Characterization of marine isoprene-degrading communities

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Running title: Identification of a marine sink for isoprene
SUMMARY

Isoprene is a volatile and climate-altering hydrocarbon with an atmospheric concentration similar to that of methane. It is well established that marine algae produce isoprene, however, until now there was no specific information about marine isoprene sinks. Here we demonstrate isoprene consumption in samples from temperate and tropical marine and coastal environments, and furthermore show that the most rapid degradation of isoprene coincides with the highest rates of isoprene production in estuarine sediments. Isoprene-degrading enrichment cultures, analyzed by DGGE and 454 pyrosequencing of the 16S rRNA gene and by culturing, were generally dominated by Actinobacteria, but included other groups such as Alphaproteobacteria and Bacteroidetes, previously not known to degrade isoprene. In contrast to specialist methane-oxidising bacteria, cultivated isoprene degraders were nutritionally versatile, and nearly all of them were able to use n-alkanes as a source of carbon and energy. We therefore tested and showed that the ubiquitous marine hydrocarbon-degrader, Alcanivorax borkumensis, could also degrade isoprene. A mixture of the isolates consumed isoprene emitted from algal cultures, confirming that isoprene can be metabolized at low, environmentally-relevant concentrations, and suggesting that, in the absence of spilled petroleum hydrocarbons, algal production of isoprene could maintain viable populations of hydrocarbon-degrading microbes. This discovery of a missing marine sink for isoprene is the first step in obtaining more robust predictions of its flux, and suggests that algal-derived isoprene provides an additional source of carbon for diverse microbes in the oceans.
INTRODUCTION

Isoprene (2-methyl-1,3-butadiene) is the second most abundant natural hydrocarbon in the atmosphere (Müller et al., 2008), with a concentration nearly equal to that of methane, and thousands of natural products derive from this pivotal five-carbon metabolite. Although terrestrial plants are the main suppliers of isoprene to the atmosphere (Sharkey et al., 2008), recent research shows that marine algae are a significant source of atmospheric isoprene (Palmer and Shaw, 2005; Meskhidze and Nenes, 2006; Liakakou et al., 2007). Isoprene biosynthesis has numerous potential physiological roles, and cellular protection from thermal stress has been conclusively demonstrated for vascular plants (Siwko et al., 2007).

Isoprene is volatile (boiling point of 34°C) and highly reactive, with numerous effects on atmospheric chemistry that are dependant on levels of solar irradiation and concentrations of other molecules in the atmosphere. For example, the production of alkylperoxy radicals starts a chain of reactions; when NOx are abundant these radicals result in the formation of tropospheric ozone (Fehsenfeld et al., 1992), which is regarded as the third most powerful greenhouse gas after methane and carbon dioxide (IPCC, 2001) and a major pollutant that can exacerbate or initiate numerous respiratory problems in animals (US EPA, 1996). Importantly, the consumption of hydroxyl radicals by isoprene reduces their capacity to oxidise volatile organic compounds that lengthen the residence time (and hence global climate-changing effects) of gases such as methane (Monson and Holland, 2001). Isoprene may encourage climate cooling by contributing 5-25% of the global IPCC estimate of natural secondary organic aerosols (Claeys et al., 2004), (with
even higher estimates for the Southern Ocean (Meskhidze and Nenes, 2006)) which *inter alia* serve as cloud-condensation nuclei and so result in reduced radiative forcing.

While current estimates of isoprene emissions to the atmosphere from marine environments (0.1 – 1.2 Tg y\(^{-1}\); [Palmer and Shaw, 2005]) are lower than from terrestrial environments (~413 Tg y\(^{-1}\); [Muller *et al*., 2008]), evidence is emerging that marine emissions could be substantially higher (Meskhidze and Nenes, 2006; Liakakou *et al*., 2007), especially when highly productive coastal zones are considered. The most intense marine emissions of isoprene to the atmosphere occur during spring (Liakakou *et al*., 2007), or in locations that have been fertilised by iron (Wingenter *et al*., 2004; Moore and Wang, 2006), both coinciding with phytoplankton blooms. It has been proposed that, in addition to enzymatic isoprene production, light-induced oxidation of dissolved organic carbon produced by marine phytoplankton, may be responsible for some of this isoprene liberated to the atmosphere (McKay *et al*., 1996).

