Molecular Ecology of Isoprene-Degrading Bacteria

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Abstract: Isoprene is a highly abundant biogenic volatile organic compound (BVOC) that is emitted to the atmosphere in amounts approximating to those of methane. The effects that isoprene has on Earth’s climate are both significant and complex, however, unlike methane, very little is known about the biological degradation of this environmentally important trace gas. Here, we review the mechanisms by which bacteria catabolise isoprene, what is known about the diversity of isoprene degraders in the environment, and the molecular tools currently available to study their ecology. Specifically, we focus on the use of probes based on the gene encoding the α-subunit of isoprene monooxygenase, isoA, and DNA stable-isotope probing (DNA-SIP) alone or in combination with other cultivation-independent techniques to determine the abundance, diversity, and activity of isoprene degraders in the environment. These parameters are essential in order to evaluate how microbes might mitigate the effects of this important but neglected climate-active gas. We also suggest key aspects of isoprene metabolism that require further investigation in order to better understand the global isoprene biogeochemical cycle.

Keywords: climate; BVOC; isoprene; isoprene monooxygenase; isoA; DNA stable-isotope probing

1. Isoprene and Climate

Isoprene (2-methyl-1,3-butadiene), with global annual emissions to the atmosphere of approximately 500 Tg, is the most abundantly produced biogenic volatile organic compound (BVOC) on Earth. This is similar in magnitude to the emissions of all other BVOCs combined and equal to global emissions of methane per year [1,2]. Due to its abundance, volatility (boiling point of 34 °C), and high reactivity (due to the presence of two C=C bonds) isoprene plays a significant and complex role in atmospheric chemistry, and hence, climate [3]. In rural environments with low levels of nitrogen oxides (NOx), isoprene reacts with hydroxyl radicals (OH), reducing the oxidising capacity of the atmosphere [1]. This in turn increases the residence time of greenhouse gases, such as methane, and thus enhances global warming [4,5]. In urban environments, nitric oxide (NO) is typically present at high concentrations and reacts with isoprene to form nitrogen dioxide (NO2). The photolysis of NO2 increases the tropospheric levels of ozone [3], a greenhouse gas which has detrimental effects on air quality and plant and animal health [6]. Conversely, the atmospheric oxidation of isoprene can also form secondary aerosols that act as cloud condensation nuclei, resulting in a global cooling effect [7,8].

2. Global Isoprene Emissions

The vast majority of isoprene (~90%) emitted globally is produced by terrestrial plants [9]. However, there is a lot of variation in isoprene production, particularly in trees, which are major sources. For example, not all trees produce isoprene, and high and low emitters can be found even among closely related species [10–12]. In isoprene-emitting plants, isoprene is synthesised in the
chloroplast from dimethylallyl diphosphate (DMAPP) in a reaction catalysed by isoprene synthase [13]. Although typically ~2% of carbon photosynthetically fixed in high isoprene-emitting trees can be diverted to the production of isoprene, and in some cases even more [14], it is striking that its role in plants is not yet fully understood. Previous studies have reported that isoprene can confer protection against thermal and oxidative stress (reviewed in [15]). However, the molecular mechanisms behind these processes have not yet been completely elucidated. Other proposed roles for isoprene include effects on plant growth, insect herbivory and, more recently, the regulation of gene expression, as well as effects on the proteome and the metabolome of plants (reviewed in [15]).

Since trees are the major source of isoprene in the biosphere, the increase in land usage dedicated to the cultivation of high isoprene-emitting trees (e.g., poplar (*Populus* spp.), willow (*Salix* spp.), eucalyptus (*Eucalyptus* spp.), and oil palm (*Elaeis guineensis*), especially for biofuel and food production, has raised serious concerns about the impact on air quality and human health [6,16,17].

The remaining 10% of isoprene emissions to the atmosphere is attributed to bacteria, fungi, algae, and animals [18–22]. Bacteria reported to synthesise isoprene to date include strains from the *Proteobacteria*, *Actinobacteria*, and *Firmicutes* phyla, with *Bacillus* being the best-characterised isoprene-producing genus [21,23–25]. However, the biosynthetic pathways for isoprene in bacteria and indeed why isoprene is produced by microbes is uncertain (reviewed in [26]).

In the marine environment, isoprene emissions are estimated to range from 0.1 to 11.6 Tg per year depending on the methodology used (reviewed in [26]) and mainly originate from phytoplankton, seaweeds, and heterotrophic bacteria [20,27,28]. The role of isoprene in marine environments is not well understood. It has been suggested that it might confer protection against high temperatures, light intensity, and oxidative stress [27,29–31]. Other environments that have been reported to emit isoprene, but remain relatively unexplored, include wetlands and freshwater lakes [32,33]. One further source of isoprene to the atmosphere is anthropogenic emissions of isoprene (estimated at ~0.8 Tg per year) [34]. These are due to the production of isoprene for use as a bulk chemical to synthesise a wide range of industrial products, including synthetic rubber, elastomers and pharmaceuticals, vehicle exhausts, and biomass burning [34–36].

3. Biological Sinks for Isoprene

It has been known for over 20 years that soils act as a biological sink for isoprene at environmentally relevant concentrations due to field chamber and continuous-flow experiments performed with temperate, tropical, and boreal soils [37–40]. In the marine environment, Acuña Alvarez et al. [41] showed the capacity for biological degradation of isoprene in estuarine, coastal, and open marine waters and sediments. They also demonstrated that isoprene-degrading bacteria isolated from these environments consumed isoprene naturally produced by microalgal cultures. More recently, experiments conducted with phyllosphere samples from high isoprene-emitting trees from temperate and tropical regions (poplar, willow, and oil palm trees) have shown that this environment is likely to be an important source of isoprene-degrading microorganisms [42–45]. However, despite these findings, the significance of the biological consumption of isoprene is still a key aspect of the global isoprene cycle that needs to be addressed.

4. Diversity of Isoprene-degrading Bacteria

Early soil enrichments with isoprene led to the isolation of bacteria that could use this trace gas as a sole carbon and energy source. These microorganisms were tentatively assigned to the genera *Rhodococcus*, *Nocardia*, *Arthrobacter*, and *Alcaligenes* [37,46–48]. Studies with rubber-contaminated soil yielded *Pseudomonas*, *Klebsiella*, and *Alcaligenes* isoprene-degrading strains [49]. Isoprene degraders of the genera *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Sphingobacterium*, *Sphingobium*, and *Pantoea* have been also isolated from soils and leaves from tropical teak (*Tectona grandis*) and mahua trees (*Madhuca latifolia*) [50]. However, none of these strains was characterised in detail. Isoprene enrichments with garden and tyre dump soils, as well as soils in the vicinity of willow trees, have also led to the isolation of
several *Rhodococcus* species and novel *Nocardioides*, *Ramlibacter*, and *Variovorax* strains, which degrade isoprene [51–56]. Recent studies exploring the phyllosphere of poplar, willow, and oil palm trees have shown that this environment harbours a considerable variety of isoprene-degrading isolates, including members of the genera *Rhodococcus*, *Gordonia*, *Sphingopyxis*, and *Variovorax* [42,51,52,55]. Moreover, Acuña Alvarez et al. [41] reported the isolation, from marine and estuarine environments, of several *Actinobacteria* (Leifsonia, Gordonia, *Mycobacterium*, *Rhodococcus*) and *Alphaproteobacteria* (*Stappia*, *Loktanella*, *Shinella*) that could grow on isoprene, with *Gordonia* and *Mycobacterium* being the most well characterised [57]. Thus far, freshwater environments have not been explored in detail, however, van Hylckama Vlieg et al. [58] isolated *Rhodococcus* sp. AD45 from freshwater sediments, which has become a key bacterial strain for the study of isoprene metabolism [43,58–61]. Details about the isoprene-degrading strains belonging to these genera are provided in [62]. Finally, to our knowledge, no anaerobic bacteria, archaea, or fungi have been reported to use isoprene as a sole carbon and energy source, although Kronen et al. [63] have recently shown that strict anaerobes can use isoprene as an electron acceptor to support homoacetogenesis, and thus anaerobic degradation of isoprene also warrants further attention.

