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Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor

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Summary

The possibility that electrodes might serve as an electron acceptor to simulate the degradation of aromatic hydrocarbons in anaerobic contaminated sediments was investigated. Initial studies with *Geobacter metallireducens* demonstrated that although toluene was rapidly adsorbed onto the graphite electrodes it was rapidly oxidized to carbon dioxide with the electrode serving as the sole electron acceptor. Providing graphite electrodes as an electron acceptor in hydrocarbon-contaminated sediments significantly stimulated the removal of added toluene and benzene. Rates of toluene and benzene removal accelerated with continued additions of toluene and benzene. [^{14}C]-Toluene and [^{14}C]-benzene were quantitatively recovered as [^{14}C]-CO_2, demonstrating that even though the graphite adsorbed toluene and benzene they were degraded. Introducing an electrode as an electron acceptor also accelerated the loss of added naphthalene and [^{14}C]-naphthalene was converted to [^{14}C]-CO_2. The results suggest that graphite electrodes can serve as an electron acceptor for the degradation of aromatic hydrocarbon contaminants in sediments, co-localizing the contaminants, the degradative organisms and the electron acceptor. Once in position, they provide a permanent, low-maintenance source of electron acceptor. Thus, graphite electrodes may offer an attractive alternative for enhancing contaminant degradation in anoxic environments.

Introduction

Microorganisms can degrade a diversity of aromatic hydrocarbons under anaerobic conditions (Widdel and Rabus, 2001; Díaz, 2004; Widdel *et al.*, 2006; Foght, 2008; Carmona *et al.*, 2009), but aromatic hydrocarbons often persist as contaminants in sedimentary environments because of a lack of suitable electron acceptors (Lovley and Chapelle, 1995; Lovley, 1997; Reddy *et al.*, 2002; Rogers *et al.*, 2002; Frysinger *et al.*, 2003). For example, benzene is an aromatic hydrocarbon contaminant of particular concern and one of the most difficult for microorganisms to degrade (Carmona and Díaz, 2005). However, microorganisms can anaerobically oxidize benzene to carbon dioxide with the reduction of Fe(III) (Lovley *et al.*, 1994; Lovley *et al.*, 1996a; Villatoro-monzón *et al.*, 2008), nitrate (Coates *et al.*, 2001; Kasai *et al.*, 2006) or sulfate (Lovley *et al.*, 1995; Vogt *et al.*, 2007; Musat and Widdel, 2008) and in some instances benzene may also be degraded under methanogenic conditions (Weiner and Lovley, 1998). The anaerobic degradation of benzene can be greatly stimulated by making Fe(III) more available with the addition of chelators which solubilize Fe(III) (Lovley *et al.*, 1994; Lovley *et al.*, 1996b; Lovley and Woodward, 1996), or with the addition of soluble electron shuttles that promote electron transfer between cells and insoluble Fe(III) oxides (Lovley *et al.*, 1996b). The addition of sulfate to hydrocarbon-contaminated sediments may also stimulate anaerobic benzene oxidation (Weiner *et al.*, 1998; Anderson and Lovley, 2000) and other hydrocarbon contaminants (Coates *et al.*, 1997; Hayes *et al.*, 1999; Rothermich *et al.*, 2002).

However, providing chelators, electron shuttles, nitrate or sulfate as electron acceptors in open environments, such as sediments or groundwater, is problematic. All of these amendments are soluble and will diffuse away from the point of application. Introduction of oxygen has the added benefit of allowing attack on the aromatic rings via oxidases (Crestini and Giovannozzi Sermanni, 1995; Harwood and Parales, 1996; Wilson and Bouwer, 1997; Baldwin *et al.*, 2009), but introduction of oxygen is even more difficult because of the low solubility of oxygen, and the fact that it is rapidly abiotically consumed by reactions with reduced species, such as Fe(II), when introduced into anoxic environments (Borden *et al.*, 1997; Tuxen *et al.* 2005). Therefore, extended treatment of contaminated sites with any of these electron acceptor options

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requires long-term continuous or semi-continuous amendment strategies and thus substantial labour, costs and energy investments.

Electrodes offer a potential alternative as an electron acceptor for promoting the anaerobic degradation of organic contaminants (Bond *et al.*, 2002; Lovley, 2006; 2008; Huang *et al.*, 2009). *Geobacter metallireducens*, which was the first pure culture found to anaerobically oxidize an aromatic hydrocarbon (Lovley *et al.*, 1989; Lovley and Lonergan, 1990), was previously reported to anaerobically oxidize benzoate with an electrode serving as the sole electron acceptor (Bond *et al.*, 2002). Current evidence suggests that *Geobacter* species, which form a biofilm on the electrode surface, directly transfer electrons to the electrode surface via outer surface *c*-type cytochromes and electrically conductive pili (Holmes *et al.*, 2005; Reguera *et al.*, 2006; Kim *et al.*, 2008; Nevin *et al.*, 2009; Richter *et al.*, 2009; Yi *et al.*, 2009). Investigations on this form of respiration have primarily focused on acetate as the electron donor and have demonstrated that acetate is oxidized to carbon dioxide with quantitative electron transfer to the electrode surface (Bond and Lovley, 2003; Nevin *et al.*, 2008). A wide diversity of other microorganisms are able to transfer electrons to electrodes (Debabov, 2008; Lovley, 2008; Logan, 2009), and therefore, it is conceivable that there are many types of organisms that could anaerobically oxidize contaminants in this manner.

