal dechlorination products. These data illustrate why

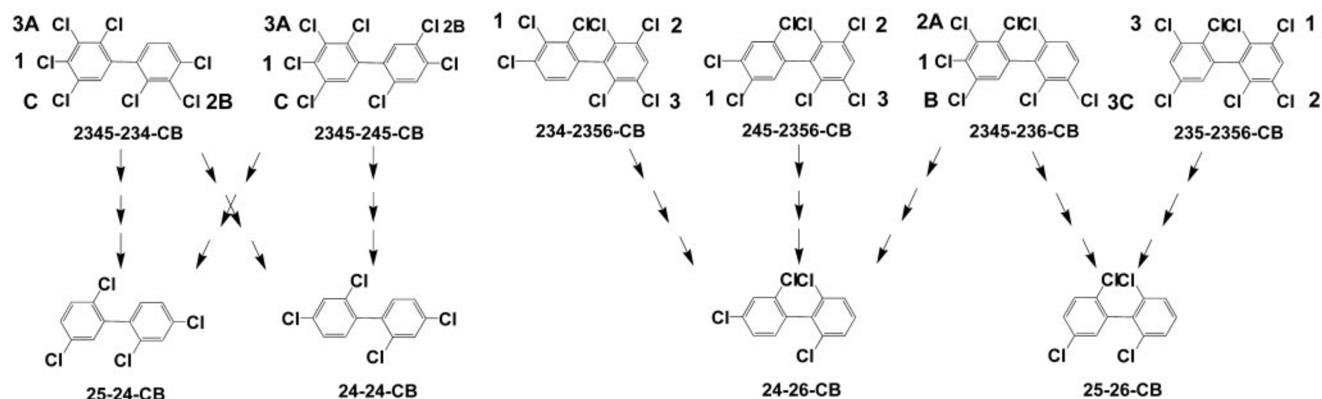

FIG. 4. Proposed pathways for dechlorination of six heptachlorobiphenyls to the four major dechlorination products observed in the enrichment cultures. We propose that the first chlorine removed from 2345-chlorophenyl rings may be either the *para* chlorine (60% of the time) or the *meta* chlorine in position 3 (40% of the time). The proposed order of removal of the chlorines for each congener is also shown. For congeners with 2345-chlorophenyl rings we indicate the preferred order of attack by 1, 2, and 3 and the less frequent pattern of attack by A, B, and C. Note that the chlorination pattern of both rings affects the order in which chlorines are removed.

24-24-CB and 24-26-CB accumulate to such high levels. They also permit a mass balance analysis. For 24-24-CB the mass balance is nearly perfect: an observed 23.9 mol% increase versus an observed 23.8 mol% decrease. For 24-25-CB and 25-26-CB the mass balances are also very good, approximately 108% and 94%, respectively. For 24-26-CB the mass balance is 139%. This could be due to an error in the calibration of 24-26-CB or to comigration of a contaminant with 24-26-CB, leading to an overestimate of this congener.

Sixty-four PCB congeners were confirmed substrates, and 47 of these have six or more chlorines. Virtually all congeners containing 34-, 234-, 235-, 236-, 245-, 2345-, 2346-, and 2356-chlorophenyl rings and some congeners with 23456-chlorophenyl rings were substrates. However, as previously observed

TABLE 4. Mass balance of penta-, hexa-, and heptachlorobiphenyls dechlorinated to the four major tetrachlorobiphenyl products

Substrates		Total decrease (mol%)	Intermediates		Products		Total increase (mol%)
Congener	Observed decrease (mol%)		Congener	Amt (mol%)	Congener	Amt (mol%)	
2345-245-CB[a]	2.7	23.8	245-24-CB	0.7	24-24-CB	23.2	23.9
245-245-CB	8.7						
2345-234-CB[a]	1.2						
234-245-CB	11.2						
2345-245-CB[b]	4.0	7.1	235-24-CB	Unknown[c]	24-25-CB	7.7	7.7
235-245-CB	1.1						
2345-234-CB[b]	1.8						
234-235-CB	0.2						
2345-236-CB[b]	1.7	8.3	235-26-CB	0.2	25-26-CB	7.5	7.7
235-236-CB	1.1						
2356-235-CB	0.5						
2356-25-CB	2.4						
236-25-CB	2.4						
2345-236-CB[a]	1.1	15.1	236-24-CB	1.0	24-26-CB	19.1	21.1
236-245-CB	6.5		2356-24-CB	1.0			
2356-245-CB	3.1						
2356-234-CB	1.3						
234-236-CB	3.1						

[a] The decrease for this congener was based on 40% *meta* dechlorination of the 2345-chlorophenyl ring to a 245-chlorophenyl ring.