5. Isoprene Degradation Pathway

In the best-characterised isoprene-degrading bacterium, *Rhodococcus* sp. AD45, isoprene is first oxidised to epoxyisoprene in a reaction catalysed by isoprene monooxygenase (IsoMO; Figure 1), a member of the soluble diiron monooxygenase (SDIMO) family [64]. Then, a glutathione-S-transferase (IsoI) conjugates the reactive epoxide with glutathione, forming 1-hydroxy-2-glutathionyl-2-methyl-3-butene (HGMB), which is further converted to glutathionyl-2-methyl-3-butenal (GMB) by a dehydrogenase (IsoH; [58,59]; Figure 1). However, the downstream catabolic pathway has not yet been elucidated. It is assumed that, after the removal of glutathione and β-oxidation, GMB intermediates enter central metabolism, thus allowing *Rhodococcus* sp. AD45 to grow on isoprene as a sole carbon and energy source.

![Figure 1. Isoprene degradation pathway in the model isoprene-utilising bacterium *Rhodococcus* sp. AD45.](image)

Enzymes: IsoMO, isoprene monooxygenase; IsoI, glutathione-S-transferase; IsoH, dehydrogenase; HGMB, 1-hydroxy-2-glutathionyl-2-methyl-3-butene; GMB, 2-glutathionyl-2-methyl-3-butenal; GMAA, 2-glutathionyl-2-methyl-3-butenonic acid; GSH, reduced glutathione. The question mark indicates uncertainty in the details of the catabolic pathway from GMB.

All isoprene degraders characterised to date contain six genes (*isoABCDEF*) encoding the $\alpha_2\beta_2\gamma_2$ oxygenase (IsoABE), reductase (IsoF), Rieske-type ferredoxin (IsoC), and coupling protein (IsoD) that form IsoMO. Four additional genes (*isoGHIF*), which encode a putative coenzyme A transferase, a dehydrogenase, and two glutathione transferases [58–60], are located immediately upstream (5') of the structural genes *isoABCDEF*, which appears to be a typical layout for iso genes in all bona fide isoprene-degrading bacteria studied in detail to date (Figure 2). It is interesting that in some isoprene degraders, such as *Rhodococcus* sp. AD45 and *Variovorax* sp. WS11, the isoprene degradation gene cluster is encoded on a megaplasmid [53,61], suggesting that these bacteria might have acquired the ability to grow on isoprene by horizontal gene transfer.
Variovorax sp. WS11 [53, 61]. Targeted mutagenesis and expression studies, along with the construction of reporter which isoprene degradation is regulated, although it has been shown that epoxyisoprene upregulates within the isoprene degradation gene cluster, whereas genes involved in the biosynthesis of glutathione (phylogenetically diverse bacteria. Therefore, the use of 16S rRNA primers alone to identify target for the design of probes to study isoprene degraders in environmental samples. Following these principles, El Khawand et al. [51] aligned sequences from bona fide positive bacteria include: aldH (encoding an aldehyde dehydrogenase), gshA (glutamate cysteine ligase), gshB (glutathione synthetase), and CoA-DSR (CoA-disulfide reductase) [61]. Three putative transcriptional regulators (marR1, marR2 and gntR) are also located within the isoprene degradation gene cluster of Rhodococcus sp. AD45 [61]. However, in Gram-negative isoprene-degrading strains, such as Variovorax sp. WS11, aldH and garB (encoding a glutathione disulfide reductase) are present within the isoprene degradation gene cluster, whereas genes involved in the biosynthesis of glutathione (gshAB) are located on the chromosome [53] (Figure 2). Finally, two putative LysR-type transcriptional regulators (dmlR4 and dmlR5) have been identified flanking the isoprene degradation gene cluster of Variovorax sp. WS11 [53].

Thus, there are still some uncertainties within the isoprene degradation pathway, such as the downstream catabolic steps from GMBA, the role of the accessory genes, and the exact mechanisms by which isoprene degradation is regulated, although it has been shown that epoxyisoprene upregulates the expression of iso genes at a higher level than isoprene itself in Rhodococcus sp. AD45 and Variovorax sp. WS11 [53, 61]. Targeted mutagenesis and expression studies, along with the construction of reporter strains, should be a fruitful approach to elucidate the full isoprene degradation pathway and to decipher the nature and mechanisms of action of putative promoters, transcriptional activators, and inducers, all of which are currently ongoing in our laboratory.

6. Molecular Techniques to Study the Ecology of Isoprene Degraders

6.1. isoA Probes

As described above, isoprene degradation seems to be a widespread trait across many phylogenetically diverse bacteria. Therefore, the use of 16S rRNA primers alone to identify isoprene degraders in environmental samples would not be successful. However, molecular ecology studies targeting genes encoding key subunits of other SDIMOs, such as mmoX (soluble methane monooxygenase; sMMO), have greatly enhanced our knowledge of the diversity and abundance of methane-oxidising bacteria in many environments [65–68]. The availability of genes essential for isoprene metabolism has enabled us to follow a similar approach. The isoA gene (a homolog of mmoX), encodes the α-subunit of the IsoMO which contains the diiron centre at the putative active site of SDIMO enzymes [64]. This isoA gene is conserved in all characterised isoprene degraders and can be readily distinguished from the α-subunits from other SDIMOs [51, 69]. Therefore, isoA constituted an obvious target for the design of probes to study isoprene degraders in environmental samples. Following these
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principles, El Khawand et al. [51] aligned isoA sequences from bona fide isoprene-degrading bacteria and designed a PCR primer set targeting this gene. This isoA primer set yielded a PCR product with DNA extracted from a wide range of isoprene degraders and environmental samples, including leaves, soils, and estuarine and marine water and sediments, but no PCR product was observed with DNA from non-isoprene-degrading strains that harboured other SDIMO enzymes [51]. In addition, the IsoA sequences retrieved from environmental samples showed >86% amino acid identity to IsoA from ratified isoprene degraders, which at the time were predominantly Actinobacteria, and could be clearly separated into two distinct groups: those retrieved from terrestrial and estuary samples of lower salinity and those recovered from marine environments, including the mouth of the estuary [51]. Therefore, this approach confirmed isoA as an excellent marker gene for isoprene degradation and contributed to the expansion of the known diversity of isoprene degraders in terrestrial, estuarine, and marine environments [57].