Electrodes are potentially attractive electron acceptors for stimulating the anaerobic degradation of aromatic hydrocarbon contaminants because they can provide a low-cost, low-maintenance, continuous sink for electrons. Although a diversity of electrode materials can serve as an electron acceptor for microbial respiration, including metals (Dumas *et al.*, 2007; Richter *et al.*, 2008; Yi *et al.*, 2009), graphite serves equally well as an electron acceptor and is inexpensive. Furthermore, graphite is durable and does not corrode or otherwise degrade in long-term deployments (Reimers *et al.*, 2006; Tender *et al.*, 2008).

Graphite electrodes have previously been deployed in marine and freshwater sediments (Holmes *et al.*, 2004; Reimers *et al.*, 2006; Tender *et al.*, 2008) in investigations designed to determine whether 'sediment microbial fuel cells' can be designed to power electronic monitoring equipment. In these systems graphite electrodes buried in the anoxic sediments serve as the electron acceptor for the microbial oxidation of fermentation intermediates in the sediments. Electrons transferred to the sediment electrode are passed to a graphite electrode suspended in the overlying aerobic water and are consumed with the reduction of oxygen. Graphite electrodes emplaced in the terrestrial subsurface electrically connected to graphite electrodes at land surface function in a similar manner and have been used to monitor microbial activity in a contaminated aquifer (Williams *et al.*, 2010).

The purpose of the studies reported here was to evaluate the potential for microbial oxidation of aromatic hydrocarbons coupled with electron transfer to electrodes and to determine whether providing an electrode as an electron acceptor could stimulate the anaerobic degradation of aromatic hydrocarbon contaminants in polluted marine sediments.

Results and discussion

Anaerobic oxidation of toluene coupled to electron transfer to an electrode by G. metallireducens

Geobacter metallireducens grown on graphite anodes with acetate as the electron donor could readily be adapted to metabolize benzoate, and then toluene with an electrode serving as the sole electron acceptor (Fig. 1). There was a substantial lag period when the culture was initially switched from benzoate to toluene, but once the culture was adapted to toluene, the rate of toluene oxidation, as indicated by the amount of current produced, was faster than for a comparable quantity of benzoate (Fig. 1). Current production with both aromatic compounds was slower than with acetate. This may reflect the higher quantity of electron equivalents of acetate added (10 mM acetate = 80 mM electron equivalents) versus the electron equivalents of toluene (1.1 mM toluene = 40 mM electron equivalents) as well as a propensity for faster acetate metabolism.

It was not possible to determine the stoichiometry of toluene consumption and current production by monitoring the loss of toluene over time because there was a rapid uptake of toluene under abiotic conditions (Fig. 2). Most of this loss could be attributed to adsorption of the

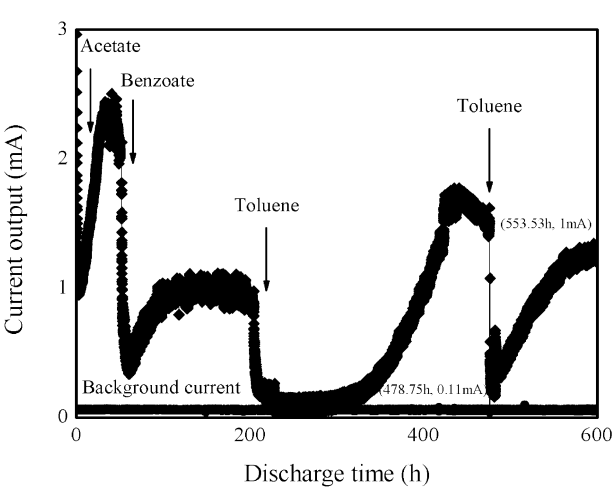

Fig. 1. Current production of *G. metallireducens* with acetate, benzoate or toluene serving as the electron donor and a graphite electrode serving as the electron acceptor. The arrows indicate when fresh acetate, benzoate and toluene medium were added. The data are representative of duplicate trials.

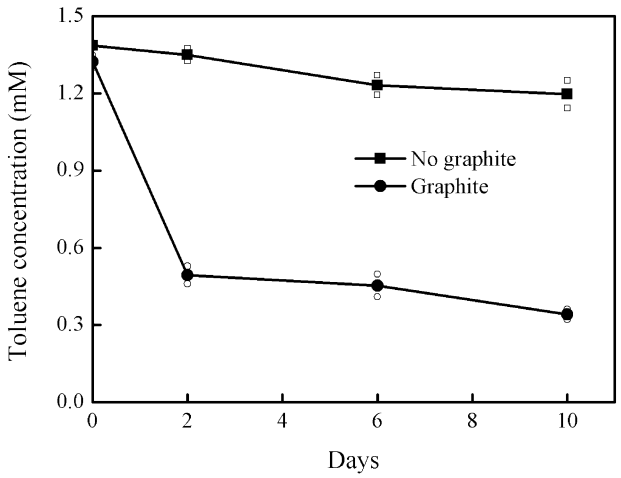

Fig. 2. Adsorption of toluene to graphite. Toluene concentrations in uninoculated microbial fuel cells with the graphite sticks used as electrodes, but not connected to the cathode, or without graphite. The closed symbols are the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