The ability of bacteria to degrade isoprene in soil and freshwater sediments has been shown (Cleveland and Yavitt, 1998; Fall and Copley, 2000); however, despite its high energy content, there are only hints of isoprene degradation in marine and coastal environments that are estimated from depth-mixing profiles (Moore and Wang, 2006). It is well established that a significant proportion of gases like methane (Reeburgh *et al*., 1993) and dimethylsulfide (Kiene and Bates, 1990) are oxidised before they can enter the atmosphere, and indeed models of marine isoprene flux assume a bacterial sink term (Palmer and Shaw, 2005), however firm evidence for this is currently lacking. Therefore, in this study we aim to determine the extent of isoprene degradation in marine and coastal environments, investigate the factors affecting degradation, identify and isolate the organisms responsible, and investigate their ability to degrade isoprene produced at low
concentrations by microalgae, thereby allowing a better understanding of how environmental perturbations and changing climate will alter isoprene flux.
RESULTS AND DISCUSSION

Degradation of isoprene occurs in tropical and temperate seawater and sediments, and along an estuary

In order to test the potential for isoprene degradation in marine environments, we added isoprene (0.1% v/v, equivalent to a concentration of 81.9 ppm in the headspace and 0.581 mM in solution) to marine and estuarine waters and sediment slurries in sealed bottles with a large aerobic headspace. Isoprene was degraded in samples taken from points along the Colne estuary, UK (Fig. 1D), with two striking trends being evident (Fig. 1A and B): degradation was at least an order of magnitude faster in sediments than water samples, and the onset and rate of biodegradation were more rapid at the head of the estuary than at the marine end (Tamhane’s T2 test, p<0.01). We measured isoprene concentration (waters) and production (sediments and waters) along the estuary to test whether there was any correspondence between consumption and production rates. Isoprene concentrations in water samples clearly decrease from the head to typical marine concentrations at the mouth (Fig. 2). At low tide, isoprene concentrations in the estuary water are higher, suggesting that the diatom-rich microphytobenthos is a major source of isoprene. No net production of isoprene was observed in waters, whereas isoprene production in sediments was reasonably uniform at all locations along the estuary, except point 3 (Fig. 2). Phototrophs are the primary suppliers of isoprene (Milne et al., 1995; Shaw et al., 2003; Broadgate et al., 2004), and the microphytobenthos of intertidal sediments are typically more productive than phytoplankton (MacIntyre et al., 1996), thus explaining the dominance of the benthic communities in estuarine isoprene production. The increase in water volume towards the mouth of the estuary, and thereby the diffusion potential for microphytobenthos-sourced isoprene, provides a possible explanation for the
variation in water isoprene concentrations both along the estuary and between high and low tides, although higher nutrient levels at the head, and thus elevated microalgal production in the water column (Kocum et al., 2002), may be a contributing factor. Therefore, areas with high algal biomass and higher in-situ concentrations of isoprene sustain a larger and better acclimated population of isoprene degraders, explaining the pattern of degradation seen in Fig. 1A and B.

We have shown that isoprene concentrations in water samples were $7 \times 10^{-4}$ to 0.9 nmol l$^{-1}$ from the marine to the freshwater end of the Colne estuary (Fig. 2). Reported concentrations in seawater measured in the Straits of Florida, the North Sea and the North Pacific range from $7 \times 10^{-4}$ to 0.054 nmol l$^{-1}$ (Milne et al., 1995; Broadgate et al., 1997; Matsunaga et al., 2002), so concentrations measured at the freshwater end of the estuary are an order of magnitude greater than the highest concentrations reported previously (0.054 nmol l$^{-1}$). To our knowledge there are no reports of production of isoprene from marine sediments. Isoprene production in the Colne estuary sediments ranged from 0.15 to 0.71 pmol cm$^{-2}$ h$^{-1}$ (Fig. 2), which would result in an isoprene flux of $2.5 \times 10^{7}$ to $1.2 \times 10^{8}$ molecules cm$^{-2}$ s$^{-1}$. Fluxes for this temperate estuary, therefore, are higher than those reported by Broadgate et al. (1997) from near shore in the North Sea ($1.7 \times 10^{7}$ molecules cm$^{-2}$ s$^{-1}$), equivalent to those reported by Milne et al. (1995) in the Gulf Stream off the Florida coast ($3.4 \times 10^{7}$ molecules cm$^{-2}$ s$^{-1}$), and the values from three out of four points in the Colne estuary are in the range of those reported by Liakakou et al. (2007) for a coastal Mediterranean site ($10^{8}$ to $6 \times 10^{9}$ molecules cm$^{-2}$ s$^{-1}$). This highlights the importance of coastal areas in contributing to the flux of isoprene to the atmosphere.

