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Biodegradation of 2,4,5-trichlorophenol by aerobic microbial communities: biorecalcitrance, inhibition, and adaptation

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Abstract Chlorinated aromatic compounds challenge our environment and wastewater treatment processes due to their biorecalcitrance and inhibition. In particular, 2,4,5-trichlorophenol (TCP) seems to demonstrate greater resistance to biodegradation than other trichlorophenols and is a known uncoupler of the electron transport chain, although little work addresses this compound specifically. Here, we investigate the biorecalcitrance, inhibition, and adaptation to 2,4,5-trichlorophenol by aerobic mixed microbial communities. We show that 2,4,5-trichlorophenol is strongly resistant to biodegradation at concentrations greater than 40 μM, demonstrates inhibition to respiration in direct proportion to 2,4,5-trichlorophenol concentration (with 50% inhibition projected near 85 μM 2,4,5-trichlorophenol), and does not sustain biomass in continuous reactors, even when all input 2,4,5-trichlorophenol is degraded. Communities showed consistent adaptation patterns to 2,4,5-trichlorophenol at concentrations of 10 μM and 20 μM, but these patterns diverged at concentrations greater than 40 μM. Finally, thermodynamic approximations were used to estimate the yield of 2,4,5-trichlorophenol as 0.165 gVSS/gCOD, a low value that partially explains why biodegradation of 2,4,5-trichlorophenol did not sustain the biomass. In particular, we estimated that the minimum concentration to support steady-state biomass (S_{min}) is approximately 180 μM, a value much larger than the 40-μM concentration that is strongly resistant to biodegradation. Thus, readily biodegradable concentrations of 2,4,5-trichlorophenol are too low to sustain the biomass that biodegrades it.

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Abbreviations

TCP	2,4,5-trichlorophenol
2,4,5-T	2,4,5-trichlorophenoxyacetic acid

Introduction

Halogenated aromatic compounds are of practical importance due to their persistence in the environment, history of large-scale use, and toxicity (Haggbloom 1992). Among the halogenated aromatics, chlorophenols have been widely used as biocides and wood preservatives, and they can be produced in the bleaching process of pulp and paper mills (Ali and Sreekrishnan 2001). Understanding the biodegradation of chlorophenols can help us better predict their fate in the environment and to develop treatment schemes to remove them from wastewaters prior to their discharge.

Key among the chlorinated phenols is 2,4,5-trichlorophenol (TCP), which is present in the environment because it has been used in the production of a variety of biocides (Hazardous Substances Data Bank). TCP has been used as a biocide itself and as an intermediate in the production of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), one of the most widely used herbicides in the 20th century. It is included in the USEPA's list of priority pollutants (Maltseva and Oriel 1997). Furthermore, TCP is the first intermediate in microbial degradation of 2,4,5-T (Daubaras et al. 1995).

Although little work has explored 2,4,5-TCP biodegradation relative to other chlorophenols, it seems that TCP is remarkably more resistant to biodegradation then other trichlorophenols. Golovleva et al. (1990) found that a *Nocardiodes simplex* strain 3E, isolated from soils contaminated with 2,4,5-T, could aerobically degrade 2,4,5-TCP at concentrations up to 40 μM, but was toxic at a concentration of 80 μM. Similar results by Madsen and Aamand (1992) showed that 2,4,5-TCP could be dechlorinated at concentrations less than or equal to 40 μM. In comparison to 2,4,5-TCP, Lora et al. (2000) found that 2,4,6-TCP was degradable at concentrations into the mM range. Maltseva and Oriel (1997) isolated an alkiphilic 2,4,6-TCP degrading strain, *Nocardiodes* sp. strain M6, that could degrade up to 1600 mg/l 2,4,6-TCP (~8 mM) and 100 mg/l 2,4,5-TCP (~500 μM) in a highly concentrated cell suspension (OD_{550} of 3.0) at pH = 9.4, a value significantly higher then other work using less concentrated suspensions. Utkin et al. (1995) showed that *Desulfitobacterium dehalogenans* JW/IU-DC1 could readily dehalogenate 2,3,4,-, 2,3,6,-, and 2,4,6,-TCP, but neither 2,4,5-TCP nor its 5-chloro substituted analogs 2,3,5- and 3,4,5-TCP. Furthermore, 2,4,5-TCP is a known uncoupler of the electron-transport chain (Strand et al. 1999), effectively reducing the yield (Y) of the bacteria in its presence. The effect of TCP in this regard is so great that TCP addition to bench-scale simulated activated sludge systems has been exploited to reduce sludge production by up to 50% (Strand et al. 1999).