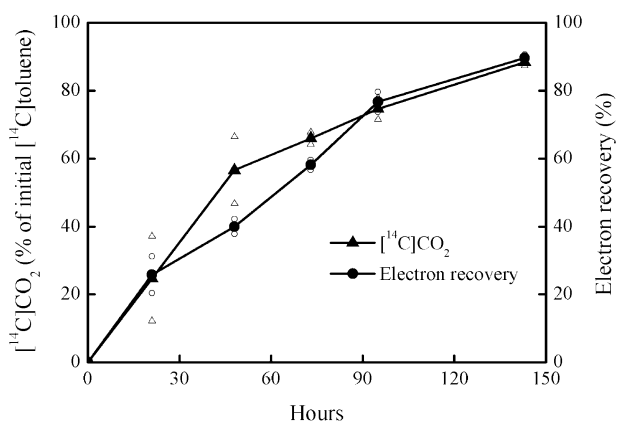

Fig. 3. [^{14}C]-CO_2 production from [^{14}C]-toluene and electron recovery by *G. metallireducens* with a graphite electrode serving as the sole electron acceptor. The closed symbols are the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

toluene onto the graphite as toluene losses were much greater in the presence of the graphite.

In order to determine whether the graphite-adsorbed toluene was available for metabolism, and to quantify the stoichiometry of toluene oxidation and electron transfer to the anode, current production was monitored in a culture that received toluene (376 μM) amended with [^{14}C]-toluene (Fig. 3). Electron recovery efficiency, calculated as: [(number of electrons recovered as current)/(number of electrons in the added toluene)], was 91% (mean of duplicate studies). This is comparable to previously reported electron recoveries for oxidation of acetate and benzoate to carbon dioxide coupled to electrode reduction by *Geobacter* species (Bond *et al.*, 2002; Bond and Lovley, 2003; Nevin *et al.*, 2008). Production of [^{14}C]-carbon dioxide tracked with the electron recovery over time, which further demonstrated that toluene was oxidized to carbon dioxide with the electrode serving as the sole electron acceptor. These results indicate that the toluene that was adsorbed on the graphite could be metabolized, but it is unknown whether the adsorbed toluene was metabolized directly on the anode surface or first desorbed into the aqueous phase to re-establish equilibrium between adsorbed and dissolved toluene as toluene was degraded.

Confocal scanning laser microscopy of *G. metallireducens* grown on anodes with toluene as the electron donor revealed clumps of cells dispersed across the surface (Fig. 4). Most of the cells appeared to be in direct contact with the anode surface. This degree of anode colonization is consistent with previous reports of biomass abundance for *Geobacter sulfurreducens* at similar current densities (Bond and Lovley, 2003; Reguera *et al.*, 2006).

Enumeration of cells on the anode sampled at the end of the incubation shown in Fig. 1 indicated that the cell density was $(57.2 \pm 8.14) \times 10^5$ cells cm^{-2}. Thus, the total cell abundance on the anode was 3.72×10^8. A current of 1 mA is equivalent to 2.25×10^{19} electrons per hour (1 mA = 1 mC s^{-1} = 6.24×10^{15} electrons $\times$ 3600 s h^{-1} = 2.25×10^{19} electrons h^{-1}) and when it is considered that each toluene yields 36 electrons, this translates into a toluene oxidation rate of 2.8×10^{-15} mol (h·cell)$^{-1}$ [2.25×10^{19} electrons h^{-1} $\times$ mol toluene/($36 \times 6.02 \times 10^{23}$ electrons)/3.72×10^8 cells = 2.8×10^{-15} mol (h·cell)$^{-1}$].

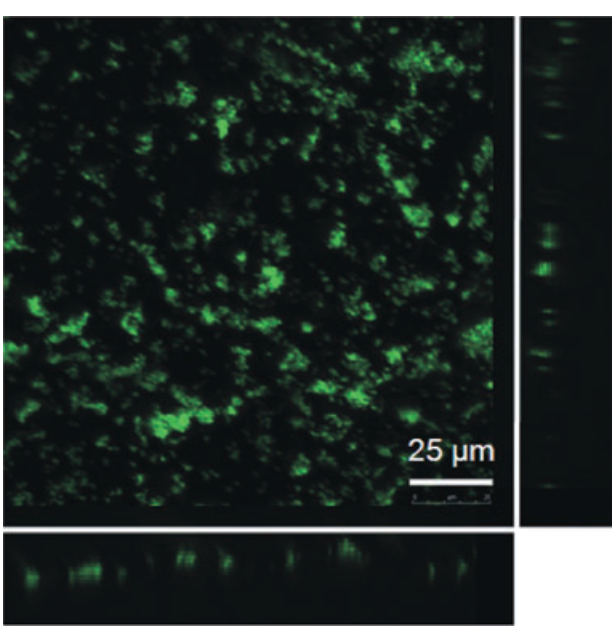

Fig. 4. Confocal laser scanning microscopy of *G. metallireducens* grown on a graphite anode surface with 1 mA current output and toluene as the sole electron donor.

