FEMS Microbiology Ecology, 2024, **100**, fiae132
DOI: 10.1093/femsec/fiae132
Advance access publication date: 26 September 2024
Minireview

Widespread occurrence of dissolved oxygen anomalies, aerobic microbes, and oxygen-producing metabolic pathways in apparently anoxic environments

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Editor: [Marcus Horn]

Abstract

Nearly all molecular oxygen (O_2) on Earth is produced via oxygenic photosynthesis by plants or photosynthetically active microorganisms. Light-independent O_2 production, which occurs both abiotically, e.g. through water radiolysis, or biotically, e.g. through the dismutation of nitric oxide or chlorite, has been thought to be negligible to the Earth system. However, recent work indicates that O_2 is produced and consumed in dark and apparently anoxic environments at a much larger scale than assumed. Studies have shown that isotopically light O_2 can accumulate in old groundwaters, that strictly aerobic microorganisms are present in many apparently anoxic habitats, and that microbes and metabolisms that can produce O_2 without light are widespread and abundant in diverse ecosystems. Analysis of published metagenomic data reveals that the enzyme putatively capable of nitric oxide dismutation forms four major phylogenetic clusters and occurs in at least 16 bacterial phyla, most notably the *Bacteroidota*. Similarly, a re-analysis of published isotopic signatures of dissolved O_2 in groundwater suggests *in situ* production in up to half of the studied environments. Geochemical and microbiological data support the conclusion that "dark oxygen production" is an important and widespread yet overlooked process in apparently anoxic environments with far-reaching implications for subsurface biogeochemistry and ecology.

Keywords: dark oxygen production; chlorite dismutation; nitric oxide dismutation; subsurface microbiome; hypoxia; cryptic O_2 cycling

Principles of light-independent oxygen production and consumption

By far the most important source of molecular oxygen (O_2) on Earth is photosynthesis, a biotic process that generates O_2 as a byproduct through the lysis of water molecules (Nelson and Ben-Shem 2004, Fischer et al. 2016). Despite the quantitative importance of photosynthesis, O_2 is additionally produced by light-independent abiotic and biotic reactions. Here, we refer to all light-independent production pathways, whether biotic or abiotic, as "dark oxygen production" (DOP; Fig. 1).

Abiotic DOP specifically proceeds via the radiolysis of water (Gutsalo 1971, Das 2013), which is driven in dark geological systems such as aquifers and bedrock by the decay of radioactive elements present in surrounding rock, and via the consumption of surface-bound radicals on Si-bearing minerals such as quartz (He et al. 2021, 2023, Stone et al. 2022). Although direct O_2 formation is likely negligible (Le Caër 2011), both pathways produce abundant reactive oxygen species (ROS) such as hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2). ROS can be subsequently disproportionated biotically by the enzymes superoxide dismutase and catalase—or abiotically by ferrous iron and other reduced metals—to form O_2 and H_2O (Xu et al. 2013, Sutherland et al. 2022). However, surface-bound radical generation requires either intense mechanical abrasion (He et al. 2021, 2023) or hydrothermal temperatures (Stone et al. 2022). Thus, while possibly important earlier in Earth's history, this mechanism is likely insignificant in most low-temperature, anoxic systems today. In contrast, water radiolysis followed by ROS disproportionation can act as a net source of O_2 to groundwater systems over geologic timescales (i.e. the timescale of radioactive decay). It was recently proposed that molecular oxygen may also be produced via the electrolysis of seawater at polymetallic nodules in the deep ocean, yet the details of this reaction are so far unknown (Sweetman et al. 2024).

Biotic DOP is carried out by microorganisms belonging to several different and generally well-known lineages within the *Archaea* and *Bacteria*. Microbial DOP proceeds via three fundamentally different microbial processes: chlorite (ClO_2^-) dismutation (Xu and Logan 2003), nitric oxide (NO) dismutation (Ettwig et al. 2012), and the lysis of water via methanobactins (Dershwitz et al. 2021). Despite this variety of microbial metabolisms producing O_2, these processes were all reported relatively recently: chlorite dismutation in the mid-1990s (van Ginkel et al. 1996), NO dismutation in 2010 (Ettwig et al. 2010), and water lysis by