The purpose of this work is to understand the bases for the biorecalcitrance and inhibition of TCP to non-adapted and adapted activated sludge microbial communities. Biorecalcitrance refers to the inability of a compound to be biodegraded. Inhibition refers to a compound interfering with the metabolic activity of microorganisms. Because TCP appears to be the most toxic of the trichlorophenols, understanding and describing its biodegradation will serve to best elaborate the potential problems these compounds pose in wastewater treatment systems. Furthermore, due to the unique behaviors observed in response to TCP, we can gain insight into the mechanisms responsible for its difficult biodegradation and perhaps apply this knowledge to other halogenated aromatics. Specifically, batch studies were used to gauge the effect of TCP concentration on its recalcitrance and its inhibition of oxygen-uptake. Continuous studies assessed the ability of a community to adapt to TCP, which includes biodegradation of TCP and supporting biomass synthesis. Finally, we predicted the yield of TCP based on a proposed 2,4,5-T biodegradation pathway and thermodynamic approximations to help explain its unique biodegradation trends.

Materials and methods

Medium

The medium for all experiments consisted of 3.6 mM NH_4Cl, 45.0 μM $CaCl_2{\cdot}2H_2O$, 0.6 μM

$FeCl_3$, 4.2 μM $MgSO_4 \cdot 7H_2O$, 1.6 μM $MnCl_2 \cdot 4H_2O$, and 24.3 nM Na_2MoO_4, prepared in MilliQ water and autoclaved. 1-M phosphate buffer was sterilized and added to the cool medium for a final concentration of 10 mM with a final pH of 7.0–7.2. The TCP stock solution had a concentration of 1.0 mM and was filter sterilized prior to adding asceptically.

Inoculum

Activated sludge from the Stickney Wastewater Treatment Plant (Calumet City, IL) was frozen in 10 ml portions at –80°C in a 1:1 mixture with 50% glycerol (vol/vol). Prior to inoculating a reactor, the inoculum was thawed, centrifuged at 3300 g for 5 min, and 18 ml of supernatant was removed and replaced with clean medium. This process was repeated two more times (3 total cycles) to remove glycerol and soluble organic matter from the inoculum.

Reactor set-up

Batch reactors were 1-l Erlenmeyer flasks initially filled to 500 ml total volume. Continuous reactors also were 1-l Erlenmeyer flasks, but were fitted with an outlet at the 500-ml level, resulting in a 325-ml liquid volume in the reactor during shaking. Medium was pumped to the reactors using a calibrated Minipuls 2 pump with Viton pump tubing. All tubing from the aspirator bottle, holding the medium, to the reactors, excepting the pump tubing, was red (opaque) Teflon to minimize light effects of the photolytically sensitive TCP. The reactors were covered in aluminum foil to prevent photolysis and were operated aerobically at room temperature (20°C) with continuous shaking at 125 rpm.

For respirometry, the reactors were 300-ml media bottles. The contents were continuously stirred and maintained at 30°C. The inoculum was added directly to medium components; therefore, the growth substrate was residual organic solids from the activated sludge. Diluted glycerol also was present (1.5% vol/vol). The community was in the early endogenous decay phase so that the oxygen-uptake rate was nearly constant over time. A glass finger containing NaOH pellets was inserted into each reactor to absorb CO_2 produced, and the rate of oxygen-uptake was monitored continuously by a respirometer system from Challenge Environmental Systems Inc. (Little Rock, AR).