In order to ascertain whether isoprene biodegradation is a global phenomenon in marine ecosystems, we also investigated Mediterranean and tropical environments.
Isoprene degradation was detected within 300 days in one out of four seawater samples from Sulawesi (Indonesia; Fig. 1A); and further enrichments from this sample showed >95% degradation within 40 days (Fig. S4B), confirming that an isoprene-degrading community had been established. When incubation time was extended beyond 300 days, isoprene degradation was seen in two of the other three samples (Fig. S4A). Isoprene biodegradation was also detected in one of four replicate sediment slurries (Fig. S2A) and water samples (Fig. S3B) from a Mediterranean brackish lagoon (Etang de Berre), with degradation occurring significantly faster in sediments than water samples.

**Degradation is favoured at low isoprene concentrations**

These experiments involving 0.1% v/v isoprene (equivalent to a concentration of 81.9 ppm in the headspace and 0.581 mM in solution) served to identify important trends. However, because isoprene and its degradation products can be toxic in a dose-dependent manner (Ensign, 2001), and potential isoprene consumers may have been inhibited/killed by this high concentration, we examined the effect of decreasing the isoprene concentration ≥100-fold in the water samples from Etang de Berre and the Colne estuary. Biodegradation was more than ten-fold faster in all the water samples collected along the Colne estuary when isoprene was added at 0.001% (equivalent to a concentration of 0.82 ppm in the headspace and 5.81 µM in solution Fig. 1C) or 0.0001% v/v (equivalent to a concentration of 0.082 ppm in the headspace and 0.581 µM in solution; data not shown) than when added at 0.1% v/v. In the Etang de Berre samples, instead of isoprene degradation occurring in ~250 days in just one out of four replicates with 0.1% v/v isoprene, complete degradation was observed in all four replicates by 55 days with 0.001% v/v isoprene (Fig. S3). It is therefore clear that microbial communities, particularly those from
environments with naturally low isoprene concentrations (e.g. seawater compared with estuarine sediments), may be inhibited by high concentrations of isoprene.

**Isoprene degrading enrichment cultures contain bacteria from diverse phylogenetic lineages, and are dominated by Actinobacteria**

In order to characterize the main microbes in, and obtain pure isoprene degraders from, isoprene enrichments, samples from the original microcosms (E0) were inoculated into seawater-based minimal medium (ONR7a) and re-supplied with isoprene. The degradation rate increased significantly in the enrichment (E1) derived from the original microcosms (E0), but generally decreased after further enrichments (E2 or E3), probably reflecting a trade-off between a larger inoculum of isoprene degraders with dilution of a growth factor from the environment (Figs. S2, S4, S5 and S6). Denaturing gradient gel electrophoresis (DGGE) of amplified fragments of 16S rRNA genes revealed a reduction in bacterial diversity and some changes in community composition at each stage of enrichment. For example, in sediments from the middle (point 2) of the Colne estuary Bacteroidetes appeared in the third enrichment (E3), Epsilonproteobacteria in E2, while Actinobacteria (100% 16S rRNA sequence similarity to strain i24; see later) were present throughout (Fig. S7A). Sequences from dominant bands in the final enrichment from the marine sediment at the mouth of the Colne estuary (point 4) were 100% similar to several *Pseudomonas* and 92% similar to *Paenibacillus* species (Fig. S7B).