Received 15 May 2024; **revised** 23 July 2024; **accepted** 25 September 2024

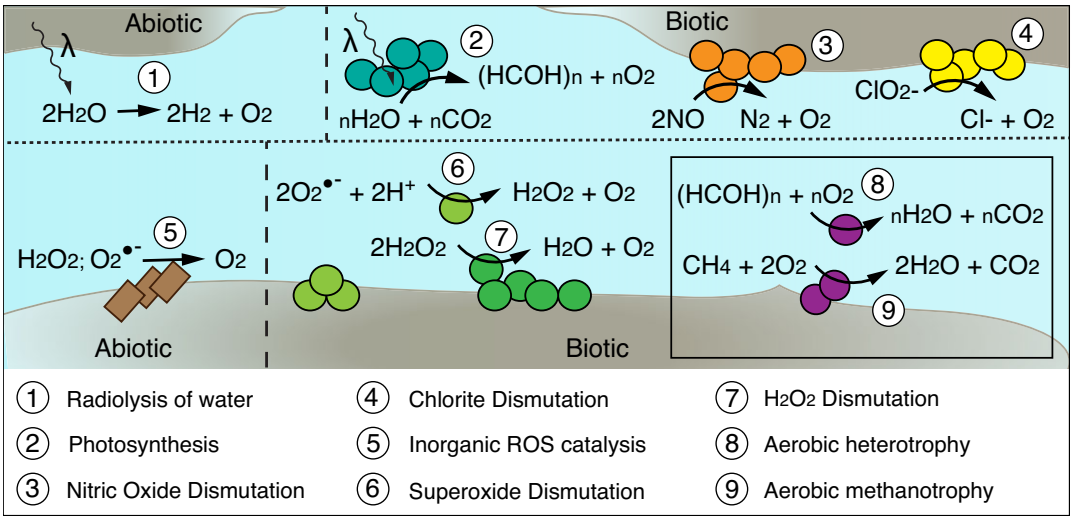

Figure 1. Processes involved in the production, recycling, and consumption of O_2 in modern environments. Reactions responsible for net O_2 production (reactions 1–4) are shown above, and those that recycle or consume O_2 (reactions 5–9) are below the dotted line. Abiotic reactions are shown to the left and biotic reactions to the right of the dashed lines. Note: intermediate reactions and electrons are not shown. This overview highlights the reactions reviewed here and is not exhaustive, e.g. most abiotic and biotic O_2-consuming redox reactions are not shown.

methanobactins only three years ago (Dershwitz et al. 2021). In both dismutation pathways, the crucial O_2-producing step is the electron-neutral dismutation of ClO_2^- or NO into O_2 and chloride (Cl^-) or O_2 and dinitrogen gas (N_2) or nitrous oxide (N_2O). Chlorite dismutase (CLD) and proposed nitric oxide dismutase (NOD) enzymes are both heme-containing oxidoreductases (Hofbauer et al. 2014, Murali et al. 2022), yet they are unrelated belonging to distant protein families. In contrast, the molecular mechanism for methanobactin-dependent lysis of water remains unclear to date (Dershwitz et al. 2021), and, unlike for both dismutation pathways, the boundary conditions for the energetic feasibility of methanobactin-dependent water lysis do not suggest a substantial occurrence in natural ecosystems.

Balancing the O_2 sources, the most important sink for O_2 is respiration. The reduction of O_2 provides the largest free energy release per electron transfer, with the exception of fluorine and chlorine, and is thus a powerful electron acceptor (Catling et al. 2005, Jørgensen 2006). Aerobic respiration is therefore widespread in the tree of life. Many single-celled eukaryotes (Zimorski et al. 2019), ∼70% of bacteria (Morris and Schmidt 2013), and essentially all macroscopic life forms respire O_2 (Hedges et al. 2004). However, in recent years, it is becoming increasingly recognized that many aerobes occur in ecosystems in which O_2 does not seem to be available or at least does not accumulate. For example, strictly aerobic methanotrophs are reported to be active in apparently anoxic sediments of hydrocarbon seeps (Ruff 2020) and wetlands (Reis et al. 2024). The presence of aerobes in such environments has historically been explained either by (i) their capability to exist in a facultative anaerobic lifestyle, (ii) their capability to remain dormant during unfavorable conditions, or (iii) using small amounts of oxygen that become available through advection, storage, or diffusion, or that are produced via photosynthesis. Indeed, recently developed dissolved O_2 sensors reveal that nanomolar (i.e. $\sim 10^{-9}$ mol l^{-1}) concentrations of O_2 are common in nature, leading to the presence of nanaerobic microbes in many apparently anoxic environments (Berg et al. 2022). In this review, we compile and provide evidence for a fourth, largely overlooked mechanism by which aerobic microbes can survive in apparently anoxic environments even when sunlight is not available, advection is not present, and diffusion from oxic systems is too slow: *in situ* DOP.