Sampling

Samples from the mixed reactors were removed periodically. They were monitored for bacterial growth via optical density (600 nm), calibrated to dry weight, using a spectrophotometer (Spectronic 20, Genesys). TCP concentrations were measured with high performance liquid chromatography (HPLC). First, the samples were filtered through a 0.2-μm PVDF membrane filter (Whatman 6872–2502). These samples were placed in HPLC vials and separated with a Hitachi D-7000 HPLC system having a Supelco SupelcoSil LC18 column (25 cm × 4.6 mm). The elution solvent was 70% pure HPLC-grade methanol plus 30% acetic acid in water (2% vol/vol). Concentrations were quantified by comparing sample peak areas to peak areas of standards.

Results and discussion

Biorecalcitrance of TCP

Based on preliminary results, we set-up batch reactors with 10-ml inocula (as outlined in the Methods section) and increasing TCP concentrations of 10, 20, 40, 65, or 90 μM. Figure 1 shows that adaptation and degradation occurred in the 10-, 20-, and 40-μM reactors, although the adaptation period increased with higher TCP concentration. The community did not adapt to concentrations greater then 40 μM within the time frame studied (45 days). This work agrees with past work (Madsen and Aamand 1992; Golovleva et al. 1990), suggesting that an upper threshold for TCP biodegradation is around 40 μM, beyond which biodegradation is strained.

Inhibition due to TCP

We operated four respirometry reactors, consisting of 1 control (no TCP) and 3 challenge reactors

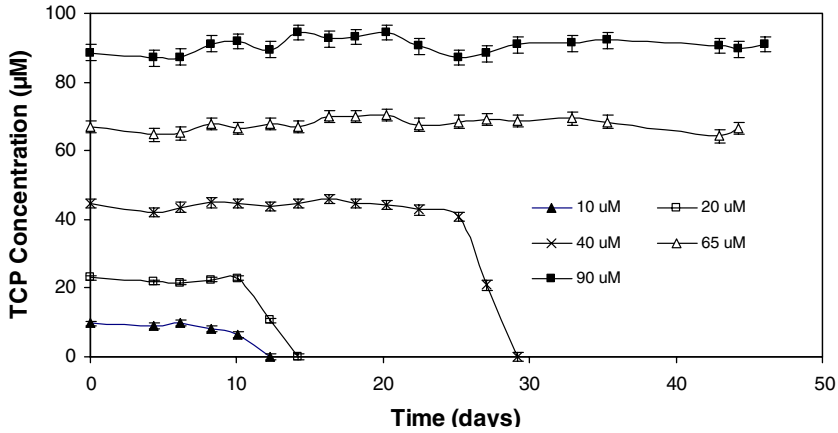

Fig. 1 Batch experiments with variable starting TCP concentrations indicate that, within the time period studied (45 days), adaptation and degradation were possible at or below 40 μM, but not at 65 μM or 90 μM

(with TCP), with non-adapted activated sludge. We sequentially added TCP in 40-μM steps to give concentrations of 40, 80, and 120 μM TCP to the three challenge reactors for approximately 2 h per concentration. Figure 2 presents the ratio of the average oxygen-uptake rate of the challenge reactors to the average oxygen-uptake rate of the control over the same time period. The relative oxygen-uptake-rate of the challenge reactors decreased in proportion to the TCP concentration, and linear regression gave a nearly perfect linear correlation. Extending the trend line to a ratio of zero predicts complete inhibition of respiration near 160 μM TCP. At 40- to 60-μM, the percent inhibition of oxygen respiration was 20–30%. Thus, inhibition of oxygen respiration probably was not the main cause of TCP's biorecalcitrance at TCP concentrations greater then 40 μM in the batch experiments.

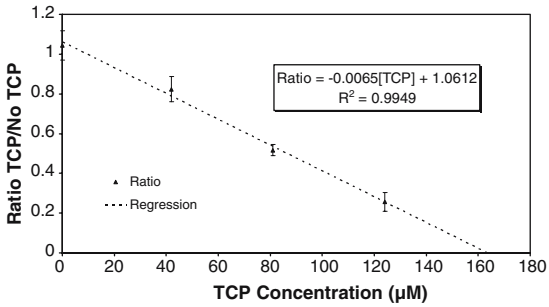

Fig. 2 Ratio of the average oxygen-uptake rate for the challenge reactors (with TCP) to the control reactor (no TCP) as a function of TCP concentration. The decrease in relative oxygen-uptake rate is directly proportional to TCP concentration