This is a much faster rate of toluene oxidation that was previously reported for *G. metallireducens* growing with Fe(III) oxide as the electron acceptor (Lovley and Lonergan, 1990). For example, in a study initiated with 1 mM toluene, during the most active phase of toluene metabolism (day 3 to day 12 in Fig. 1; Lovley and Lonergan, 1990), *G. metallireducens* oxidized toluene with the reduction of Fe(III) oxide at a maximum rate of only 2.5×10^{-17} mol (h·cell)$^{-1}$ [9 mmol l^{-1} of Fe(II) produced during this period with 46×10^9 cells per litre at the mid-point yields: 9 mmol l^{-1} Fe(II) × (mmol toluene/36 mmol Fe(II)) × (mol toluene/1000 mmol toluene) × 1/(46×10^9 cells)/216 h = 2.5×10^{-17} mol (h·cell)$^{-1}$]. In a study initiated with 10 mM toluene, during the most active phase of toluene metabolism (day 10 to day 45 in Fig. 1; Lovley and Lonergan, 1990), the rate was similar, 1.9×10^{-17} mol (h·cell)$^{-1}$ [33 mmol l^{-1} of Fe(II) produced with 60×10^9 cells l^{-1} at the midpoint yields: 33 mmol l^{-1} Fe(II) × (mmol toluene/36 mmol Fe(II)) × (mol toluene/1000 mmol toluene) × 1/(60×10^9 cells)/792 h = 1.9×10^{-17} mol (h·cell)$^{-1}$]. Thus, the per cell rate of toluene oxidation with an electrode serving as the electron acceptor was more than 100-fold faster than previously reported rates with Fe(III) oxide serving as the electron acceptor.

Stimulation of anaerobic hydrocarbon degradation in petroleum-contaminated sediments

Studies were conducted with petroleum-contaminated sediments from Boston Harbor in order to evaluate whether the stimulation of anaerobic aromatic hydrocarbon oxidation with electrodes observed in pure culture could be replicated with the natural community in contaminated sediments. Inserting a graphite stick into heat-killed sediments resulted in a significant decrease in toluene (Fig. 5), consistent with the finding from the pure culture study that the graphite adsorbs toluene. An adsorption of toluene was also apparent in live sediments in which an electrode was added, but could not serve as an electron acceptor because it was not connected to a potentiostat (Fig. 6A). Toluene loss in this treatment was comparable to that in sediments that contained a connected electrode, but in which the sediments had been heat-killed. In both instances, an initial adsorption phase was followed by no further toluene loss. However, in live sediments with a connected electrode that could function as an electron acceptor toluene continued to be removed after the initial adsorption phase (Fig. 6A), indicating that providing an electrode as an electron acceptor accelerated toluene removal beyond that due to mere adsorption. Once toluene was completely removed, more toluene was added and it was removed even faster (Fig. 6B). Continued additions of toluene were associated with increased rates of toluene removal (Fig. 6B and C), suggesting that the microbial community was adapting for enhanced toluene removal.

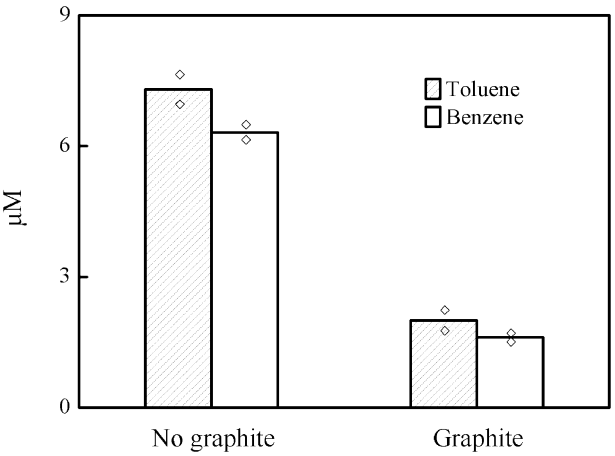

Fig. 5. Adsorption of toluene and benzene to graphite. Toluene and benzene concentrations in heat-killed sediments with the graphite sticks used as electrodes, but not connected to the cathode, or without graphite after 12 days. The bars represent the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

When [^{14}C]-toluene was added along with the unlabelled toluene, there was a steady production of [^{14}C]-CO_2 over time and almost all of the added radiolabel was recovered as [^{14}C]-CO_2 (Fig. 6D). There was no production of [^{14}C]-CO_2 in a control in which the electrode was not connected. These results confirmed that the removal of added toluene could be attributed to toluene oxidation and that the graphite-adsorbed toluene was available for microbial oxidation.

Toluene is generally considered to be the aromatic hydrocarbon that is most readily degraded under anaerobic conditions (Holliger and Zehnder, 1996; Foght, 2008). Benzene is typically more recalcitrant, which is problematic due to benzene's higher toxicity (Carmona and Díaz, 2005). As with toluene, there was a substantial loss of added benzene in heat-killed sediments that could be attributed to adsorption to the graphite (Fig. 5). Providing an electrode as an electron acceptor greatly stimulated the continued loss of benzene (Fig. 7A). The rate of benzene removal increased with subsequent additions to the system (Fig. 7B and C). [^{14}C]-benzene was quantitatively oxidized to [^{14}C]-CO_2 when an electrode was provided as an electron acceptor (Fig. 7D), demonstrating that benzene removal could be attributed to anaerobic benzene degradation and that benzene adsorbed to the electrode was also degraded.