[b] The decrease for this congener was based on 60% *para* dechlorination of the 2345-chlorophenyl ring to a 235-chlorophenyl ring.

[c] This congener coelutes with 245-25-CB and could not be resolved. It is a likely intermediate, but its contribution could not be determined.

TABLE 5. Phylogenetic analysis of sediment-free PCB-dechlorinating enrichment

Clone distribution			Bergey's classification (RDP II) of OTU		No. of 16S rRNA genes[c]	Closest match in GenBank database as determined by RDP Sequence Match and BLAST tools	% Identity
Clone(s)	n[a]	Normalized %[b]	Phylogenetic group	Closest classified relative (% certainty)			
JN18 V62 Y, JN18 V2 A*, JN18 V17 A3	23	18–26	*Betaproteobacteria*	*Thauera* (94)	4[d]	*Azoarcus* strain *LU1* (AJ007007)	98–99
JN18 A94 J	2	3–4.5	*Deltaproteobacteria*	*Geobacter* (96)	2	Uncultured bacterium clone W31 (AY770971)	96
JN18 A91	2	1.5–2	*Betaproteobacteria*	*Burkholderiales* (95)	3–6	Uncultured bacterium clone B44 (AF407722)	95
JN18 A13 Q, JN18 A60 A4, JN18 A17R	17	11–13	*Gammaproteobacteria*	*Pseudomonas* (100)	4–7	Uncultured bacterium clone 69-7G (AY955095)	99
JN18 107 G	42	27–32	*Bacteroidales*	*Porphyromonadacae* (100)	5–6	*Bacteroides* sp. strain Z4 (AY949860)	>99
JN18 A30 B, JN18 A96 B*	4	13–18	*Chloroflexi*	*Dehalococcoides* (100)	1	*Dehalococcoides* strains CBDB1 and FL2 (AF230641 and AF357918)	>99
JN18 A14 H	7	2.5–7	*Clostridiales*	*Sedimentibacter* (100)	3–12	Uncultured bacterium clone PL-5B8 (AY570591)	96
JN18 V41 S	3	1–3	*Clostridiales*	*Lachnospiracae* (94)	3–12	*Anaerotruncus colihominis* HKU19 (DQ080188)	94
JN18 A56 K, JN18 A89 K*	3	1–3	*Clostridiales*	*Clostridiacae* (64)	3–12	Uncultured rumen bacterium clone 4C28d-15 (AB034128)	87
JN18 A24 M	1	0.5–1	*Clostridiales*	*Syntrophomonadaceae* (96)	3–12	Uncultured bacterium clone Vadin CA02 (UEU81706)	97
JN18 A7 F*	3	2–10	*Eubacteria*	*Proteobacteria* (38)	1–7[e]	Uncultured bacterium clone SHA-53 (AJ249111)	99

[a] Number of clones.
[b] Percentage of clones normalized for estimated rRNA copy number.
[c] Number of 16S rRNA genes based on numbers reported for the same genus or closest relatives in reference 1 and in the online databases described in references 27 and 39 (see text).
[d] Value for *Azoarcus*, the closest relative whose 16S rRNA copy number is known.
[e] Value based on the range of *rrn* copy numbers for the *Proteobacteria* found in this study.

for Process N, the *meta* chlorines on 23- and 25-chlorophenyl groups were not substrates.

Analysis of sediment-free PCB-dechlorinating enrichment culture. We amplified and cloned nearly complete 16S rRNA genes from genomic DNA extracted from our culture after eight transfers without sediment. RFLP analysis of 107 clones and subsequent sequencing of the 16S rRNA genes of all distinct RFLP groups revealed 11 operational taxonomic units (OTUs) (Table 5). Each OTU was phylogenetically classified using the naïve Bayesian rRNA classifier hosted on the RDP II site (20). This classifier is trained on all known type strain 16S rRNA sequences. The query sequence is assigned a taxonomic hierarchy, and the calculation is repeated for 100 trials to assign an estimate of the certainty of the taxonomic assignment. Table 5 shows the major phylogenetic group and the most precise taxonomic unit that could be assigned for each OTU and also shows the closest sequence match in the GenBank database as determined by using both BLAST and the RDP II Sequence Match tool (4, 20). All but one of the OTUs had close sequence matches ($\geq$94 to $>$99% identity), but only four of those including *Dehalococcoides* matched isolated strains.