Adaptation to TCP

We tested adaptation by operating 4 batch reactors with a starting concentration of 10 μM TCP. Upon complete TCP degradation, more TCP was added, and this process was repeated with a range of increasing TCP concentrations. Figure 3 shows the course of adaptation for the 4 batch reactors and an abiotic control, which showed a stable TCP concentration of 10 μM for the 40-day duration of the experiment. The communities began to biodegrade TCP after about 10 days. When TCP was initially restored to 10 μM, it was removed within 2 days for all 4 reactors. Another addition of 10 μM TCP showed complete removal in less then 1 day. At this point, all 4 reactors showed the same pattern of adaptation.

We then spiked 3 of the 4 reactors to 40 μM TCP. Unlike the identical results for 10-μM spikes, the reactors spiked to 40 μM behaved quite differently from each other. Reactor 1 required 25 days to degrade the TCP, reactor 2 required 6 days, and Reactor 3 required 11 days. Subsequent additions of TCP to 50 μM in reactors 2 and 3 required an additional 17 days (reactor 2) and 7 days (reactor 3) for complete removal.

The fourth reactor was spiked with 20 μM TCP and took roughly 3 days for complete removal of the TCP. Repeating the 20-μM spikes four more times gave somewhat faster removal kinetics, but total removal still took about 3 days for each spike. Then, we spiked TCP to 20 μM, and, approximately 1 day after this spike, increased TCP to 45 μM. Although the community was

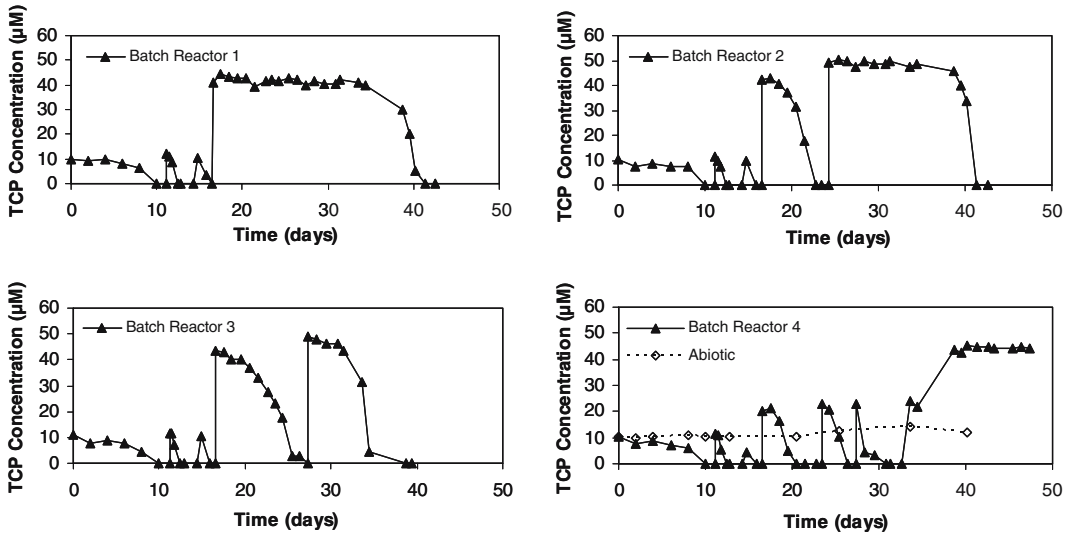

Fig. 3 Adaptation to TCP demonstrated reproducible behavior in batch reactors at 10μM and 20 μM TCP, but began to diverge from each other at higher concentrations

active in biodegrading the 20-μM spike, no TCP degradation occurred at 45 μM, even after 24 days. This result underscores the increased biorecalcitrance of TCP at concentrations greater then 40 μM.

Continuous degradation of TCP and community response

Figure 4 presents the TCP concentrations for four continuously operated reactors. We started all reactors in batch mode with 10 μM TCP to allow adaptation. Continuous operation began on day 15 with a hydraulic retention time (HRT) of 2.5 days. From day 15 until day 21, the TCP concentration in the feed was 25 μM. TCP was completely removed, but the biomass declined steadily. The inlet TCP concentration was then increased to 60 μM for 4 days (days 21–25) and finally to the final inlet TCP concentration of 80 μM.