Biochemistry and physiology of microbial light-independent oxygen production

Of the enzymes involved in microbial DOP, CLD is the best understood. CLD is a relatively small heme-dependent enzyme (∼120 Da) that consists of two (Celis et al. 2015) or four identical subunits (van Ginkel et al. 1996, Mehboob et al. 2009). It is highly specific for the dismutation of chlorite to chloride and O_2 with no other side products (Lee et al. 2008). The organisms capable of chlorite dismutation are specialized on the reductive dissimilation of (per)chlorate into chloride, with chlorite being a strongly oxidative and toxic intermediate (Xu and Logan 2003, Coates and Achenbach 2004). Although being facultative anaerobes, some of these organisms can use the generated O_2 for aerobic degradation of organics (Carlström et al. 2015). The details concerning the biochemical properties of CLD and the physiology of (per)chlorate reducers are thoroughly discussed and reviewed elsewhere (Coates and Achenbach 2004, Mlynek et al. 2011, Hofbauer et al. 2014, Schaffner et al. 2015, 2017) and are not the focus of this review.

Less is known about the inner workings of the enzymes involved in NO dismutation because the putative NOD has not been biochemically or structurally characterized. A putative NOD was first postulated as a key enzyme in the nitrite-driven anaerobic oxidation of methane performed by *Candidatus* Methylomirabilis oxyfera (*Ca.* M. oxyfera; Ettwig et al. 2010). While living in anoxic ecosystems, these organisms generate oxygen internally from nitrite—a process that involves NOD—then use the generated O_2 to oxidize methane via an aerobic metabolic pathway (Wu et al. 2011). It seems clear that the putative NOD evolved from the closely related quinol-dependent nitric oxide reductase (qNOR). Both enzymes belong to the heme-copper oxidoreductase (HCO) superfamily and differ in only few amino acid residues (Ettwig et al. 2012, Murali et al. 2022). NOD seems to have lost a quinol-binding site and thus cannot take up external electrons (Ettwig et al. 2012), indicating its adaptation for a purpose different from its ancestral scaffold.

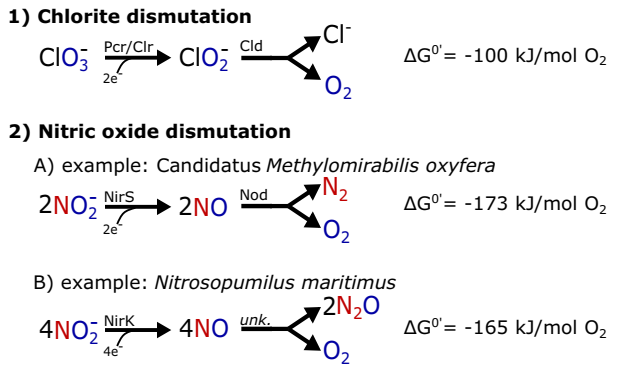

Figure 2. Overview of the different microbial DOP metabolisms. Reported Gibbs free energies (ΔG) only refer to the O_2-generating step of each process. (1) Chlorite dismutation (Mehboob et al. 2009). (2a) NO dismutation to N_2 and O_2 (Ettwig et al. 2010). (2b) NO dismutation to N_2O and O_2 (Kraft et al. 2022). Pcr: perchlorate reductase, Clr: chlorate reductase, Cld: chlorite dismutase, NirS: Fe-nitrite reductase, Nod: NO-dismutase, NirK: Cu-nitrite reductase, unk: unknown.