The potential for microbial degradation of polycyclic aromatic hydrocarbons (PAHs) is typically first evaluated with naphthalene (Coates *et al.*, 1997; Hayes *et al.*, 1999; Rothermich *et al.*, 2002). Although naphthalene is often the most rapidly degraded PAH in contaminated marine

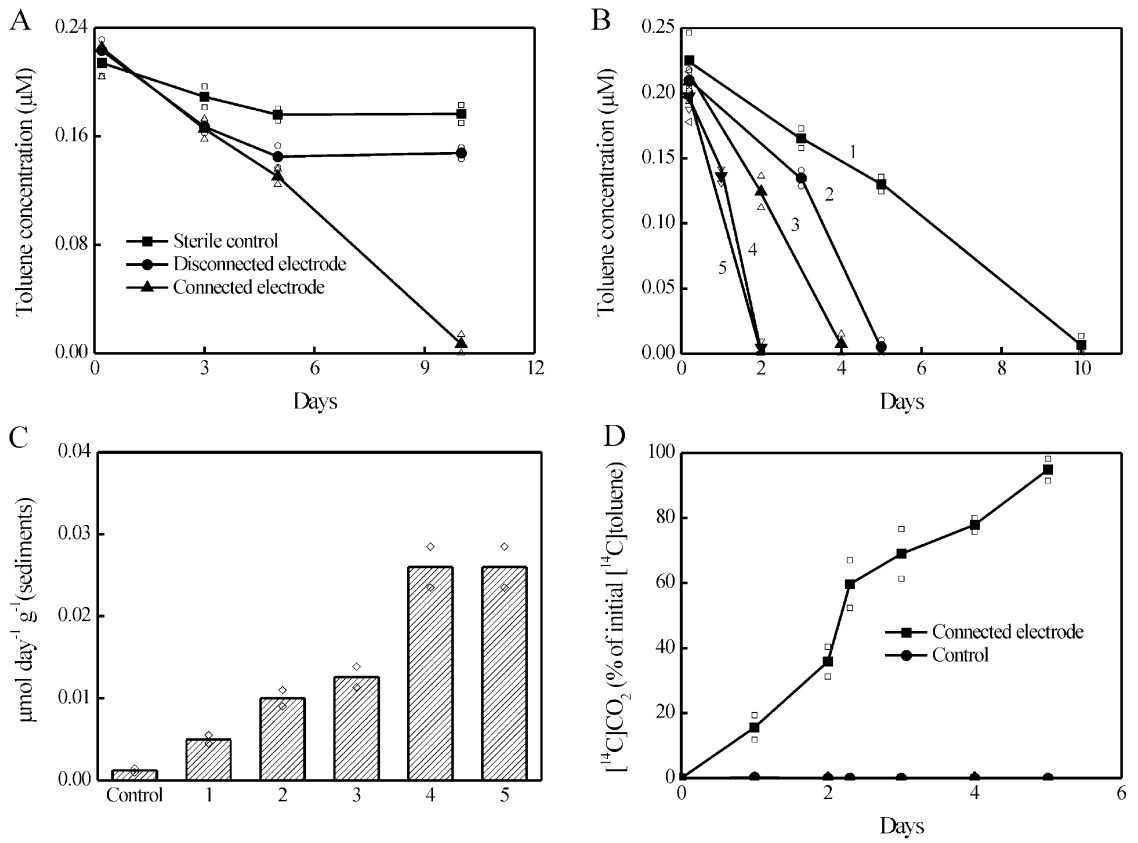

Fig. 6. Toluene degradation in Boston Harbor sediments.
A. Headspace concentrations of toluene in live sediments with a connected electrode, or in live sediments and heat-killed sediments (sterile control) with a disconnected electrode.
B. Toluene loss following successive additions of toluene to live sediments that contained a connected electrode. Numbers designate the order of the toluene additions.
C. Toluene removal rates of following successive additions calculated from data in (B) compared with the rate of removal in a control in which live sediments were incubated with an unconnected graphite stick.
D. [^{14}C]-CO_2 production from [^{14}C]-toluene.
The closed symbols and bars represent the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

sediments (Coates *et al.*, 1997; Hayes *et al.*, 1999), this is not always the case (Rothermich *et al.*, 2002). When sediments were amended with naphthalene almost all of the naphthalene was removed within 9 days when an electrode was provided as a potential electron acceptor (Fig. 8). This contrasted with virtually no removal of naphthalene in incubations of heat-killed sediments without an electrode. Inserting an electrode into heat-killed sediments or putting an electrode in live sediments, but not connecting the electrode to a cathode, resulted in some naphthalene loss, probably due to adsorption, that was more extensive than that for toluene or benzene (Fig. 8A). [^{14}C]-CO_2 was produced from [^{14}C]-naphthalene that was added into the sediments with an electrode as the electron acceptor (Fig. 8B). Slightly less than 40% of the [^{14}C]-naphthalene added was recovered as [^{14}C]-carbon dioxide. The mechanisms preventing complete recovery of the added [^{14}C]-naphthalene as [^{14}C]-carbon dioxide are not known, but this recovery is comparable to that observed in long-term (*c.* 75) day incubations of these same sediments during naphthalene degradation coupled to sulfate reduction (Hayes *et al.*, 1999). Therefore, the relatively low recovery of [^{14}C]-carbon dioxide from [^{14}C]-naphthalene compared with the higher recovery from [^{14}C]-toluene and [^{14}C]-benzene could reflect differences in the relative amount of assimilation of the compounds into cell carbon during metabolism. An alternative explanation is that less of the naphthalene that was removed from the sediments via adsorption to the graphite was available for microbial metabolism. The degradation of PAHs in contaminated marine sediments can only quantitatively be studied by measuring the decrease in the concentration of the PAH of interest in sediments over time (Rothermich *et al.*, 2002), but such studies require incubations of a year or more, which were outside the scope of this study.