Figure 4 shows that adaptation took place within 15 days, but even with consistent TCP removal from days 15–35, biomass accumulation was negative. Within a short amount of time thereafter, reactor 3 failed. Reactors 1, 2, and 4 also exhibited signs of failure near day 45. At this point, we shifted the reactors back to batch mode. Upon the shift to batch mode, the remaining TCP was rapidly degraded, and additional input spikes of 20 μM TCP were also rapidly degraded except in reactor 3, whose TCP concentration was well above the 40 μM threshold.

We restarted the reactors in continuous mode, except that the flow rate was halved and the input TCP concentration doubled, so that the TCP loading to the reactor was the same. Increasing HRT by a factor of 2 should help slow-growing microorganisms accumulate. However, reactors 1 and 4 quickly failed after the shift back to continuous mode, reactor 2 exhibited some capacity for removing TCP, and no reactor gave an indication of positive growth. Additionally, the rate of accumulation of TCP also increased over time, indicating that the communities' ability to degrade TCP was failing.

Yield and S_{min} analysis

We estimated the biomass yield for aerobic biodegradation of TCP by using the proposed microbial degradation pathway for 2,4,5-T, since TCP is its first intermediate (Ellis et al. 2003; Daubaras et al. 1996). The pathway is shown in Fig. 5. The pathway shows that 14 electrons are invested in oxygenations of TCP before it is transformed to 3-oxoadipate. Then, the remaining 24 electrons in oxoadipate contribute to supplying