Bacterial communities in several final isoprene enrichments were analyzed in detail by 454 pyrosequencing of the V3 region of the 16S rRNA gene, and 9643 reads with an average length of 155 bp were obtained. Two main trends were observed: water enrichments had a markedly different community composition between geographical locations (Fig. 3A; Table 1), and also differed from the sediment enrichments from the
same site (Fig. 3B; Table 1). In contrast, isoprene-degrading enrichments from the Colne estuary and Etang de Berre sediments were very similar and dominated by *Rhodococcus* spp. (Fig. 3B). Colne estuary water enrichments were dominated by *Mycobacterium* spp., while *Rhodococcus* spp. constituted around 80% of sequences from Etang de Berre water and sediment enrichments. Actinobacteria therefore seem to be the main organisms responsible for isoprene degradation in coastal samples, although Indonesian water enrichments were dominated by the alphaproteobacterial genus *Stappia*, with the actinobacterium, *Rhodococcus*, making up 30% of the community. Interestingly, communities in isoprene-enriched microcosms of water samples from Etang de Berre were remarkably similar with both of the isoprene concentrations used in the enrichments (0.1 or 0.001% v/v of isoprene; Table 1) and were dominated by *Rhodococcus*.

**Isolated isoprene-degrading bacteria are nutritionally versatile and most degrade n-alkanes**

Some of the main microbes identified above were cultured and their ability to degrade isoprene was confirmed. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that most isolates were Actinobacteria (Fig. 4), with several belonging to the most abundantly detected genus *Rhodococcus*. Alphaproteobacteria, including a *Xanthobacter* sp. were also isolated. Representatives of these genera have been shown, respectively, to degrade (Ewers et al., 1990) or modify (van Ginkel et al., 1986) isoprene in terrestrial environments. However, this is the first demonstration of isoprene degradation by other Actinobacteria (*Gordonia* and *Leifsonia*) and Alphaproteobacteria (a *Shinella*-like organism) as well as any representatives of the Gammaproteobacteria (*Rhodanobacter*)...
and Bacteroidetes (*Dyadobacter*) (Fig. 4). Bacteroidetes were consistently detected in DGGE profiles, pyrosequencing libraries and obtained by cultivation. The phylum Bacteroidetes is commonly associated with phytoplankton blooms (O’Sullivan et al., 2004) and diatom aggregates (Knoll et al., 2001). Moreover, a *Dyadobacter* strain induced capsule formation in a co-cultured freshwater diatom (Bruckner et al., 2008). These studies generally propose that Bacteroidetes benefit from the extracellular polymeric substances produced by algae, while our study suggests that isoprene is also an important algal product that supports bacterial growth.

It is thus apparent that isoprene-degrading capacity is widespread in diverse phyla. This raises the question of whether such microbes are specialist isoprene-degraders or generalists. We tested the ability of seven representative strains to grow on 24 carbon and energy sources (final concentration: 0.1%). All strains grew on a wide range of carbon sources including sugars, amino acids, organic acids and ethanol (Fig. 4; Table S1). Six out of seven grew on the *n*-alkane tetradecane, but none used low molecular-weight compounds like ethene, propene, dimethylsulfide and methane. As well as being nutritionally versatile, all strains tested could grow over a range of salinities and temperatures consistent with their provenance (Table S1). Therefore, in contrast to specialist methane-oxidising microbes (Trotsenko and Murrell, 2008) and like many coastal microorganisms (Mou et al., 2008), these isoprene degraders opportunistically utilize a wide range of compounds from the dissolved organic carbon pool.

Intrigued by the ability of most isolates to degrade tetradecane, we tested and confirmed that the most abundant and widespread marine hydrocarbonoclastic bacterium, *Alcanivorax borkumensis* strain SK2 (Kasai et al., 2001), could also degrade isoprene. This raises the possibility that, in the absence of spilled petroleum hydrocarbons in the...
sea, oil-degrading microbes survive by utilising algal products like isoprene. This is consistent with the commonly observed abundance of hydrocarbon degraders on the surface of algae (Radman et al., 2002; Green et al., 2004; Kaczmarska et al., 2005). Moreover, some of the most abundant microbes detected in isoprene-enrichment cultures by pyrosequencing, such as *Rhodococcus* and *Mycobacterium*, are known for their metabolic versatility and ability to degrade hydrocarbons. *Stappia* spp., which have also been found as epibionts of marine microalgae (Green et al., 2004; Kaczmarska et al., 2005) and which were abundant in isoprene enrichments of Indonesian seawater (Fig. 3), constituted 2.3-4.2% of the community in pristane and tetradecane-enriched microcosms from the same location (B. McKew, personal communication).