In pure cultures, O_2 generation coupled to N_2 or N_2O production from nitrite is indicative of NO dismutation. As O_2 production and consumption may be tightly coupled, inhibitors of the O_2-consuming processes (e.g. respiration and ammonia or methane oxidation) may be required to observe oxygen accumulation (Ettwig et al. 2010, Kraft et al. 2022). In most cases, the ability of microorganisms—including the ammonia-oxidizing archaeon *Nitrosopumilus maritimus* (Kraft et al. 2022) and *Pseudomonas aeruginosa* (Lichtenberg et al. 2021)—to produce O_2 from nitrite or NO was shown via ^{15}N-isotope labeling combined with low-concentration O_2 measurements. However, neither the genome of *N. maritimus* nor of *P. aeruginosa* encodes a *nod* gene, and the enzyme that is responsible for catalyzing NO dismutation in these organisms remains to be discovered. The presence of two distinct pathways to dismutate NO, which involve different key enzymes, indicates that the capability of NO-dismutation evolved independently at least twice.

Several key differences exist in the physiologies of the NO dismutation pathways of *Ca.* M. oxyfera, *N. maritimus*, and *P. aeruginosa*. Firstly, *Ca.* M. oxyfera consumes all O_2 that it produces directly for its own metabolism, whereas O_2 accumulates up to concentrations of several hundred nanomoles per liter in *N. maritimus* cultures (Kraft et al. 2022). Secondly, although both pathways reduce nitrite to NO that is then dismutated, *Ca.* M. oxyfera dismutates NO to O_2 and N_2 directly, whereas *N. maritimus* dismutates NO to O_2 and N_2O that is subsequently reduced to N_2 (Fig. 2). Thirdly, *P. aeruginosa* cultures produce a short-lived O_2 peak that occurs immediately after O_2 is depleted through respiration. This peak transiently exceeds O_2 concentrations accumulated by *N. maritimus*. The products, stoichiometry, and responsible enzyme remain to be resolved.

In summary, while O_2 is produced in all NO dismutation pathways, the byproducts differ. One implication is that ammonia-oxidizing archaea (AOA) produce half the amount of O_2 per nitrite consumed as compared to *Ca.* M. oxyfera. In environments in which nitrite is limiting and NO-dismutating microbes compete with other nitrite-consuming microbes, such as denitrifiers, this could constitute a substantial disadvantage. Finally, recent research indicates that the number of potential dismutation substrates discovered to date may not be exhausted. For example, it was suggested that novel *Methylomirabilota* methanotrophs potentially couple methane oxidation to iodate reduction via the production of O_2 from hypoiodous acid (Zhu et al. 2022). Future work is therefore needed to explore additional possible dismutating metabolisms.

Diversity and phylogeny of *nod* genes and of organisms potentially mediating DOP

Organisms that have the metabolic capabilities to dismutate chlorite or NO are found across the tree of life. For example, it has long been known that CLD is widespread in *Pseudomonas* sp. (Mehboob et al. 2009), *Nitrospira* sp. (Maixner et al. 2008), *Nitrobacter* sp. (Mlynek et al. 2011), *Klebsiella* sp. (Celis et al. 2015), as well as in all (per)chlorate respiring organisms such as *Dechloromonas* sp. and *Dechlorosoma* sp. (Coates et al. 1999, Achenbach et al. 2001). Furthermore, a recent publication showed the presence of *cld* genes in organisms affiliated with >60 genera within 13 phyla, mostly among *Proteobacteria* (Barnum and Coates 2023).

The list of organisms that are known to contain a putative *nod* gene is not quite as extensive as that for *cld* yet has rapidly expanded in recent years. In particular, there are at least five *Candidatus* species within the *Methylomirabilota* that contain *nod* genes: *Ca.* M. oxyfera, *Ca.* M. lanthaniphila, *Ca.* M. sinica, *Ca.* M. limnetica, and *Ca.* M. iodofontis (Ettwig et al. 2010, He et al. 2016, Graf et al. 2018, Versantvoort et al. 2018, Zhu et al. 2022). In addition to *Methylomirabilota*, *nod* genes were detected in *Sediminibacterium* sp., *Algoriphagus* sp., and *Muricauda* sp.—all within the phylum *Bacteroidota* (Ettwig et al. 2012, Murali et al. 2022, Ruff et al. 2023)—as well as in *Planctomycetota* and *Proteobacteria* (Hanke et al. 2016, Murali et al. 2022, Elbon et al. 2024, Lv et al. 2024). The alkane-oxidizing *Gammaproteobacteria* HdN1 contains a putative *nod* and may use oxygen for the activation of alkanes, although the process and activity of this NOD remain unclear (Zedelius et al. 2011). An even larger diversity of putative *nod* genes has been found through PCR-based studies and environmental surveys (Zhu et al. 2017, 2019, 2020, Zhang et al. 2018, Hu et al. 2019). Furthermore, *nod*-like genes have been reported in viral genomes in oceanic oxygen-deficient zones (Gazitúa et al. 2021) as well as in oxygen-depleted sediments colonized by foraminifera (Gomaa et al. 2021).