However, the isolation and characterisation of novel isoprene-degrading Proteobacteria, such as Variovorax, Sphingopyxis, or Ramlitibacter [56], revealed a higher diversity of isoA sequences than previously thought and emphasised the need to refine the isoA probes to cover all the isoprene degraders characterised to date. To that end, Carrión et al. [69] aligned 38 isoA genes from confirmed isoprene degraders, along with 18 sequences from metagenomes obtained from environmental samples enriched with isoprene that had ≥98% coverage and ≥85% amino acid identity to ratified IsoA sequences. Sixteen genes encoding α-subunits from other groups of SDIMOs were also included in the alignment to guide the specific amplification of isoA. After identifying the conserved regions within isoA, a primer set that spans the first iron centre of the IsoMO α-subunit was designed [69]. This new primer set yielded a specific PCR product of 497 bp with DNA from all 30 positive control isoprene-degrading strains tested, but no amplification products were obtained with DNA from the 12 non-isoprene-degrading strains used in this study as negative controls. To further validate the specificity of the new isoA probes, clone libraries from phyllosphere, soils, freshwater, and marine samples enriched with isoprene were made. Sequences retrieved by PCR with DNA from these environmental samples showed ≥84% amino acid identity to IsoA from ratified isoprene degraders but were ≤70% identical to α-subunits from other closely related SDIMOs, confirming that these isoA probes are a valuable tool to study the ecology of isoprene degraders [69]. Subsequently, Carrión et al. [69] used the new isoA primer set to further investigate the diversity of isoA genes in a wider range of environmental samples enriched with isoprene by isoA amplicon sequencing. The sequencing data revealed that phyllosphere samples tested harboured the highest diversity of isoA genes, with sequences homologous to IsoA from Rhodococcus, Gordonia, Mycobacterium, Sphingopyxis, and Variovorax (Figure 3). Soils collected from beneath high isoprene-emitting trees, such as oil palm and willow, and also rubber-contaminated soil, were dominated by isoA genes closely related to IsoA from Rhodococcus and Variovorax. The most abundant sequences from freshwater sediment were similar to Rhodococcus and Sphingopyxis IsoA, whereas coastal and marine sediments were overwhelmingly dominated by sequences closely related to IsoA from Rhodococcus (Figure 3). However, it is worth noting that the phylogeny of these isoA sequences might need to be reassessed when new genera of isoprene degraders are isolated, since the number of fully characterised isoprene-degrading strains to date is still limited. The existence of novel isoprene degraders with diverse isoA genes in the environments studied by Carrión et al. [69] is supported by the fact that some sequences recovered in this study, especially those from freshwater sediment, showed only 83–91% amino acid identity to IsoA from confirmed isoprene-degrading bacteria [69]. This information provided by the new isoA probes can be used now to design targeted isolation strategies for novel isoprene-utilising strains. The characterisation of new genera of isoprene degraders will contribute to a more robust IsoA database, which in turn will enable a more accurate phylogenetic assessment of the isoA genes retrieved from environmental samples, and thus a more precise picture of the main players in isoprene degradation in a particular habitat.
were similar to those found in leaves from an ash tree (Fraxinus spp.), a low isoprene emitter [69,70]. Therefore, estimations of isoprene production and consumption rates, coupled with surveys of isoA genes (diversity, quantity, and activity), will enable researchers to obtain a better understanding of the role of microbes in the global isoprene biogeochemical cycle. Nevertheless, soils in the vicinity of high isoprene-emitting trees, such as oil palm and willow, contained the highest numbers of isoA genes when normalised to the abundance of 16S rRNA genes (~200 to 300 isoA genes per million copies of 16S rRNA genes) [69]. This value was approximately 10-fold higher than the relative abundance of isoA observed in leaves taken from the same trees, indicating that soils might be a more important sink for isoprene than previously thought. Another interesting observation from this study was that the numbers of isoA genes in the phyllosphere of high isoprene-emitting trees were similar to those found in leaves from an ash tree (Fraxinus spp.), a low isoprene emitter [69,70]. However, a larger number of samples are required to confirm these results statistically. Finally, although several isoprene-degrading strains have been isolated from freshwater and marine samples [41,57,58], it was surprising that these environments contained similar numbers of isoA sequences per million copies of 16S rRNA genes to those found in the phyllosphere samples tested, since the leaves of trees are considered to be the main source of isoprene globally [69] (Figure 4). These preliminary results are from a very small sample of different environments and there is a considerable need to study in more detail isoprene production and consumption in terrestrial, freshwater, and marine environments. Therefore, estimations of isoprene production and consumption rates, coupled with surveys of isoA genes (diversity, quantity, and activity), will enable researchers to obtain a better understanding of the role of microbes in the global isoprene biogeochemical cycle.

Figure 3. Relative abundance and diversity of isoA genes in environmental samples enriched with isoprene analysed by amplicon sequencing. Amplicon sequence variants (ASVs) closely related to IsoA from Gordonia are shown in orange; ASVs with the highest homology to IsoA from Mycobacterium are represented in light blue; ASVs encoding proteins homologous to Rhodococcus IsoA are shown in dark blue; ASVs with the highest homology to Sphingopyxis IsoA are coloured in yellow; ASVs closely related to IsoA from Variovorax are represented in purple. Data from [69].
Although both studies revealed Rhodococcus, Nocardioides, and members of the Comamonadaceae family (Comamonas, Ramlibacter, and Variovorax) as the main isoprene degraders in soils surrounding willow trees, Rhodococcus dominated the isoprene-utilising bacterial community at high concentrations of isoprene, whereas Comamonadaceae were more abundant when low concentrations were used (Table 1). In addition, the metagenomic sequencing of the $^{13}$C-labelled DNA allowed the identification of isoprene degradation gene clusters closely related to those found in Rhodococcus, Nocardioides, Ramlibacter, and Sphingopyxis [56]. This information was subsequently used to isolate representative isoprene-degrading strains from these genera [54,56], which in turn have significantly expanded the existing database of isoprene degradation genes and paved the way for the design of the new isoA probes are a relevant and successful tool to study the ecology of isoprene degraders, however, it is important to continuously refine them based on new information, and combine them with other cultivation-independent techniques, such as DNA stable-isotope probing (DNA-SIP), to enhance the detection of uncultivated isoprene-degrading bacteria with more diverse isoA genes or with novel isoprene catabolic pathways.

6.2. DNA Stable-Isotope Probing (DNA-SIP)

DNA-SIP can be a powerful technique to link the identity and function of microbes in the environment [65,71]. It relies on the incubation of environmental samples with $^{13}$C-labelled isoprene for a sufficient time for the active isoprene-degrading community to assimilate enough $^{13}$C-isoprene into their biomass to allow the detection and analysis of $^{13}$C-labelled DNA. The $^{13}$C-labelled DNA from the active isoprene degraders can be separated from the unlabelled DNA from the rest of the microbial community (the non-isoprene-consuming population) by isopycnic ultracentrifugation. Once separated, the $^{13}$C-labelled DNA can be analysed using a variety of techniques, such as amplicon sequencing or metagenomics [72].

Several DNA-SIP experiments with $^{13}$C-labelled isoprene have been conducted with samples from terrestrial, phyllosphere, and marine environments [42,43,45,51,56,57]. El Khawand et al. [51] and Larke-Mejía et al. [56] investigated the composition of the active isoprene-degrading community from soil beneath willow trees using DNA-SIP with high and low concentrations of isoprene (Table 1). Although both studies revealed Rhodococcus and members of the Comamonadaceae family (Comamonas, Ramlibacter, and Variovorax) as the main isoprene degraders in soils surrounding willow trees, Rhodococcus dominated the isoprene-utilising bacterial community at high concentrations of isoprene, whereas Comamonadaceae were more abundant when low concentrations were used (Table 1). In addition, the metagenomic sequencing of the $^{13}$C-labelled DNA allowed the identification of isoprene degradation gene clusters closely related to those found in Rhodococcus, Nocardioides, Ramlibacter, and Sphingopyxis [56]. This information was subsequently used to isolate representative isoprene-degrading strains from these genera [54,56], which in turn have significantly expanded the existing database of isoprene degradation genes and paved the way for the design of the new isoA genes or with novel isoprene catabolic pathways.