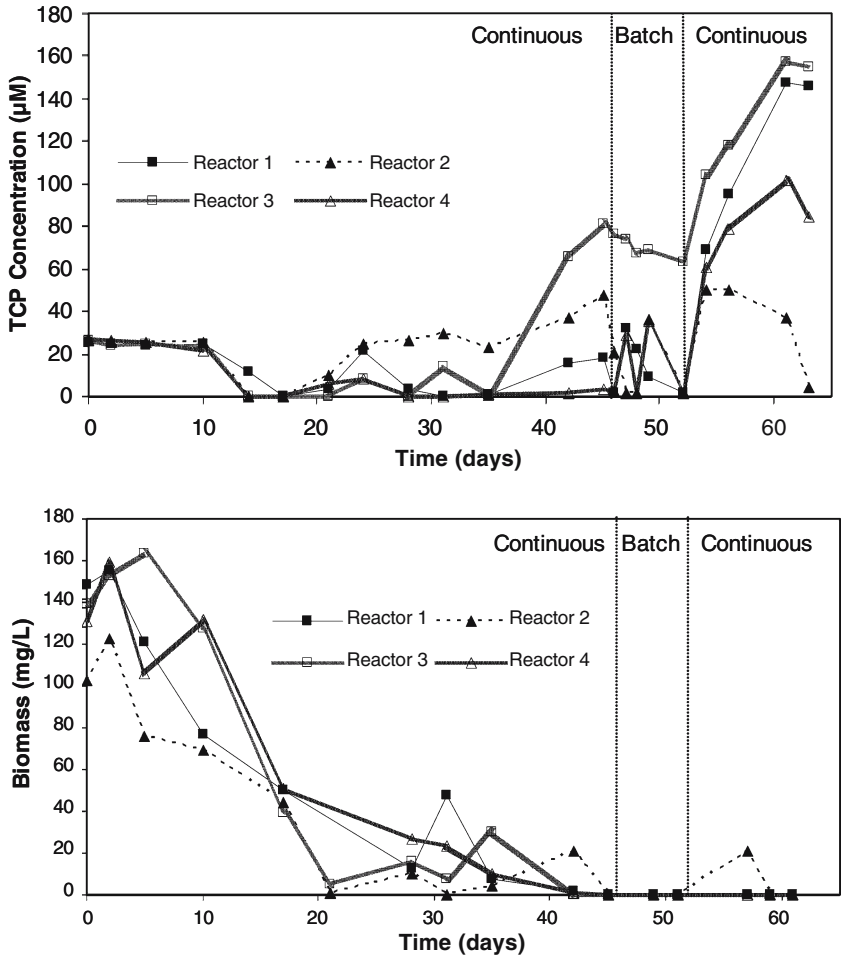

Fig. 4 TCP concentrations (top) in quadruplicate continuous bioreactors fed TCP as the sole carbon source. Days 0–15 are in batch mode for adaptation, followed by continuous operation (HRT—2.5 days,). The TCP influent was raised from 30 μM (days 15–21) to 60 μM (days 21–25) to 80 μM (days 25–46). TCP began to accumulate near day 45 in 3 of the 4 reactors, and so they were shifted to batch mode, where reactors 1, 2, and 4 recovered. Returning to continuous operation with a slower flow rate (HRT—5 days), but higher TCP influent concentration (170 μM) did not result in biomass accumulation. Biomass decreased steadily throughout the experiment (bottom)

the 14 oxygenase electrons, energy-yielding reactions, and cell synthesis. Therefore, the net electrons available for energy production and synthesis are only 10 electrons per TCP molecule. To determine the true yield (Y), we followed the method of Dahlen and Rittmann (2000), who found good agreement by partitioning the electrons in the energy consuming and energy producing reactions for dichlorophenol. In this case, the true yield is 10/24 of the theoretical yield for oxoadipate.

To calculate the yield of 3-oxoadipate, we followed the energetics and stoichiometric method developed originally by McCarty (1971) and updated by Rittmann and McCarty (2001). This method estimates true yield from the thermodynamics of intracellular electron transfer. By determining the Gibbs free energy change for the electron-donor half reaction (ΔG_d°), electron-acceptor half reaction (ΔG_a°), and cell synthesis half reaction (ΔG_c°), we can estimate the partitioning of electrons between energy and synthesis and so also predict the yield. The Gibbs free energy changes are reported in Table 1. We used group-contribution theory (Mavrovouniotis 1990, 1991; Woo and Rittmann 2000) to estimate the free energy of formation for 3-oxoadipate, as it is not listed in the literature. This value is –793.7 kJ/mol. From this analysis, we computed the theoretical true yield for 3-oxoadipate as 0.396 gVSS/gCOD. Therefore the theoretical yield for TCP would be 10/24 of this value, or 0.165 gVSS/gCOD. Taking into account the toxic effects of TCP and the fact it is an uncoupler of the electron transport chain, the actual yield may be even lower.