**Isoprene-producing algae support the growth of a mixture of isolates**

Natural isoprene concentrations in seawater (10 to 90 pM; [Milne et al., 1995; Matsunaga et al., 2002]) are several orders of magnitude lower than those used in our experiments (0.001% v/v ≈ 5.8 × 10^6 pM). Therefore, in order to test whether our isolates could consume concentrations of isoprene found in marine environments we created a mixture of seven isolates (those in Table S1) and tested whether it could consume the isoprene naturally produced by microalgae. We firstly screened a series of phototrophic marine species for isoprene production, and then selected two with relatively high levels of production: *Dunaliella tertiolecta*, not previously reported to produce isoprene, and *Phaeodactylum tricornutum*, already shown to produce isoprene (Milne et al., 1995).

Bacteria and microalgae were incubated in different minimal media in bottles that were connected to allow exchange of volatile components (Fig. S8). The isoprene concentration in the headspace of a *Dunaliella tertiolecta* culture incubated with a mixture
of isoprene-degrading isolates was significantly lower than when incubated without bacteria (Fig. 5A). The overall cell numbers of *Dunaliella* incubated with and without bacteria were not significantly different (t-test, $p>0.05$; Fig. 5B), suggesting that bacterial consumption is responsible for the decrease in isoprene. (We note that in a previous study (Shaw *et al.*, 2003) no heterotrophic consumption of isoprene in phytoplankton cultures was found, though, to our knowledge, the bacteria involved were not known to be isoprene-degraders.) Additionally, the presence of isoprene-producing microalgae maintained higher cell densities of bacteria during the earlier phases of algal growth (t-test, $p \leq 0.05$; Fig. 5C). The emitted isoprene, however, did not result in an increase in bacterial cell numbers (Fig. 5C). Similar results were obtained when using the benthic diatom *Phaeodactylum tricornutum* (Fig. S9). This experiment confirms that environmentally relevant levels of isoprene can be consumed by heterotrophic marine bacteria. It also suggests that natural levels of isoprene in marine environments may not be sufficient to allow rapid growth of such bacteria, which explains why the isolates, despite being able to use isoprene as a sole source of carbon and energy, are nutritionally versatile.

Diverse bacteria, including Actinobacteria, Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes, can supplement their nutrition with isoprene, and in-so-doing play an important role as sinks for algal-derived isoprene in marine ecosystems. Armed with this knowledge we are better placed to address many important questions, notably the nature and extent of the relationship between consuming bacteria and producing algae, the proportion of isoprene emitted that reaches the atmosphere in different marine and coastal environments, and how this may be affected by global climate change and ocean acidification.
EXPERIMENTAL PROCEDURES

Sampling

All samples used for enrichments were taken in 2006. The upper 2 cm of sediment or top 10 cm of seawater were sampled and either used immediately (Colne estuary) or stored at 4°C for about 1 week (Etang de Berre and Indonesia). In the Colne estuary, south-east England, mudflat sediment and estuary/sea water were sampled in the locations indicated in Fig. 1D (Point 1, the Hythe, 51°52’47.3”N; 0°55’43.1”E, salinity = 1‰; point 2, Wivenhoe, 51°52’8.7”N; 0°56’32.8”E; point 3, Alresford, 51°51’6.2”N; 0°57’50.7”E, salinity = 17‰; point 4, Brightlingsea, 51°47’28.7”N; 1°1’29”E, salinity = 34‰). Mudflat sediment samples were taken from within 5 m of the water samples. A preliminary sediment sample was taken from point 2 on 23 March. On 7 June, samples were taken from the freshwater end of the estuary (point 1), an intermediate location (point 3) and at the marine mouth of the estuary (point 4).

In the Berre lagoon (Etang de Berre), southern France (Fig. S1B), on 31 May, eight sediment samples were taken (Paissé et al., 2008), from a brackish retention basin, combined and mixed. Water samples were collected 500 m north east of the retention basin. Tropical seawater was sampled from a channel between the islands of Hoga and Kaledupa in southeast Sulawesi, Indonesia (Fig. S1C) on 14 August.