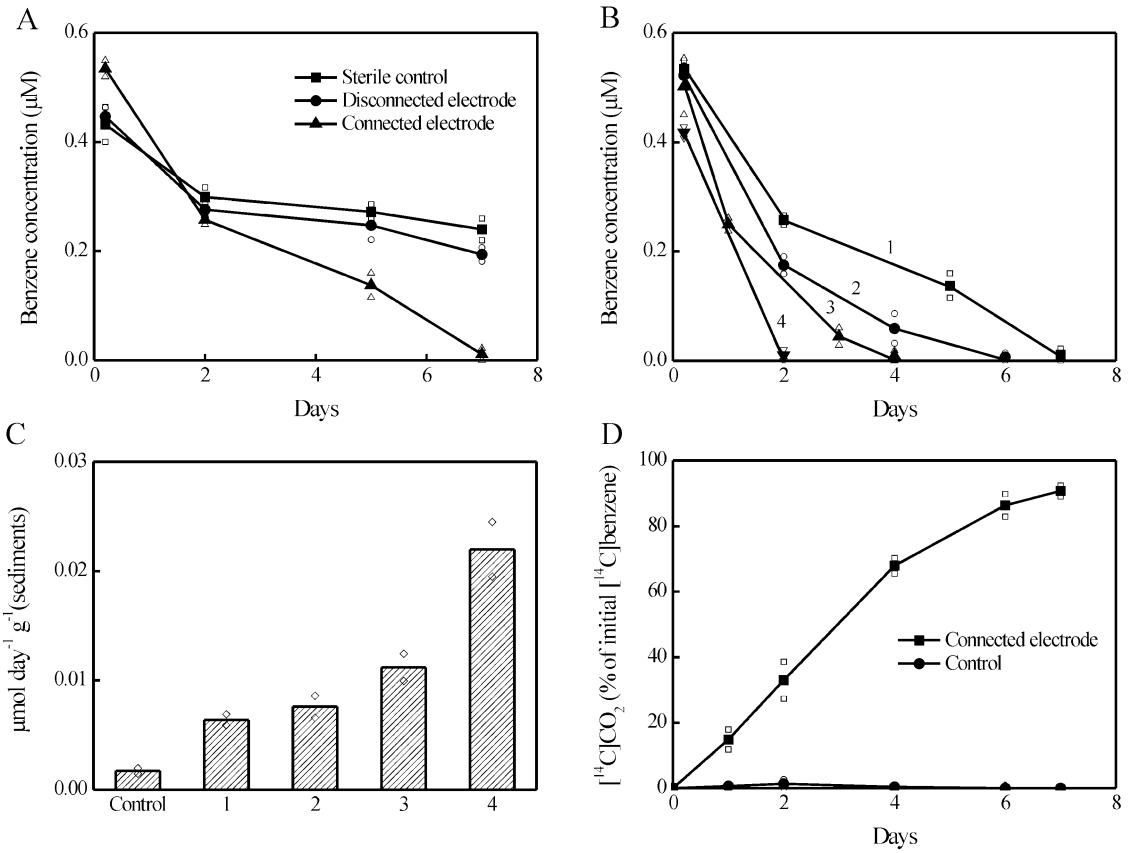

Fig. 7. Benzene degradation in Boston Harbor sediments.
A. Headspace concentrations of benzene in live sediments with a connected electrode, or in live sediments and heat-killed sediments (sterile control) with a disconnected electrode.
B. Benzene loss following successive additions of benzene to live sediments that contained a connected electrode. Numbers designate the order of the benzene additions.
C. Benzene removal rates following successive additions of benzene to live sediments that contained a connected electrode, compared with the rate of removal in a control in which live sediments were incubated with an unconnected graphite stick.
D. [^{14}C]-CO_2 production from [^{14}C]-benzene.
The closed symbols and bars represent the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

Implications

These results demonstrate that electrodes can serve as an electron acceptor for anaerobic oxidation of aromatic hydrocarbons and that providing an electrode as an electron acceptor can stimulate the anaerobic oxidation of aromatic hydrocarbons in contaminated sediments. Electrodes have several possible advantages over other, more commonly considered, electron acceptors that might be added to sediments. One significant advantage is that, once emplaced, electrodes represent a continuous long-term electron acceptor. This alleviates the need for repeated applications of the electron acceptor and monitoring to determine when more electron acceptor amendment is necessary. Soluble electron acceptors, such as oxygen, nitrate, sulfate or chelated Fe(III), rapidly diffuse away from the point of application. In contrast, electrodes can be permanently located specifically at the point of application.

Furthermore, as observed in this study with aromatic hydrocarbons, graphite readily adsorbs a diversity of organic contaminants (Shi *et al.*, 1995; Abu-salah *et al.*, 1996; Zimmerman *et al.*, 2004; Wang and Xing, 2007). Thus, when a graphite electrode is provided as an electron acceptor it has the additional benefit of concentrating the contaminant at the source of the electron acceptor.