To expand upon the diversity of microorganisms that contain *nod* genes, we identified all *nod* gene sequences within the Genome Taxonomy Database (GTDB; Parks et al. 2022) using a recently published *nod*-specific hidden Markov Model (HMM; Murali et al. 2024). In doing so, we show that at least 16 phyla and 162 genera contain NOD (Supplementary Table 1). Remarkably, more than half of the identified putative NODs are affiliated with lineages within the phylum *Bacteroidota*. Our phylogenetic analysis of NOD sequences shows that this protein family—belonging to the larger HCO superfamily—diverges into four subclades, two of which almost exclusively comprise sequences affiliating with *Bacteroidota* (Fig. 3). All four of these NOD subclades retain amino acids that are conserved across the HCO scaffold—the putative low-spin heme ligand (H358, numbering according to NOD in *Ca.* M. oxyfera, CBE69502.1), the high-spin heme ligand (H662), and two out of three histidine ligands to the non-heme metal in the active site. In all NOD, one of the histidines that bind a non-heme active site metal in other HCO is replaced by an asparagine (N564) residue (Murali et al. 2022). Despite these similarities, these divergent subclades appear to be robust, sharing no more than 40%–50% sequence similarity between the groups. To assess the biochemical differences that drove diversification of NODs, characterization of homologs from each of these groups will be necessary.