Additional samples from points 1, 3 and 4 were taken on 27 October 2008 to test the effect of lower isoprene concentrations, and from all four points between 10 and 17 March 2009 to determine isoprene concentrations and production.
Microcosms

Wet sediments from points 1, 3 and 4 on the Colne estuary were mixed in equal amounts with sterile phosphate buffered saline (pH 7.4); sediment samples from point 2 were mixed in equal amounts with water collected at the same site. Approximately 1.86 g of dry weight of sediment was used in each enrichment. Water and sediment samples from Etang de Berre had no additions. Colne estuary water from points 1, 3 and 4 were tested with no additions and also by mixing 2 ml of the estuary water with 8 ml of ONR 7a (a seawater-based minimal medium (Dyksterhouse et al., 1995)). For the Indonesian seawater sample, 20 mg l⁻¹ of NH₄NO₃ and 10 mg l⁻¹ of KH₂PO₄ were added to the water in order to achieve a level of nutrients similar to the other water samples.

Ten ml of sediment slurries or water samples were added to 125 ml serum bottles, sealed with polytetrafluoroethylene (PTFE)-lined butyl septa (Agilent). Liquid isoprene (solubility X, 10 µl, 99% purity, Aldrich) was injected through the septa using a gas-tight syringe, and mixed thoroughly. A 1:100 dilution of isoprene (prepared by adding 0.1 ml of pure isoprene to 9.9 ml of sterile ultrapure water, stirred overnight) was also used to inoculate a set of water samples from Etang de Berre, and a set of sediment and water samples collected from the Colne estuary in October 2008. Another set of the latter samples were inoculated with a 1:1000 dilution of isoprene. Samples were incubated with shaking (110 rpm) at 12°C (Colne estuary and Etang de Berre) or 30°C (Indonesia), and the concentration of isoprene in the headspace was monitored by gas-chromatography (GC). For every location and sample type, four replicates were prepared, and these microcosms were designated as E0. Four killed control microcosms were prepared for each sample type by autoclaving (121°C for 20 min). The same size bottles and volume of sample were used; controls were incubated at the same temperature as the test samples,
and isoprene in the headspace was measured alongside the equivalent quadruplicate live samples at each time point.

When isoprene was consumed, a first enrichment (E1) was prepared by diluting 2 ml from the original microcosms with 8 ml of ONR7a minimal medium. For the second (E2) and third (E3) enrichments, 1 ml of the previous culture was diluted with 9 ml of ONR7a. Isoprene was added to each new enrichment.

**Measuring isoprene concentrations**

In enrichments 50 (for the single bottles) or 250 (for the double bottles) µl of the headspace was collected with a gas-tight syringe and injected into a Philips PU 4500 GC with a 1.5-m glass column packed with 10% apiezon on chromosorb W and a flame-ionization detector (injector 160°C, column 100°C, detector 160°C). The retention time for isoprene was 0.74 min. Between 10 and 17 March 2009 water samples from Points 1 to 4 were collected at both high and low tide in triplicate from the centre of the Colne estuary in 1 liter glass containers 30 cm below the surface. From each sample, 60 ml was filtered through a 0.2 µm glass fibre filter (Chromacol Ltd., UK) into a glass purge vessel. The vessel was then purged for 20 min with helium gas (BOC Gases, UK) at a flow rate of 80 ml min⁻¹, and the released gases passed through a stainless steel cryo-trap held at -160°C using a liquid nitrogen boiler. Isoprene was quantified by GC-FID, calibrated using an isoprene standard gas (Scientific and Technical Gases Ltd., UK).

To measure production from sediments, cores (10 cm diameter, 8 cm depth) were collected at each site in triplicate, taken from the exposed intertidal zone at a mid-tide elevation. Four sub-cores (2 cm diameter, 2 cm depth) from each were incubated in gas-tight glass purge vessels at ambient temperature (14°C) and light (400 µmol m⁻² sec⁻¹) for
2 – 4 h, purged with helium at 80 ml min\(^{-1}\) for 30 min and stored in a cryo-trap before GC-FID analysis.

**Isolation and characterization of isoprene-degrading bacteria**

After the third enrichment, ten-fold serial dilutions in ONR7a minimal medium were performed, aliquots were streaked onto plates of ONR7a medium containing washed agar and incubated in an isoprene-saturated atmosphere. Isolates were tested for their ability to degrade isoprene in ONR7a medium as described above for the enrichments. The purity of the isolates was determined by repeated subculture of single colonies, microscopic analysis to see if cells had a uniform morphology, and collection of single clean sequences.