It is expected that the microorganisms utilizing the contaminants will also attach to the electrode surface (Lovley, 2006; 2008). Such attachment was apparent in the studies with *G. metallireducens*. Therefore, graphite electrodes have the unique capability of co-localizing the contaminants, electron acceptor and degradative microorganisms on the same surface.

The ultimate sink for electrons derived from anaerobic aromatic hydrocarbon degradation coupled to electron transfer to electrodes is the oxygen which receives the electrons at the cathode. Introducing oxygen into otherwise anoxic environments is a commonly considered

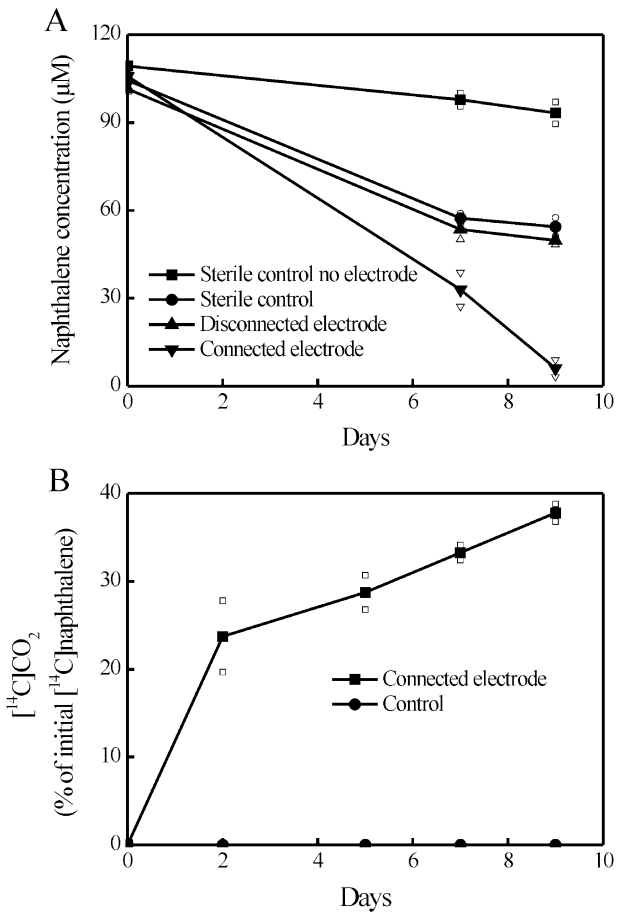

Fig. 8. Naphthalene degradation in Boston Harbor sediments.
A. Naphthalene concentrations in live sediments that contained a connected electrode or disconnected electrode or in heat-killed sediments with or without a disconnected electrode (sterile control).
B. [^{14}C]-CO_2 production from [^{14}C]-naphthalene.
The closed symbols are the means of duplicate trials, the open symbols indicate the range of values for the duplicates.

bioremediation strategy, but is technically difficult and expensive. Electrodes provide a link to oxygen as an electron acceptor in a simple and inexpensive manner.

It has previously been proposed that electrodes poised at a potential low enough to serve as an electron donor for respiration may be used to stimulate microbial reduction of nitrate (Gregory *et al.*, 2004), uranium (Gregory and Lovley, 2005) or chlorinated solvents (Aulenta *et al.*, 2008; Strycharz *et al.*, 2008). Attempts to engineer such systems for large-scale field deployment are underway (Williams *et al.*, 2010). Knowledge from these studies may also aid in the design of strategies in which electrodes can be used as electron acceptors to promote contaminant removal. Although studies of sediment microbial fuel cells powering electronic equipment have suggested that these systems can run indefinitely (Tender *et al.*, 2008), long-term field trials on rates of contaminant degradation are warranted to ensure that fouling of either the anode or cathode with biofilms or chemical precipitates will not limit the effectiveness of contaminant removal.

In summary, these studies expand the range of known electron acceptors that can support anaerobic oxidation of aromatic hydrocarbons. In addition to the implications of these results for bioremediation of hydrocarbon-contaminated environments, it is often suggested that electrodes might serve as alternative electron acceptors for oxygen in wastewater treatment systems (Chen, 2004; Du *et al.*, 2007). The finding that compounds such as aromatic hydrocarbons, which are often considered to be recalcitrant to anaerobic degradation, can readily be oxidized with electron transfer to electrodes further emphasizes the potential for electrode-based systems to be an effective waste treatment option in the absence of oxygen.

Experimental procedures

Organism and sediment sources

Geobacter metallireducens (ATCC 53774 and DSM 7210) (Lovley *et al.*, 1993) was obtained from our laboratory culture collection and was routinely cultured as previously described (Lovley and Phillips, 1988).

Hydrocarbon-contaminated Boston Harbor sediments were collected as previously described (Hayes *et al.*, 1999). Sediments were visually black with a strong odour and a thin layer of oil. Buckets of grab samples taken from a depth of *ca.* 8 m were transported directly to the laboratory, stored at 16°C in the dark under strict anaerobic conditions. Seawater was collected from a depth of about 0.5 m at the same site and stored at 4°C in the dark.