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probes. Recent studies have also shown that soils in the vicinity of oil palm trees harbour a very diverse isoprene-utilising bacterial community, with *Aquabacterium, Novosphingobium, Pelomonas, Rhodoblastus, Saccharibacter, Sphingomonas,* and *Rhodococcus* being the main genera enriched in samples incubated with $^{13}$C-isoprene [42,45,56] (Table 1). However, no isolates from these genera were obtained in these studies and the pathway that these $^{13}$C-labelled bacteria might use to catabolise isoprene has not yet been elucidated, except for *Novosphingobium,* for which a metagenome-assembled genome (MAG) containing a full isoprene degradation gene cluster with 76.2–100% identity at the derived amino acid level with the corresponding polypeptides from *Sphingopyxis* sp. OPL5 was recovered [42].

| Study                     | Environment         | Isoprene Concentration (ppm) | Active Isoprene Degraders                                      |
|---------------------------|---------------------|------------------------------|-----------------------------------------------------------------|
| El Khawand et al., 2016   | Willow soil         | 5000                         | *Rhodococcus,* *Variovorax,* *Comamonas*                        |
| Johnston et al., 2017     | Estuarine water and sediment | 2000                         | *Microbacterium,* *Rhodococcus,* *Mycobacterium,* *Gordonia*    |
| Crombie et al., 2018      | Poplar leaves       | 500                          | *Rhodococcus,* *Xanthomonadaceae,* *Comammadaceae*              |
| Larke-Mejíia et al., 2019 | Willow soil         | 25                           | *Ramlibacter,* *Variovorax,* *Rhodococcus*                      |
| Carrion et al., 2020      | Oil palm leaves     | 25                           | *Gordonia,* *Zoogloe*                                          |
| Larke-Mejíia et al.,      | Oil palm leaves     | 25                           | *Pelomonas,* *Novosphingobium,* *Rhodoblastus,* *Sphingomonas*  |
| unpublished               |                     |                              | *Gordonia,* *Sphingomonas,* *Aquincola*                         |

In addition to soils, DNA-SIP has also been used to identify active isoprene degraders in the phyllosphere. DNA-SIP experiments performed with leaf washings from white poplar showed that *Rhodococcus* and *Variovorax* played a major role in isoprene degradation in this environment (Table 1). Transcriptomic analyses also revealed the presence of RNA encoding *iso* genes of both genera in microcosms set up with the same phyllosphere samples incubated with $^{13}$C-isoprene [43]. Moreover, *Rhodococcus,* *Pseudonocardia,* and *Variovorax* MAGs containing genes involved in isoprene metabolism were reconstructed from the metagenomic sequencing of the $^{13}$C-labelled DNA. The *Rhodococcus* MAG was the most abundant bin recovered and was phylogenetically close to *Rhodococcus* sp. ACPA4 (98.7% average nucleotide identity), an isoprene degrader isolated from poplar leaves [52]. The *Pseudonocardia* MAG contained six genes homologous to those involved in isoprene degradation, including *isoA,* with 75–93% identity at the derived amino acid level to the corresponding proteins from *Rhodococcus* sp. AD45 [43], which is interesting because there are no known isoprene degraders from this genus. However, the isolation of representative strains from *Pseudonocardia* spp. is needed to confirm their ability to consume isoprene. Conversely, the *Variovorax* MAG contained a complete isoprene degradation gene cluster in an identical layout to those of many ratified isoprene-utilising isolates [43] but had a low amino acid identity (42–71%) to the homologous Iso enzymes from *Rhodococcus* sp. AD45. The IsoMO from the *Variovorax* MAG was subsequently shown to oxidise isoprene when cloned and expressed in a non-isoprene-degrading *Rhodococcus* strain, confirming that it is a functional enzyme, and illustrating how the heterologous expression of putative *iso* gene clusters can be a good way to test for activity [43]. These types of DNA-SIP experiments have guided the targeted isolation of isoprene-degrading *Variovorax* strains and have provided a new “workhorse” Gram-negative strain, *Variovorax* sp. WS11, which is currently being characterised in detail [53,56], and is being compared to the Gram-positive *Rhodococcus* sp. AD45. In addition to poplar leaves, the phyllosphere of oil palm, one of the most prolific isoprene-producing trees, has also been explored using DNA-SIP. These experiments have thus far revealed that the main players in isoprene degradation from this environment appear to be members of the genera *Aquincola,* *Gordonia,* *Sphingomonas,* and *Zoogloea* [42,45] (Table 1).
Finally, in the marine environment, Johnston et al. [57] used DNA-SIP to study isoprene degradation in surface estuarine sediments and showed that the active isoprene-utilising bacteria in this environment were mainly Actinobacteria, including Mycobacterium, Microbacterium, Gordonia, and Rhodococcus (Table 1). However, no members of Proteobacteria were highly enriched in the $^{13}$C-labelled microbial community [57].

Table 1 summarises the main cultivation-independent studies focusing on isoprene degradation, highlighting that, although Rhodococcus is ecologically flexible and widespread across terrestrial, phyllosphere, and estuarine samples, each environment that has been examined so far has its own specific isoprene-degrading community. Moreover, the limited studies carried out to date suggest that genera other than Rhodococcus dominate the isoprene-utilising bacterial community when lower concentrations of isoprene are added (Table 1). Thus, further cultivation-independent studies exploring contrasting environments are required to expand our understanding of the diversity of isoprene degraders and, consequently, to better assess their contribution to the isoprene biogeochemical cycle. Coupling these studies with cultivation, pathway analysis, and probe refinement will further improve our understanding of isoprene degradation.

7. Outlook

Despite isoprene being released to the atmosphere in large amounts every year and having major impacts on the climate, we are only starting to understand its biogeochemical cycle, and there are several questions that need to be addressed. Firstly, more accurate estimations of isoprene production in the biosphere are required, especially in marine environments, in order to improve global models of isoprene fluxes and to predict how fluxes are going to be affected by climate change. Secondly, although the isoprene synthesis pathway in plants has been fully elucidated, the isoprene synthase from bacteria and algae is not yet characterised and should be a target for investigation in order to better assess its contribution to the global isoprene cycle.