Seven isolates that degraded isoprene were tested for utilization of different carbon sources and their salinity and temperature range for growth, defined by visual detection of turbidity. Colonies were suspended in 5 ml of ONR7a minimal medium into 50 ml serum bottles. For the carbon source tests, 5 ml of ONR7a was supplemented with 0.1% of the compounds listed in the legend to Fig. 4. Stock solutions containing 100 g l\(^{-1}\) of glucose, fructose, sucrose, alanine, arginine and pyruvate or 10 g l\(^{-1}\) of methionine, benzoate and acetate were prepared. For ethanol, glycerol, methanol, tetradecane, pristane, benzene, toluene, squalane, DMS and isoprene, 5 µl of a 99% pure solution of each compound was added directly. For methane, ethene and propene (≥99% pure), a bladder was emptied and filled three times with the gas before adding 5 µl with a gas-tight syringe. In the case of biphenyl (99% pure) and polyisoprene, 5 mg was added directly. Homogeneous poly-cis-isoprene was obtained by grinding a natural rubber latex condom in a sterile mortar with liquid nitrogen. To determine the salinity range for growth, ONR7a containing 0.1% v/v
isoprene was prepared by adjusting the NaCl concentration between 3.37-140‰. Temperature requirements were determined by incubating in 5 ml of ONR7a medium with 0.1% v/v isoprene at 4, 12, 20, 25, 30 and 37°C. Tests were performed in duplicate; negative controls without any carbon source and positive controls with isoprene were also prepared. Incubations for the salinity and carbon source tests were performed at 20°C, and all were shaken in the dark at 110 rpm.

DNA extraction, 16S rRNA gene amplification, sequencing, and phylogenetic analysis of pure cultures was carried out as described previously (Fahy et al., 2008), and a neighbor-joining tree, using Jukes-Cantor distances, was constructed, and 1000 replicates were bootstrapped, using the Phylip package (Felsenstein et al., 2004).

**Nucleotide sequence accession numbers**

Nucleotide sequences for the isolates have been deposited with the EMBL Nucleotide Sequence Database under the accession numbers FN298498-FN298506.

**DNA extraction, 16S rRNA gene amplification and denaturing gradient gel electrophoresis (DGGE) and sequencing from microcosms**

Cells were collected by centrifugation from 2 ml of the enrichments (E0, E1, E2, E3) when all the isoprene had been degraded. DNA extraction using bead-beating was performed as previously described (McKew et al., 2007). Primers for positions 341 to 534 in *Escherichia coli* (Muyzer et al., 1993) were used for PCR amplification of the variable V3 region of the 16S rRNA bacterial gene, and DGGE was performed as previously described (McKew et al., 2007), except that gels were silver stained (Nielsen et al., 1999) with a modified fixation protocol of 30 min in the developing solution without NaBH₄ and formaldehyde,
and no final fixing step in NaCO$_3$. Selected DGGE bands were excised with a sterile razor blade and soaked for seven days in 20 µl of water in the case of silver-stained gels. Re-amplification, cleaning and sequencing were as previously described (McKew et al., 2007). Sequences were compared with those in the nucleotide database at the National Centre for Biotechnology Information using BLAST (Altschul et al., 1990).

**Pyrosequencing**

PCR products were obtained as described above, except that the forward primers for the V3 region of the 16S rRNA gene had a 5´modification that consisted of a 454 amplicon adapter followed by a unique ten-nucleotide barcode (Parameswaran et al., 2007). The last enrichment showing isoprene degradation from each location was selected for pyrosequencing. PCR products were quantified with a Nanodrop ND-1000 spectrophotometer and pooled in equal amounts. Approximately 200 ng of this pooled sample was analysed by pyrosequencing using the Roche/454 Amplicon sequencing platform.

Sequences were separated according to barcodes. Barcode and primer sequences were removed and sequences of at least 150 bp were processed using the Greengenes pyrosequencing pipeline (DeSantis et al., 2006a). Sequences from each sample were aligned against the Greengenes 16S rRNA gene database using NAST (Nearest Alignment Space Termination) (DeSantis et al., 2006b). Parameters used were minimum length of 100 bp and 85% of minimum identity. The taxonomic composition of each set of aligned sequences was obtained by comparison against the Ribosomal Database Project using the Greengenes classification tool.
Incubations of isoprene-degrading isolates with phytoplankton species

Incubations were performed in triplicate in 125 ml double borosilicate serum bottles sealed with PTFE-lined butyl septa (Agilent). The double container consisted of two serum bottles connected near the top so that only volatiles were exchanged (Fig. S8). A side arm at the bottom of each bottle sealed with a septa union valve allowed sampling of the liquid phase. Subsamples of 0.1 ml were taken in triplicate 1, 3, 5, 7 and 10 days after incubations were started and cells numbers determined using a Helber counting chamber. Headspace samples were taken with a gas-tight syringe through the tops of the bottles and analyzed by GC-FID as described before.