Studies with an electrode serving as the electron acceptor

All studies were carried out at room temperature (*ca.* 25°C) in three-electrode, dual-chambered microbial fuel cells as previously described (Strycharz *et al.*, 2008). Both anode and cathode were comprised of solid graphite sticks (surface area of 65 cm^2, grade G10, Graphite Engineering and Sales, Greenville, MI). The anode was poised with a potentiostat (ECM8, Gamry Instruments, PA, USA) at +300 mV (versus Ag/AgCl). Data were autorecorded with a computer that connected to the potentiostat. The gas phase was N_2/CO_2, 80:20 (v/v) for the pure culture studies and 95:5 for the sediment studies. The anodic 470 ml anode chamber was filled with 250 ml of medium (Strycharz *et al.*, 2008) or 200 g of sediment slurry [1:4 (v/v) sediment–seawater]. The cathodic chamber was filled with 250 ml of medium for the pure culture studies or 200 ml of seawater for the sediment studies and was continuously bubbled with the appropriate N_2/CO_2 gas mixture throughout the study.

Cultures of *G. metallireducens* were established in the anode chamber in a medium that contained acetate (10 mM) and both Fe(III) citrate (11 mM) and the poised electrode as electron acceptors. When Fe(II) concentrations reached

8 mM, the medium was replaced with fresh medium that contained acetate but no Fe(III) citrate. When stable current outputs were reached, the medium was subsequently replaced with fresh medium that contained benzoate (1 mM) or toluene (1.129 mM) as the electron donor. Filter-sterilized toluene (30 μl) was added to the fresh medium with a sterile syringe to provide a concentration of 1.129 mM.

[Ring-Ul-^{14}C]-toluene (2.17×10^9 Bq mmol^{-1}; Sigma Chemical Corporation, St. Louis, MO) stock was prepared in sterile anoxic toluene to provide 3.7×10^3 Bq μl^{-1}. Anode chamber with *G. metallireducens* grown on electrode was amended 10 μl above stock in 250 ml of medium to give a total of 3.7×10^4 Bq ^{14}C and 376 μM toluene.

Sediment incubations included controls in which the microorganisms in the sediments were killed with heat by autoclaving at 121°C on two separate occasions. Additional controls consisted of live sediments but with the graphite stick not connected to a potentiostat to prevent it from serving as an electron acceptor. Toluene or benzene was added to the sediments to give a total concentration of 10 μM or 9 μM from stocks prepared with sterile anoxic water. Naphthalene was dissolved in the seawater mixed with the sediments to provide an initial concentration of 100 μM.

For studies with [^{14}C]-labelled tracers the toluene and benzene stocks were amended with [ring-Ul-^{14}C]-toluene (2.17×10^9 Bq mmol^{-1}) or [ring-Ul-^{14}C]-benzene (2.78×10^9 Bq mmol^{-1}; Moravek Biochemicals, Brea, CA), respectively, to provide *ca*. 6.7×10^4 Bq when added to the sediments. The sediments exposure to naphthalene was amended with 4.8×10^4 Bq [ring-Ul-^{14}C]-naphthalene (1.16×10^9 Bq mmol^{-1}; American Radiolabelled Chemicals, St. Louis, MO). As previously described (Coates *et al*., 1997), 1.5 μl of a methanolic stock (3.7×10^7 Bq per ml) was dispensed onto small pieces of sterile glass fibre filters (Whatman GF/A glass microfibre filters). The methanol was evaporated for 5 min at room temperature, and the filter pieces were added to the sediment slurries under an N_2/CO_2 gas phase.

Confocal laser scanning and electron microscopy

The biofilms of *G. metallireducens* on the graphite anodes were stained with the LIVE/DEAD BacLight Viability Kit and imaged with confocal laser microscopy as previously described (Reguera *et al*., 2006). The average cell number was calculated by examining at least five fields of view.

Analytical methods

Headspace concentrations of toluene and benzene were quantified with a gas chromatograph equipped with a flame ionization detector. The hydrocarbons were separated with a Supelco VOCOL fused silica capillary column (60 m × 0.25 mm × 1.5 μm) held at 50°C for 0.5 min, followed by an increase to 200°C at 10°C min^{-1}. The concentrations of toluene and benzene in the aqueous phase were calculated with Henry's law using the constants at 25°C of 0.27 for toluene and 0.25 for benzene (Staudinger and Roberts, 1996).

Benzoate concentrations in the medium were determined with high-performance liquid chromatography (Agilent 1100 HPLC Series) with an Alltima HP C18 HL (250 mm × 4.5 mm × 5 μm) column. An eluent of MeOH–H_2O (60:40) and 0.1% H_2PO_4 was used with an isocratic flow rate of 1.0 ml min^{-1} and detection was by absorbance at 280 nm. Acetate concentrations in the medium were determined with high-performance liquid chromatography as previously described (Nevin *et al*., 2008).

For sediments degradation with naphthalene and adsorption studies, sediment sample aliquots (3 ml) were extracted with acetonitrile as previously described (Coates *et al*., 1997). The hydrocarbons were separated with a Supelco Supelcosil SC-PAH column with an eluent of MeOH–H_2O (60:40) at 2 ml min^{-1} and the hydrocarbons were quantified by measuring absorbance at 220 nm.

[^{14}C]-carbon dioxide in headspace samples was quantified with a gas proportional counter as previously described (Coates *et al*., 1997; Hayes *et al*., 1999).

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