Table 5 shows the actual number of clones observed for each OTU for the 107 clones analyzed. However, due to potential PCR bias (5, 38, 41), cloning bias, and different numbers of rRNA gene copies for different bacteria (1, 27, 39), these numbers do not provide an accurate representation of the population distribution. PCR bias and artifact formation occur at higher rates during the last few cycles (38); therefore, PCR amplification was carried out for only 25 cycles in an effort to minimize such bias. Also, in order to minimize the rRNA copy number bias, we used data for 16S rRNA gene copy numbers from the closest phylogenetic relatives of our 11 OTUs for which such data are available to calculate percent distributions normalized by rRNA copy number. (The sources of these data were reference 1 and the online databases Ribosomal RNA Operon Copy Number Database, release 2.5 [posted 1 February 2004; http://rrndb/cme.msu.edu/rrndb/servlet/controller] and Genome Atlas Database [updated 8 November 2005; http://www.cbs.dtu.dk/services/GenomeAtlas/], as described in references 27 and 39.) Table 5 also shows the rRNA copy numbers for the genera most closely related to our OTUs, as well as the normalized percent distributions of the various OTUs.

The normalized data suggest that the most abundant bacteria in the PCB-dechlorinating culture were members of the *Bacteroidales* (27 to 32%) and *Thauera*-like *Betaproteobacteria* (18 to 26%), followed by *Dehalococcoides* spp. (13 to 18%) and *Pseudomonas* spp. (11 to 13%).

Figure 5 is a bootstrapped neighbor-joining tree showing the relationships of 18 sequences representing the 11 OTUs in the PCB-dechlorinating enrichment culture with their closest known type strains. The tree topologies were essentially identical for the neighbor-joining, maximum-parsimony, and maximum-likelihood trees.

DISCUSSION

Establishment of sediment-free cultures that extensively dechlorinate Aroclor 1260. Others have succeeded in sustaining PCB dechlorination activity in sediment-free media, but the reported activity of the cultures was restricted to a few PCB