In addition, the microbial ecology studies on isoprene degradation summarised here clearly indicate that isoprene degraders are widespread across a wide range of ecosystems, but to assess the contribution of microbes to the global isoprene biogeochemical cycle, it is essential to estimate their abundance, activity, and diversity in the environment. Cultivation-independent techniques, such as DNA-SIP and isoA probes, have been successfully applied to terrestrial, marine, freshwater, and phyllosphere samples to study the abundance and diversity of isoprene degraders. However, we cannot discard the possibility that other phylogenetically diverse isoA genes or different and novel pathways of isoprene degradation from uncultured microorganisms have been missed. To that end, the combination of DNA-SIP with other cultivation-independent techniques, such as protein-SIP [73] or Raman microspectroscopy coupled to single-cell genomics [74,75], will be key approaches in future studies to elucidate novel isoprene metabolic pathways, identify the active isoprene degraders in a microbial community, and extend the currently known diversity of isoprene-utilising bacteria. For example, protein-SIP has proven to be a successful technique not only to ratify the phylogeny of the most active microorganisms in the bacterial community revealed by DNA-SIP, but also to reconstruct complete metabolic pathways [76,77]. In addition, protein-SIP studies performed on environmental samples have allowed the identification of uncultivated bacteria and novel uncharacterised proteins involved in processes of interest, such as anaerobic methane oxidation or antibiotic degradation, which in turn can be targeted for functional biochemical studies [77,78]. Finally, protein-SIP can also inform subsequent focused proteomics studies to quantify key enzymes of metabolic processes. Such data could then be integrated into models of carbon, nitrogen, or sulfur fluxes to assess the environmental significance of a metabolic pathway of interest [77]. Similarly, Raman microspectroscopy performed with $^{13}$C-labelled substrates, alone or in combination with heavy water, is a very promising next-generation physiology approach to the link structure and function of a microbial community in its natural habitat [74,75,79,80]. One of its many advantages is that, as a rapid and non-destructive technique, Raman microspectroscopy can be combined with Raman-activated cell sorting (RACS)
to separate isotopically labelled cells from the rest of the microbial community. In turn, sorted
cells can be used for single-cell genomics or as an inoculum for cultivation, which in turn enables
in-depth studies of the physiology and biochemistry of isolates [81–83]. However, although Raman
microspectroscopy has proven to be a very powerful technique to reveal the function and interactions
of complex microbial communities in their natural environments, it still faces some challenges to
be broadly applied in microbial ecology studies. Firstly, the appropriate instrumentation required
for Raman microspectroscopy is rather expensive, and consequently is not widely available [74,79].
Secondly, cells have to be separated from the sample matrix prior to Raman analysis, which can lead to
preferential cell recovery. Therefore, the cell extraction protocol should be optimised for each specific
sample to minimise the risk of preferential recovery [84]. In addition, genome amplification from single
cells is commonly achieved by multiple displacement amplification, which can lead to, e.g., chimera
formation, erroneous nucleotide incorporation, or uneven genome coverage. However, most of these
issues can be solved with long mate-pair libraries, high sequence coverage, and post-sequencing
normalisation [75,79]. Finally, RT-qPCR assays on environmental samples using the isoA probes and
metatranscriptomic experiments [43,85,86] will be useful approaches to elucidate who are the most
active isoprene degraders in a particular environment.

There is evidence of the enrichment of particular taxa when soils are subjected to atmospherically
relevant concentrations of isoprene [39,87], and the cultivation of such bacteria might allow potentially
different catabolic pathways to be discovered, and thus broaden the base from which primers/probes
can be refined or new ones designed. In addition, there is a culture of a homoacetogen that can use
isoprene as an electron acceptor [63], and there may well be more anaerobes that can metabolise isoprene
that have evaded cultivation. Undoubtedly, such anaerobes would have catabolic pathways that are
different from aerobes and potentially distinct from, e.g., alkene-oxidising anaerobes. Therefore, the
identification of these pathways, combined with primer/probe design, would provide a means to explore
the relative contribution of anaerobic and aerobic isoprene-degrading microbes in diverse environments.

Thus, now that an improved molecular toolbox to study the ecology of isoprene degraders is
available, what is required is a systematic survey of isoprene-utilising microorganisms in contrasting
environments. Of particular interest will be soils and canopies from high isoprene-emitting trees used
as biofuel crops, such as willow, poplar, oil palm, or eucalyptus, since the large amounts of NOx and
VOCs released by large plantations of these trees could have a detrimental effect on air quality, human
health, and impede progress towards the United Nations sustainable development goals [6,16,88].
Therefore, studying the production and biological degradation of isoprene in depth in large plantations
of these crops would provide data to assess the extent to which microbes might mitigate the effects of
this climate-active gas in these regions. It will be also interesting to compare the abundance, diversity,
and activity of isoprene degraders between high and low isoprene-emitting trees to study if and
how isoprene shapes the microbial community associated with plants. Other environments that
have been reported to house isoprene-producing organisms, but remain relatively unexplored, e.g.,
marine and freshwater environments, wetlands, or mosses [33,89–91], also need to be surveyed with a
multidisciplinary approach, including flux measurements and molecular ecology techniques, to inform
global models of isoprene cycling. In particular, production and consumption should be teased apart
by using 13C-labelled isoprene [92]. Diurnal and seasonal isoprene flux measurements and transcript
analyses will provide a more complete picture of environments as isoprene sources or sinks, and how
production and consumption are influenced by environmental parameters.

Another key aspect that requires further investigation is how isoprene metabolism is regulated,
both with model isoprene degraders in the laboratory and subsequently in the environment. Although
putative transcriptional regulators within the isoprene degradation gene clusters of different isoprene
utilisers have been identified, the exact mechanisms by which they control isoprene degradation
are not known. Targeted mutagenesis and expression studies, along with reporter strains and
transcriptomic experiments will provide valuable data to identify the promoters, regulatory proteins,
and environmental conditions that regulate isoprene metabolism.
Finally, a detailed characterisation of IsoMO from representative isolates, including kinetics, affinities, substrate specificities, and the effects of inhibitors, will provide valuable information about the existence of “high affinity” and “low affinity” isoprene degraders in the environment, as is the case for other SDIMO-containing microorganisms, such as methanotrophs [93], while additionally recognising that there may be multiple pathways for isoprene oxidation. These data will also allow the comparison of the activity of IsoMO with that of related oxygenases that can co-oxidise isoprene, such as sMMO [53,94], and establish what is the contribution of these enzymes to the biological sink for isoprene.

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References

1. Atkinson, R.; Arey, J. Gas-phase tropospheric chemistry of biogenic volatile organic compounds: A review. Atmos. Environ. 2003, 37, 197–219. [CrossRef]
2. Guenther, A.B.; Jiang, X.; Heald, C.L.; Sakulyanontvittaya, T.; Duhl, T.; Emmons, L.K.; Wang, X. The model of emissions of gases and aerosols from nature version 2.1 (MEGAN2.1): An extended and updated framework for modelling biogenic emissions. Geosci. Model Dev. 2012, 5, 1471–1492. [CrossRef]
3. Paciﬁco, F.; Harrison, S.P.; Jones, C.D.; Sitch, S. Isoprene emissions and climate. Atmos. Environ. 2009, 43, 6121–6135. [CrossRef]
4. Collins, W.J.; Derwent, R.G.; Johnson, C.E.; Stevenson, D.S. The oxidation of organic compounds in the troposphere and their global warming potentials. Clim. Chang. 2002, 52, 453–479. [CrossRef]
5. Folberth, G.A.; Hauglustaine, D.A.; Lathière, J.; Brocheton, F. Interactive chemistry in the Laboratoire de Météorologie Dynamique general circulation model: Model description and impact analysis of biogenic hydrocarbons on tropospheric chemistry. Atmos. Chem. Phys. 2006, 6, 2273–2319. [CrossRef]
6. Ashworth, K.; Wild, O.; Hewitt, C.N. Impacts of biofuel cultivation on mortality and crop yields. Nat. Clim. Change. 2013, 3, 492–496. [CrossRef]
7. Carlton, A.G.; Wiedinmyer, C.; Kroll, J.H. A review of secondary organic aerosol (SOA) formation from isoprene. Atmos. Chem. Phys. 2009, 9, 4987–5005. [CrossRef]
8. Engelhart, G.J.; Moore, R.H.; Nenes, A.; Pandis, S.N. Cloud condensation nuclei activity of isoprene secondary organic aerosol. J. Geophys. Res. 2011, 116, D02207. [CrossRef]
9. Sharkey, T.D.; Wiberley, A.E.; Donohue, A.R. Isoprene emission from plants: Why and how. Ann. Bot. 2008, 101, 5–18. [CrossRef]
10. Loreto, F.; Ciccioli, P.; Brancaleoni, E.; Valentini, R.; De Lillis, M.; Csiky, O.; Seufert, G. A hypothesis on the evolution of isoprenoid emission by oaks based on the correlation between emission type and Quercus taxonomy. Oecologia 1998, 115, 17430–17435.
11. Monson, R.K.; Jones, R.T.; Rosenstiel, T.N.; Schnitzler, J.P. Why only some plants emit isoprene. Plant Cell Environ. 2013, 36, 503–516. [CrossRef] [PubMed]
12. Sharkey, T.D. Is it useful to ask why plants emit isoprene? Plant Cell Environ. 2013, 36, 517–520. [CrossRef] [PubMed]
13. Rohmer, M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat. Prod. Rep. 1999, 16, 565–574. [CrossRef] [PubMed]
14. Sharkey, T.D.; Singsaaas, E.L.; Vanderveer, P.J.; Geron, C. Field measurements of isoprene emission from trees in response to temperature and light. Tree Physiol. 1996, 16, 649–654. [CrossRef]
15. Lantz, A.T.; Allman, J.; Weraduwage, S.M.; Sharkey, T.D. Isoprene: New insights into the control of emission and mediation of stress tolerance by gene expression. Plant Cell Environ. 2019, 42, 2808–2826. [CrossRef]
16. Hewitt, C.N.; MacKenzie, A.R.; Di Carlo, P.; Di Marco, C.F.; Dorsey, J.R.; Evans, M.; Fowler, D.; Gallagher, M.W.; Hopkins, J.R.; Jones, C.E.; et al. Nitrogen management is essential to prevent tropical oil palm plantations from causing ground-level ozone pollution. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18447–18451. [CrossRef]