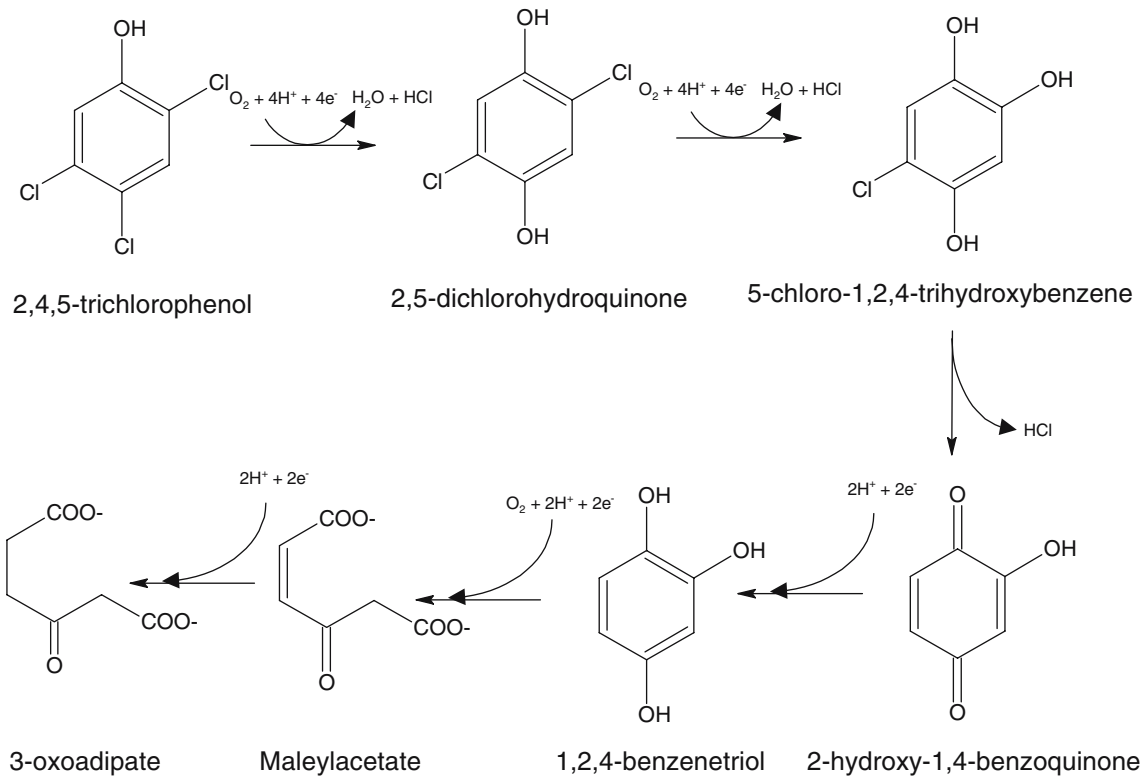

Fig. 5 Proposed degradation pathway for 2,4,5-TCP derived from the biodegradation pathway of 2,4,5-T, where 2,4,5-TCP is the first intermediate (Ellis et al. 2003; Daubaras et al. 1996). Electron flow has been added to show the electron investment required to turn TCP into oxoadipate, after which all the reactions are electron and energy yielding

The yield, the maximum specific growth rate (q_{max}), the half-maximum rate concentration (K) for a specific growth substrate, and the endogenous decay rate (b) can be combined to estimate the minimum substrate required to sustain growth in a continuous reactor, S_{min}:

$$S_{min} = Kb/(Yq_{max} - b)$$

Since we could find no reported values for the maximum specific growth rate and half-maximum rate concentration for TCP, we used the values obtained by Dahlen and Rittmann (2000) for 2,4-dichlorophenol, 2.4 gCOD/gVSS·d, and 15 mgCOD/l respectively, with a decay constant of 0.27 day^{-1}.

Using these 2,4-dichlorophenol kinetic parameters, we estimated S_{min} as 182 μM TCP, or 32 mgCOD/L. This value probably underestimates S_{min} for TCP, since TCP is more difficult to biodegrade than is 2,4-dichlorophenol, and it also exhibits toxicity. Even so, the S_{min} value of 182 μM is high compared to the concentration of TCP that is stably biodegradable (~40 μM), a

Table 1 Donor and acceptor half-reactions and their $\Delta G°'$ values used to calculate the yield of 3-oxoadipate

	Half-reactions	$\Delta G°'$ (kJ/e^- eq.)
R_{donor}	$3/11\ CO_2 + e^- + 10/11\ H^+ \rightarrow 1/22\ C_6H_6O_5^{2-}$ (oxoadipate) $+ 7/22\ H_2O$	–32.25
$R_{acceptor}$	$1/4\ O_2 + H^+ + e^- \rightarrow 1/2\ H_2O$	–78.72
$R_{Cell\ Synthesis}$	$1/5\ CO_2 + 1/20\ NH_4^+ + 1/20\ HCO_3^- + H^+ + e^- \rightarrow 1/20\ C_5H_7O_2N + 9/20\ H_2O$	Not defined independently

factor that helps explain why we saw only negative growth in the continuous reactors.

Conclusions

This work explored the biorecalcitrance, inhibition, adaptation, and continuous degradation of 2,4,5-trichlorophenol in a mixed aerobic microbial community using a combination of batch, respirometric, continuous, and modeling approaches. TCP exhibited strong biorecalcitrance at concentrations greater than 40 μM and was inhibitory to respiration in direct proportion to its concentration. TCP adaptation and biodegradation were reproducible at 10 and 20 μM, but inconsistent at higher concentrations. Continuous biodegradation of TCP could not be sustained in the system, because biomass did not accumulate. Analysis of the yield and S_{min} for TCP helps explain why no biomass accumulated, since the estimated S_{min} for TCP was at least 182 μM, a strongly inhibitory concentration.

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