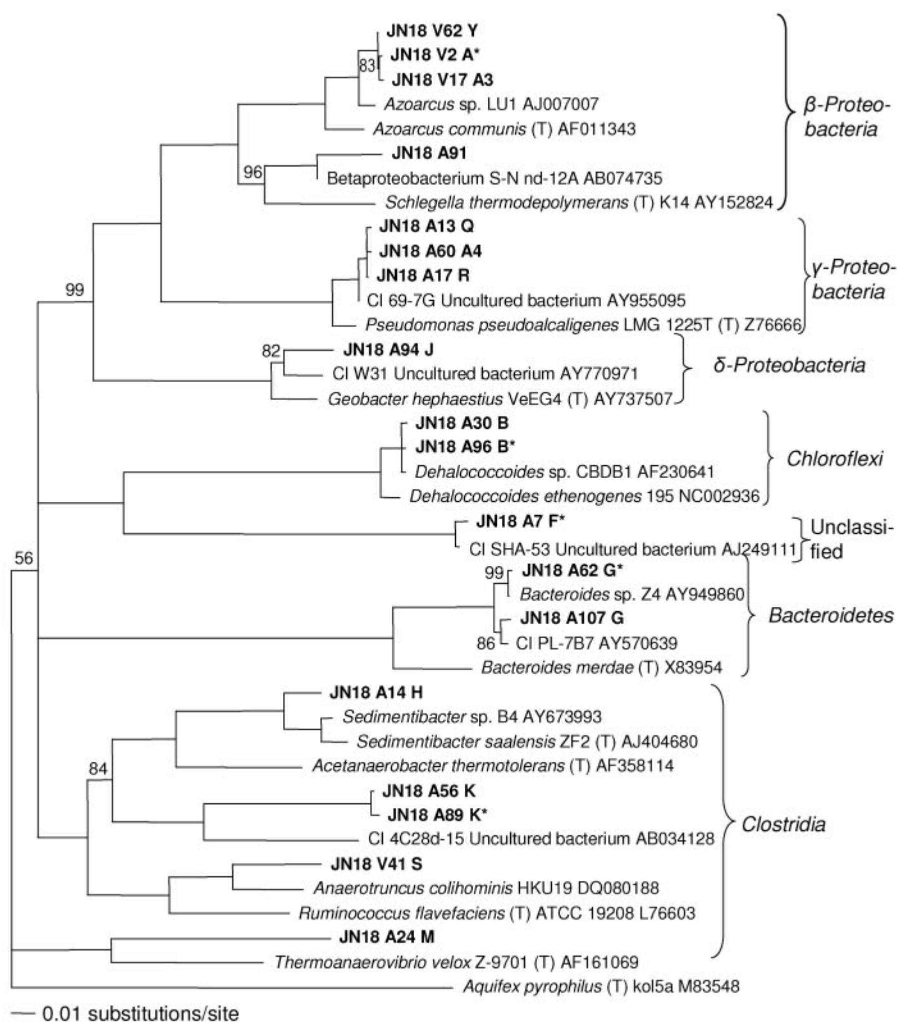

FIG. 5. Bootstrapped neighbor-joining tree of OTUs in sediment-free PCB-dechlorinating enrichment cultures and their closest relatives and type strains. The numbers at the nodes indicate the percentages of times that nodes appeared in 1,000 trials. Nodes that are not labeled appeared 100% of the time. Sequences cloned from DNA extracted from the enrichments after eight transfers without sediment are indicated by boldface type. JN18 refers to the experiment, and the additional letters and numbers indicate the clone name and RFLP designation where applicable. Type strains are indicated (T).

congeners that are chlorinated on only one ring and are not significant components of PCB mixtures found in the environment (31, 68). This is the first time that a sediment-free culture capable of degrading a complex Aroclor mixture has been developed. The cultures dechlorinate at least 64 PCB congeners, all of which are chlorinated on both rings and are present in Aroclor 1260 and 47 of which have six or more chlorines.

Our sediment-free enrichment cultures dechlorinated 76% of the hexa- through nonachlorobiphenyls in Aroclor 1260 (250 μg/ml) to tri- through pentachlorobiphenyls in 110 days at 22 to 24°C. These products are less toxic and less persistent in vertebrates (16). Nearly all congeners in Aroclor 1260 were substrates, including congeners with three or four *ortho* chlorines. The latter congeners exist as stable enantiomers, and we wondered whether their dechlorination would be limited. Stereoisomers of PCBs exist as racemic mixtures in commercial PCBs, and evidence for stereoselective dechlorination of some PCB congeners has been reported (52, 67). Our data show that there was well over 50% dechlorination of 236-236-CB, 236-245-CB, 2356-245-CB, and 2345-236-CB, demonstrating that both stereoisomers of each of these congeners are dechlorinated. However, since we did not separate the enantiomers, we do not know whether they were dechlorinated with the same efficiency. We thought that perhaps the presence of four *ortho* chlorines would sterically block access of dehalogenase enzymes, but the dechlorination of 236-236-CB shows that this clearly was not the case. This congener decreased from 1.56 mol% to 0.14 mol%, demonstrating that there was no steric hindrance or enantiomer selectivity.

Observed PCB dechlorination matches that seen in situ. It is especially significant that the PCB dechlorination observed in our enrichment culture, Process N, is the same dechlorination process that occurs in situ in the Housatonic River (7, 10), the site from which the sediment used for inoculum was obtained. This means that we have retained all of the bacteria critical for Process N in our culture and are now positioned to begin to

identify the particular bacteria and reductive dehalogenase gene(s) responsible for it. The breadth of the substrate specificity, 64 penta- through nonachlorobiphenyls and nine different chlorophenyl rings, indicates that the activity results from either multiple dechlorinators, multiple reductive dehalogenase genes in a single dechlorinator, or both. Raising the concentration of PCBs 10- to 100-fold increased the rate of dechlorination, eliminated the lag, and greatly improved the reproducibility of the transfers. This indicates that the dechlorinators are likely using the PCBs as terminal electron acceptors for halorespiration.

Process N dechlorination has also been observed in laboratory experiments with sediment slurries using microbial inocula from Silver Lake (Pittsfield, MA) (53), the Hudson River (New York) (9), and Baltimore Harbor (Maryland) (69); hence, the bacteria that carry out this process are apparently widespread in the environment.