Bacteria were cultured in ONR7a (Dyksterhouse et al., 1995) and microalgae in ESAW (Berges et al., 2001). Between 10 and 30% of an exponential-phase bacterial or algal culture was used as the inoculum for the experiment. Two experiments were performed with different microalgal species, *Dunaliella tertiolecta* CCAP 19/27 and *Phaeodactylum tricornutum* CCMP 632. In both cases the bacterial mixture consisted of a mixture of the seven characterized isoprene-degrading isolates grown separately to an OD$_{600}$ of 0.6 to 0.7 and mixed equally.
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FIGURE LEGENDS

Fig. 1 Isoprene degradation, concentration and production in water and sediment samples. Degradation of isoprene in dark microcosms as indicated by the headspace concentration relative to killed controls, in: A, water from Indonesia and Colne estuary (2006) from three locations: head (point 1), intermediate (point 3) and mouth (point 4); B, sediments from Colne estuary (2006) from points 1, 2, 3 and 4; C, water from Colne estuary, points 1, 3 and 4 (2008). The mean of four replicates is plotted, and bars represent standard errors. Note the difference in the time scales and the different concentrations of added isoprene. D, Sampling points along Colne estuary, Essex, UK.

Fig. 2 Isoprene in-situ concentrations in estuarine water samples and isoprene production rates in light-incubated sediment samples along Colne estuary.

Fig. 3 Bacterial communities in isoprene-enriched microcosms analyzed by 454 pyrosequencing of partial 16S rRNA genes. Bacterial genera comprising more than 5% of the total community in isoprene-enriched microcosms from A, water and B, sediment. Others: sum of abundances for genera representing less than 5% of the community composition.

Fig. 4 Phylogenetic tree based on almost complete 16S rRNA gene sequences and carbon sources utilized by selected isoprene-degrading isolates. Isoprene-degrading strains are colour-coded according to their origin (see Fig. 1, black for Etang de Berre). All strains grew with the following as sole source of carbon and energy: glucose, fructose, alanine, acetate, benzoate, pyruvate, ethanol. None of the strains utilized: methionine,
biphenyl, toluene, ethane, propene, poly-cis-isoprene, dimethylsulfide, methane. Growth substrates differentially utilized by the strains are indicated in square brackets by the codes: S sucrose, A arginine, G glycerol, M methanol, T tetradecane, P pristane, Sq squalane, B benzene. Branches supported by ≥70% bootstrap resampling are indicated by a dot. The bar represents 0.1 average nucleotide substitutions per base. γProt = Gammaproteobacteria.

**Fig. 5** Isoprene concentration and cell numbers for isoprene-producing *Dunaliella tertiolecta* and isoprene-consuming bacteria incubated in separate vessels that allow exchange of volatile compounds. A, Isoprene concentration in the headspace. Cell numbers of B, *Dunaliella tertiolecta* and C, bacteria. Three experiments were performed (Fig. S8): green lines, *Dunaliella tertiolecta* minus isoprene-consuming bacteria; black lines, *Dunaliella tertiolecta* plus bacteria; blue line, bacteria alone.

**Table 1. Community composition of isoprene-enriched microcosms from water and sediment samples analysed by 454 pyrosequencing of the 16S rRNA gene.** Phylum or class is indicated on the left by: A: Actinobacteria; αP: Alphaproteobacteria; βP: Betaproteobacteria; γP: Gammaproteobacteria; B: Bacteroidetes; F: Firmicutes. Enrichments are from samples: CW1, 3: Colne water points 1, 3; EWH, EWL: Etang de Berre water with 0.1% v/v, 0.001% v/v isoprene, respectively; IW: Indonesia water; CS1, 2, 3, 4: Colne sediment points 1, 2, 3, 4, respectively; ES: Etang de Berre sediment. Others are those genera that represented less than 0.5% of the community. Bold black: percentage above 5%.