17. Monson, R.K.; Winkler, B.; Rosenstiel, T.N.; Block, K.; Merl-Pham, J.; Strauss, S.H.; Ault, K.; Maxfield, J.; Moore, D.J.P.; Trahan, N.A.; et al. High productivity in hybrid poplar plantations without isoprene emission to the atmosphere. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 1596–1605. [CrossRef]

18. Bäck, J.; Aaltonen, H.; Helén, H.; Kajos, M.K.; Patokoski, J.; Taipale, R.; Pumpajan, J.; Heinonsalo, J. Variable emissions of microbial volatile organic compounds (MVOCs) from root-associated fungi isolated from Scots pine. *Atmosph. Environ.* **2010**, *44*, 3651–3659. [CrossRef]

19. Dani, S.K.G.; Benavides, A.M.S.; Michelozzi, M.; Peluso, G.; Torzillo, G.; Loreto, F. Relationship between isoprene emission and photosynthesis in diatoms, and its implications for global marine isoprene estimates. *Mar. Chem.* **2017**, *189*, 17–24. [CrossRef]

20. Exton, D.A.; McGenity, T.J.; Steinke, M.; Smith, D.J.; Suggett, D.J. Uncovering the volatile nature of tropical coastal marine ecosystems in a changing world. *Glob. Chang. Biol.* **2015**, *21*, 1383–1394. [CrossRef] [PubMed]

21. Fall, R.; Copley, S.D. Bacterial sources and sinks of isoprene, a reactive atmospheric hydrocarbon. *Environ. Microbiol.* **2000**, *2*, 123–130. [CrossRef] [PubMed]

22. Gelmont, D.; Stein, R.A.; Mead, J.F. Isoprene—the main hydrocarbon in human breath. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1456–1460. [CrossRef]

23. Kuzma, J.; Nemecek-Marshall, M.; Pollock, W.; Fall, R. Bacteria produce the volatile hydrocarbon isoprene. *Curr. Microbiol.* **1995**, *30*, 97–103. [CrossRef] [PubMed]

24. Schöller, C.; Molin, S.; Wilkins, K. Volatile metabolites from some Gram-negative bacteria. *Chemosphere* **1997**, *35*, 1487–1495. [CrossRef]

25. Schöller, C.E.G.; Gürtl, H.; Pederson, R.; Molin, S.; Wilkins, K. Volatile metabolites from Actinomycetes. *J. Agric. Food Chem.* **2002**, *50*, 2615–2621. [CrossRef]

26. McGinity, T.J.; Crombie, A.T.; Murrell, J.C. Microbial cycling of isoprene, the most abundantly produced biological volatile organic compound on Earth. *ISME J.* **2018**, *12*, 931–941. [CrossRef]

27. Dani, S.K.G.; Loreto, F. Trade-off between dimethyl sulfide and isoprene emissions from marine phytoplankton. *Trends Plant Sci.* **2017**, *22*, 361–372. [CrossRef]

28. Shaw, S.L.; Gantt, B.; Meskhidze, N. Production and emissions of marine isoprene and monoterpenes: A review. *Adv. Meteorol.* **2010**, [CrossRef]

29. Exton, D.A.; McGinity, T.J.; Steinke, M.; McGinity, T.J. Spatial and temporal variability of biogenic isoprene emissions from a temperate estuary. *Glob. Biogeochim. Cycl.* **2012**, *26*, GB2012. [CrossRef]

30. Hackenberg, S.C.; Andrews, S.J.; Airs, R.; Arnold, S.R.; Brewin, R.J.W.; Chance, R.J.; Cummings, D.; Dall’Omo, G.; Lewis, A.C.; et al. Potential controls of isoprene in the surface ocean. *Glob. Biogeochim. Cycles* **2017**, *31*, 644–662. [CrossRef]

31. Meskhidze, N.; Sabolis, A.; Reed, R.; Kamykowski, D. Quantifying environmental stress-induced emissions of algal isoprene and monoterpenes using laboratory measurements. *Biogeosciences* **2015**, *12*, 637–651. [CrossRef]

32. Ekberg, A.; Arneth, A.; Hakota, H.; Hayward, S.; Holst, T. Isoprene emission from wetland sedges. *Biogeosciences* **2009**, *6*, 601–613. [CrossRef]

33. Steinke, M.; Hodapp, B.; Subhan, R.; Bell, T.G.; Martin-Creuzburg, D. Flux of the biogenic volatiles isoprene and dimethyl sulfide from an oligotrophic lake. *Sci. Rep.* **2018**, *8*, 630. [CrossRef] [PubMed]

34. Morais, A.R.C.; Dworakowska, S.; Reis, A.; Gouveia, L.; Matos, C.T.; Bogdal, D.; Bogel-Lukasik, R. Chemical and biological-based isoprene production: Green metrics. *Catal. Today* **2015**, *239*, 38–43. [CrossRef]

35. Borbon, A.; Fontaine, H.; Veillerot, M.; Locoige, N.; Galloo, J.C.; Guillermo, R. An investigation into the traffic-related fraction of isoprene at an urban location. *Atmos. Environ.* **2001**, *35*, 3749–3760. [CrossRef]

36. Chen, J.; Li, C.; Ristovski, Z.; Milic, A.; Gu, Y.; Islam, M.S.; Whang, S.; Hao, J.; Zhang, H.; He, C.; et al. A review of biomass burning: Emissions and impacts on air quality, health and climate in China. *Sci. Total Environ.* **2017**, *579*, 1000–1034. [CrossRef]

37. Cleveland, C.C.; Yavitt, J.B. Consumption of atmospheric isoprene in soil. *Geophys. Res. Lett.* **1997**, *24*, 2379–2382. [CrossRef]

38. Cleveland, C.C.; Yavitt, J.B. Microbial consumption of atmospheric isoprene in a temperate forest soil. *Appl. Environ. Microbiol.* **1998**, *64*, 172–177. [CrossRef]
39. Gray, C.M.; Helmig, D.; Fierer, N. Bacteria and fungi associated with isoprene consumption in soil. *Elem. Sci. Anth.* 2015, 3, 000053. [CrossRef]