Microbial community analysis with respect to halorespiration. Even after normalization, *Bacteroidales* appeared to be the most abundant bacteria in our enrichment culture. No members of the *Bacteroidales* are known to be halorespirers, and the prominent presence of these bacteria in our culture may have more to do with the citrate used to chelate the Ti(III) reductant in our medium than with any role of these bacteria in dechlorination. *Bacteroides* spp. are known to carry out fermentation of citrate to formate, acetate, and bicarbonate (36; see Table 17.8 in reference 46). Clostridia have frequently been found in dechlorinating enrichments, and members of four clostridial families were present in our culture. These organisms are typically fermenters and may have been present due to the citrate and the yeast extract in our medium. Several clostridia are known to carry out halorespiration, although PCB dechlorination has not been demonstrated. The dechlorinating clostridia include *Desulfitobacterium* spp., *Dehalobacter* spp., and *Clostridium bifermentans* DPH1 (45, 57). The first two taxa belong to the family *Peptococcaceae*, which was not detected in our enrichment culture.

Beta-, *Delta*-, and *Gammaproteobacteria* were all present. No dehalogenating bacteria have been identified in the *Beta*- or *Gammaproteobacteria*, and the role of the *Pseudomonas* and *Thauera*-like bacteria in the enrichment culture is unclear. Diverse *Azoarcus* and *Thauera* spp. are known to degrade a wide variety of aromatic compounds using oxygen or nitrate as a terminal electron acceptor (49). There was probably still some biphenyl present in our culture from the dehalogenation of 26-BB in earlier transfers, but there was no nitrate in the medium. The prominent presence of *Thauera*-like bacteria in our enrichment culture suggests that they may have another means of anaerobic growth.

There are several halorespiring species in the *Deltaproteobacteria*. These include *Desulfuromonas* spp., *Desulfomonile* spp., *Desulfovibrio dechloroacetovorans* SF3, *Anaeromyxobacter dehalogenans* (45, 57), *Geobacter lovleyi* (60), and *Trichlorobacter thiogenes* (22), a very close relative of *Geobacter* (59). Clone JN18 A94 J in our enrichment culture is closely related to *Geobacter*; thus, we cannot rule out the possibility that it could be involved in the dechlorination.

We did not find any sequences that belong to the DF-1 or *o*-17 group of *Chloroflexi* (65). DF-1 and *o*-17 are distant relatives of *Dehalococcoides* (87 to 89% identity for 16S rRNA sequences) that are known to dechlorinate several individual PCB congeners (21, 70).

We cannot rule out the possibility that our culture contains previously unidentified dechlorinating bacteria. However, the most likely candidates for PCB dechlorinators in our enrichment culture are members of the genus *Dehalococcoides*. The two *Dehalococcoides* sequences that we found have 16S rRNA sequences that are nearly identical to those of *Dehalococcoides* spp. strains CBDB1, FL2, and BAV1, which belong to the Pinellas group of *Dehalococcoides* (30). Strain CBDB1 can use hexa- and pentachlorobenzenes as terminal electron acceptors (37) and can also dechlorinate 1,2,3,7,8-pentachloro-*p*-dibenzodioxin (19). Strain FL2 can use trichloroethene and *cis*-1,2-dichloroethene as electron acceptors, and strain BAV1 can use *cis*-1,2-dichloroethene and vinyl chloride as electron acceptors (28, 29). Our *Dehalococcoides* sequences are also closely related to the sequence of *Dehalococcoides ethenogenes* strain 195 (Fig. 5), which has been shown to dechlorinate 23456-CB to 2346-CB/2356-CB and 246-CB (23). *Dehalococcoides* strains 195, FL2, BAV1, and CBDB1 contain 18, 14, 7, and 32 nonidentical reductive dehalogenase genes (32, 40, 56). Together, these reductive dehalogenase genes comprise 42 different orthologous clusters. We found two different *Dehalococcoides* 16S rRNA gene sequences in our culture, and we expect that each of the corresponding strains also has multiple reductive dehalogenase genes. It is intriguing to speculate that two or more *Dehalococcoides* strains with different sets of reductive dehalogenase genes act together to achieve the broad PCB congener specificity characteristic of Process N dechlorination. However, definitive proof of this requires firm evidence that the dechlorination of Aroclor 1260 is linked to the growth of *Dehalococcoides*. Studies to determine if this is the case are in progress.

Conclusion. For the first time, this research has identified and developed robust, highly enriched, sediment-free cultures of the bacteria that carry out Dechlorination Process N, a major microbial PCB dechlorination activity which occurs naturally in the Housatonic River. This is a major and unprecedented breakthrough in the field which will, for the first time, make it possible to conclusively identify the bacterial strains and reductive dehalogenase genes responsible for a major dechlorination process that actually occurs in the environment. The insights that we gain about these bacteria and their gene expression should also be useful in the development of effective remediation applications.

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