40. Pegoraro, E.; Abrell, L.; Van Haren, J.; Barron-Gafford, G.; Grieve, K.A.; Yadavinder, M.; Murthy, R.; Lin, G. The effect of elevated atmospheric CO$_2$ and drought on sources and sinks of isoprene in a temperate and tropical rainforest mesocosm. *Cloth. Chang. Biol.* 2005, 11, 1234–1246. [CrossRef]

41. Acuña Alvarez, L.; Exton, D.A.; Timmis, K.N.; Suggett, D.J.; McGenity, T.J. Characterization of marine isoprene-degrading communities. *Environ. Microbiol.* 2009, 11, 3280–3291. [CrossRef] [PubMed]

42. Carrió, O.; Gibson, L.; Elias, D.M.O.; McNamara, N.P.; Van Alen, T.A.; Op den Camp, H.J.M.; Supramaniam, C.V.; McGenity, T.J.; Murrell, J.C. Diversity of isoprene-degrading bacteria in phyllosphere and soil communities from a high isoprene-emitting environment: A Malaysian oil palm plantation. *Microbiome* 2020, 8, 81. [CrossRef] [PubMed]

43. Crombie, A.T.; Larke-Mejia, N.L.; Emery, H.; Dawson, R.; Pratscher, J.; Murphy, G.P.; McGenity, T.J.; Murrell, J.C. Poplar phyllosphere harbors disparate isoprene-degrading bacteria. *Proc. Natl. Acad. Sci. USA* 2018, 115, 13081–13086. [CrossRef] [PubMed]

44. Gibson, L.; Crombie, A.T.; Emery, H.; McGenity, T.J.; Murrell, J.C. Community profiling of isoprene-degrading bacteria associated with the phyllosphere of a Salix tree. Manuscript in preparation.

45. Larke-Mejia, N.L.; Carrió, O.; Crombie, A.T.; McGenity, T.J.; Murrell, J.C. *Sphingopyxis* sp. OPL5, an isoprene-degrading bacterium from the Sphingomonadaceae family isolated from oil palm leaves. Manuscript in preparation.

46. Ewers, J.; Freier-Schroder, D.; Knockmuss, H.J. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. *Arch. Microbiol.* 1990, 154, 410–413. [CrossRef]

47. Van Ginkel, C.G.; de Jong, E.; Tilanus, J.W.R.; de Bont, J.A.M. Microbial oxidation of isoprene, a biogenic foliage volatile and of 1,3-butadiene, an anthropogenic gas. *FEMS Microbiol. Lett.* 1987, 45, 275–279. [CrossRef]

48. Van Ginkel, C.G.; Welten, H.G.I.; de Bont, J.A.M. Oxidation of gaseous and volatile hydrocarbons by selected alkene-utilizing bacteria. *Appl. Environ. Microb.* 1987, 53, 2903–2907. [CrossRef]

49. Srivastava, N.; Shukla, A.K.; Singh, R.S.; Upadhyay, S.N.; Dubey, S.K. Characterisation of bacterial isolates from rubber dump site and their use in biodegradation of isoprene in batch and continuous bioreactors. *Bioresour. Technol.* 2015, 188, 84–91. [CrossRef]

50. Singh, A.; Srivastava, N.; Dubey, S.K. Molecular characterization and kinetics of isoprene degrading bacteria. *Bioresour. Technol.* 2019, 278, 51–56. [CrossRef]

51. El Khawand, M.; Crombie, A.T.; Johnston, A.; Vavlline, D.V.; McAuliffe, J.C.; Latone, J.A.; Primak, Y.A.; Lee, S.K.; White, G.M.; McGenity, T.J.; et al. Isolation of isoprene degrading bacteria from soils, development of isoA gene probes and identification of the active isoprene-degrading soil community using DNA-stable isotope probing. *Environ. Microbiol.* 2016, 18, 2743–2753. [CrossRef] [PubMed]

52. Crombie, A.T.; Emery, H.; McGenity, T.J.; Murrell, J.C. Draft genome sequences of three terrestrial isoprene-degrading *Rhopococcus* strains. *Genome Announc.* 2017, 5, e01256-17. [CrossRef] [PubMed]

53. Dawson, R.A.; Larke-Mejia, N.L.; Crombie, A.T.; Ul Haque, M.F.; Murrell, J.C. Isoprene oxidation by the Gram-negative model bacterium *Variovorax* sp. WS11. *Microorganisms* 2020, 8, 349. [CrossRef] [PubMed]

54. Gibson, L.; Larke-Mejia, N.L.; Murrell, J.C. Complete genome of isoprene degrading *Nocardioides* sp. WS12. *Microorganisms* 2020, 8, 889. [CrossRef]

55. Larke-Mejia, N.L. Microbial Ecology of Isoprene degraders in the Terrestrial Environment. Ph.D. Thesis, University of East Anglia, Norwich, UK, 2018.

56. Larke-Mejia, N.L.; Crombie, A.T.; Pratscher, J.; McGenity, T.J.; Murrell, J.C. Novel isoprene-degrading *Proteobacteria* from soil and leaves identified by cultivation and metagenomics analysis of stable isotope probing experiments. *Front. Microbiol.* 2019, 10, 2700. [CrossRef]

57. Johnston, A.; Crombie, A.T.; El Khawand, E.; Sims, L.; White, G.M.; McGenity, T.J.; Murrell, J.C. Identification and characterisation of isoprene-degrading bacteria in an estuarine environment. *Environ. Microbiol.* 2017, 19, 3526–3537. [CrossRef]

58. Van Hylckama Vlieg, J.E.T.; Kingma, J.; van den Wijngaard, A.J.; Janssen, D.B. A glutathione S-transferase with activity towards cis-1, 2-dichloroepoxyethane is involved in isoprene utilization by *Rhodococcus* sp. strain AD45. *Appl. Environ. Microbiol.* 1998, 64, 2800–2805. [CrossRef]
59. Van Hylckama Vlieg, J.E.T.; Kingma, J.; Kruizinga, W.; Janssen, D.B. Purification of a glutathione S-transferase and a conjugate-specific dehydrogenase involved in isoprene metabolism in *Rhodococcus* sp. strain AD45. *J. Bacteriol.* 1999, 181, 2094–2101. [CrossRef]

60. Van Hylckama Vlieg, J.E.T.; Leemhuis, H.; Spellberg, J.H.L.; Janssen, D.B. Characterization of the gene cluster involved in isoprene metabolism in *Rhodococcus* sp. strain AD45. *J. Bacteriol.* 2000, 182, 1956–1963. [CrossRef]

61. McDonald, I.R.; Bodrossy, L.; Chen, Y.; Murrell, J.C. Molecular ecology techniques for the study of aerobic methanotrophs. *FEMS Microbiol. Rev.* 2003, 27, 449–479. [CrossRef]

62. McDonald, I.R.; Kenna, E.M.; Murrell, J.C. Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl. Environ. Microbiol.* 1995, 61, 116–121. [CrossRef]

63. Kronen, M.; Lee, M.; Jones, Z.L.; Manefield, M.J. Reductive metabolism of the important atmospheric gas sulfur. *Microbiology 2020*. [CrossRef]

64. Leahy, J.G.; Batchelor, P.J.; Morcomb, S.M. Evolution of the soluble diiron monooxygenases. *Methods Enzymol.* 2005, 397, 413–427. [PubMed]

65. Dumont, M.G.; Murrell, J.C. Community-level analysis: Key genes of aerobic methane oxidation. *Appl. Environ. Microbiol.* 2008, 74, 1305–1315. [CrossRef]

66. McDonald, I.R.; Kenna, E.M.; Murrell, J.C. Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl. Environ. Microbiol.* 1995, 61, 116–121. [CrossRef]

67. McDonald, I.R.; Bodrossy, L.; Chen, Y.; Murrell, J.C. Molecular ecology techniques for the study of aerobic methanotrophs. *Appl. Environ. Microbiol.* 2008, 74, 1305–1315. [CrossRef]

68. Farhan Ul Haque, M.; Crombie, A.T.; Ensminger, S.A.; Baciu, C.; Murrell, J.C. Facultative methanotrophs are abundant at terrestrial natural gas seeps. *Microbiome* 2018, 6, 118. [CrossRef]

69. Carrió, O.; Larke-Mejía, N.L.; Gibson, L.; Farhan Ul Haque, M.; Ramiro-García, J.; McGenity, T.J.; Murrell, J.C. Gene probing reveals the widespread distribution, diversity and abundance of isoprene-degrading bacteria in the environment. *Microbiome* 2018, 6, 219. [CrossRef]

70. Kesselmeier, J.; Staudt, M. Biogenic volatile organic compounds (VOC): An overview on emission, physiology and ecology. *J. Atmos. Chem.* 2003, 43, 23–88. [CrossRef]

71. Radajewski, S.; Ineson, P.; Parekh, N.R.; Murrell, J.C. Stable-isotope probing as a tool in microbial ecology. *Nature* 2000, 403, 646–649. [CrossRef]

72. Neufeld, J.D.; Vohra, J.; Dumont, M.G.; Lueders, T.; Manefield, M.; Friedrich, M.W.; Murrell, J.C. DNA stable-isotope probing. *Nat. Protoc.* 2007, 2, 860–866. [CrossRef] [PubMed]

73. Jehmlich, N.; Schmidt, F.; Taubert, M.; Seifert, J.; Bastida, F.; von Bergen, M.; Richnow, H.H.; Vogt, C. Protein-based stable isotope probing. *Nat. Protoc.* 2010, 5, 1957–1966. [CrossRef] [PubMed]

74. Wagner, M. Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. *Curr. Opin. Biotechnol.* 2016, 41, 34–42. [CrossRef] [PubMed]

75. Wang, Y.; Huang, W.E.; Cui, L.; Wagner, M. Single cell stable isotope probing in microbiology using Raman microspectroscopy. *Curr. Opinion. Biotechnol.* 2016, 41, 26–33.

76. Jehmlich, N.; Vogt, C.; Lüsmann, V.; Richnow, H.H.; von Bergen, M. Protein-SIP in environmental studies. *Curr. Opinion. Biotechnol.* 2016, 41, 26–33.

77. Lüsmann, V.; Kappelmeyer, U.; Benndorf, R.; Martinez-Lavanchy, P.M.; Taubert, A.; Adrian, L.; Duarte, M.; Pieper, D.H.; von Bergen, M.; Müller, J.A.; et al. In situ protein-SIP highlights *Burkholderiaceae* as key players degrading toluene by para ring hydroxylation in a constructed wetland model. *Environ. Microbiol.* 2016, 18, 1176–1186. [CrossRef]

78. Ouyang, W.Y.; Su, J.Q.; Richnow, H.H.; Adrian, L. Identification of dominant sulfamethoxazole-degraders in pig farm-impacted soil by DNA and protein stable isotope probing. *Environ. Int.* 2019, 126, 118–126. [CrossRef]

79. Hatzenpichler, R.; Krukenberg, V.; Spietz, R.L.; Jay, Z.J. Next-generation physiology approaches to study microbiome function at single cell level. *Nat. Rev. Microbiol.* 2020, 18, 241–256. [CrossRef]

80. Singer, E.; Wagner, M.; Woyke, T. Capturing the genetic makeup of the active microbiome in situ. *ISME J.* 2017, 11, 1949–1963. [CrossRef]

81. Berry, D.; Mader, E.; Lee, T.K.; Woebken, D.; Wang, Y.; Zhu, D.; Palatinszky, M.; Schindlmeister, A.; Schmid, M.C.; Hanson, B.T.; et al. Tracking heavy water (D₂O) incorporation for identifying and sorting active microbial cells. *Proc. Natl. Acad. Sci. USA* 2015, 112, 194–203. [CrossRef]
82. Huang, W.E.; Ward, A.D.; Whiteley, A.S. Raman tweezers sorting of single microbial cells. *Environ. Microbiol.* 2009, 1, 44–49. [CrossRef] [PubMed]

83. Jing, X.; Gou, H.; Gong, Y.; Su, X.; Xu, L.; Yuetong, J.; Song, Y.; Thompson, I.P.; Xu, J.; Huang, W.E. Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean. *Environ. Microbiol.* 2018, 20, 2241–2255. [CrossRef] [PubMed]

84. Eichorst, S.A.; Strasser, F.; Woyke, T.; Schintlmeister, A.; Wagner, M.; Woebken, D. Advancements in the application of NanoSIMS and Raman microspectroscopy to investigate the activity of microbial cells in soils. *FEMS Microbiol. Ecol.* 2015. [CrossRef] [PubMed]

85. Dumont, M.G.; Pommerenke, B.; Casper, P. Using stable isotope probing to obtain a targeted metatranscriptome of aerobic methanotrophs in lake sediment. *Environ. Microbiol. Rep.* 2013, 5, 757–764. [CrossRef]

86. Fortunato, C.S.; Huber, J.A. Coupled RNA-SIP and metatranscriptomics of active chemolithoautotrophic communities at a deep-sea hydrothermal vent. *ISME J.* 2015, 10, 1925–1938. [CrossRef]

87. Murphy, G.P.; Uttarotai, T.; Crombie, A.T.; Lawson, T.; Chitov, T.; Murrell, J.C.; Steinke, M.; McGenity, T.J. Soil as a sink for isoprene: The effects of isoprene concentration on consumption, and the identification of isoprene-degrading bacteria. Manuscript in preparation.

88. Griggs, D.; Stafford-Smith, M.; Gaffney, O.; Rockström, J.; Ohman, M.C.; Shyamsundar, P.; Steffen, W.; Glaser, G.; Kanie, N.; Noble, I. Sustainable development goals for people and planet. *Nature* 2013, 495, 305–307. [CrossRef]

89. Janson, R.; De Serves, C. Isoprene emissions from boreal wetlands in Scandinavia. *J. Geophys. Res.* 1998, 103, 513–517. [CrossRef]

90. Hanson, D.T.; Swanson, S.; Graham, L.E.; Sharkey, T.D. Evolutionary significance of isoprene emission from mosses. *Am. J. Bot.* 1999, 86, 634–639. [CrossRef]

91. Lindwall, F.; Svendsen, S.S.; Nielsen, C.S.; Michelsen, A.; Rinnan, R. Warming increases isoprene emissions from an arctic fen. *Sci. Total Environ.* 2016, 553, 297–304. [CrossRef]

92. Rinnan, R.; Albers, C. Soil uptake of volatile organic compounds: Ubiquitous and underestimated? *JGR Biogeosciences* 2020. [CrossRef]

93. Bender, M.; Conrad, R. Kinetics of CH4 oxidation in oxic soils exposed to ambient air or high CH4 mixing ratios. *FEMS Microbiol. Lett.* 1992, 101, 261–269. [CrossRef]

94. Crombie, A.T. Metabolism of Methane and Propane and the Role of the Glyoxylate Bypass Enzymes in *Methylocella silvestris* BL2. Ph.D. Thesis, University of Warwick, Coventry, UK, 2011.