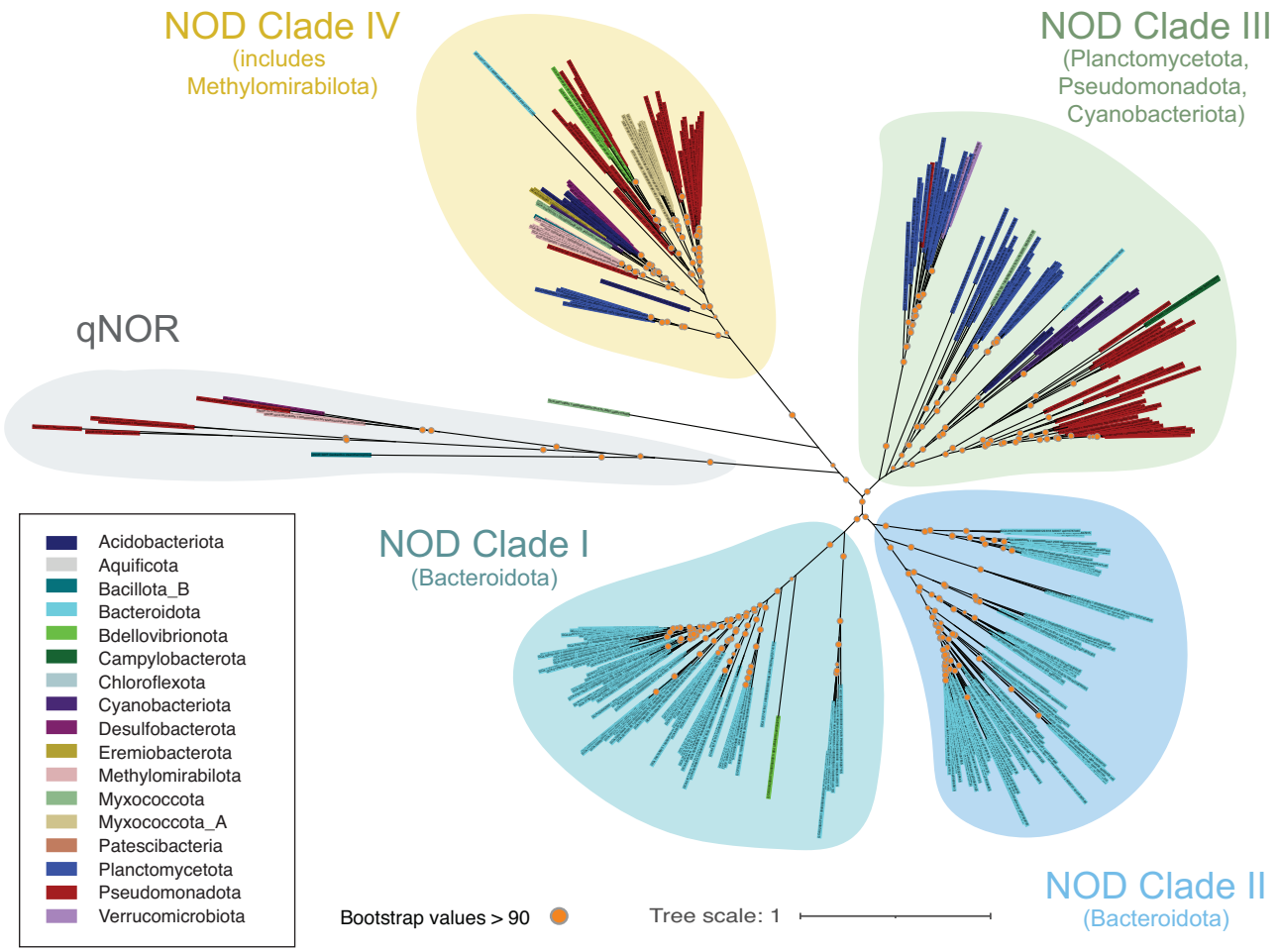

Figure 3. Phylogenetic diversity and taxonomic distribution of NOD. NOD sequences were retrieved from release 214 of the GTDB (Parks et al. 2022) using an HMM, built with curated NOD sequences (Murali et al. 2022), available on GitHub (https://github.com/ranjani-m/HCO). These sequences were aligned using MUSCLE (Edgar 2004)), and a phylogenetic tree was inferred using IQ-TREE (Minh et al. 2020) with qNOR sequences as outgroup. A substitution model was identified with IQ-TREE's ModelFinder, and the tree was validated with 1000 ultrafast bootstraps. The tree was visualized using iTOL, and the label background of each leaf on the tree was colored according to the phylum of the bacteria or archaea that contained the NOD (Supplementary Table 1). The phylum-level classification was made according to GTDB taxonomy. The tree appeared to diverge into four clades, each of which is color-coded here. The protein accession ID of each sequence in the tree according to GTDB is available in Supplementary Table 1.

We also supplemented environmental surveys from PCR-based studies (Fig. 4A; Supplementary Table 2) by categorizing the environments in which we identified *nod*-containing metagenome-assembled genomes (MAGs) in our GTDB analysis (Fig. 4B; Supplementary Table 2). Our analysis shows that *nod* genes were found in metagenomes from both natural environments such as freshwater and marine sediments, groundwaters, soils, hydrothermal vents, and methane seeps, as well as in man-made environments such as wastewater treatment plants and bioreactors. This supports earlier marker gene-based studies in which *nod* genes were found in lakes , wetlands, aquifers, soils, oil reservoirs, and wastewater treatment plants (Zhu et al. 2017, 2020, Zhang et al. 2018, Hu et al. 2019). Interestingly, the *nod* genes occurred in such artificial environments at higher abundances than in natural environments, and there existed no trend between the phylogenetic affiliation of *nod* genes and their environmental distribution. This most likely reflects the diverse lifestyles of microorganisms capable of *nod*-mediated DOP. For example, microbes affiliating with *Bacteroidota* thrive in many anoxic environments ranging from marine sediments to bioreactors. Their presence in these environments is largely consistent with biogeochemical evidence for microbial DOP in these environments.

Occurrence of O_2-dependent enzymes, metabolisms, and microbes in anoxic environments

In addition to the widespread occurrence of microorganisms that have the metabolic capabilities to produce O_2 in the absence of light, there are many records of the presence of O_2-consuming, strictly aerobic organisms and metabolic pathways inapparently anoxic environments. Despite not being detectable using traditional methods, O_2 is likely produced and consumed in these environments as indicated, e.g. by the occurrence and expression of O_2-dependent oxygenases. Below, we outline evidence for active O_2 cycling in a non-exhaustive list of select environments.

Marine environments

In the marine environment, gene transcripts of oxygenases and the presence of strict aerobes were reported from oxygen-deficient water bodies. In particular, O_2-dependent monooxygenases (Hayashi et al. 2007, Tavormina et al. 2013, Padilla et al. 2017) and aerobic respiration were found in the oxygen-deficient waters off the coast of Namibia, Mexico, and Peru (Tiano et al. 2014,

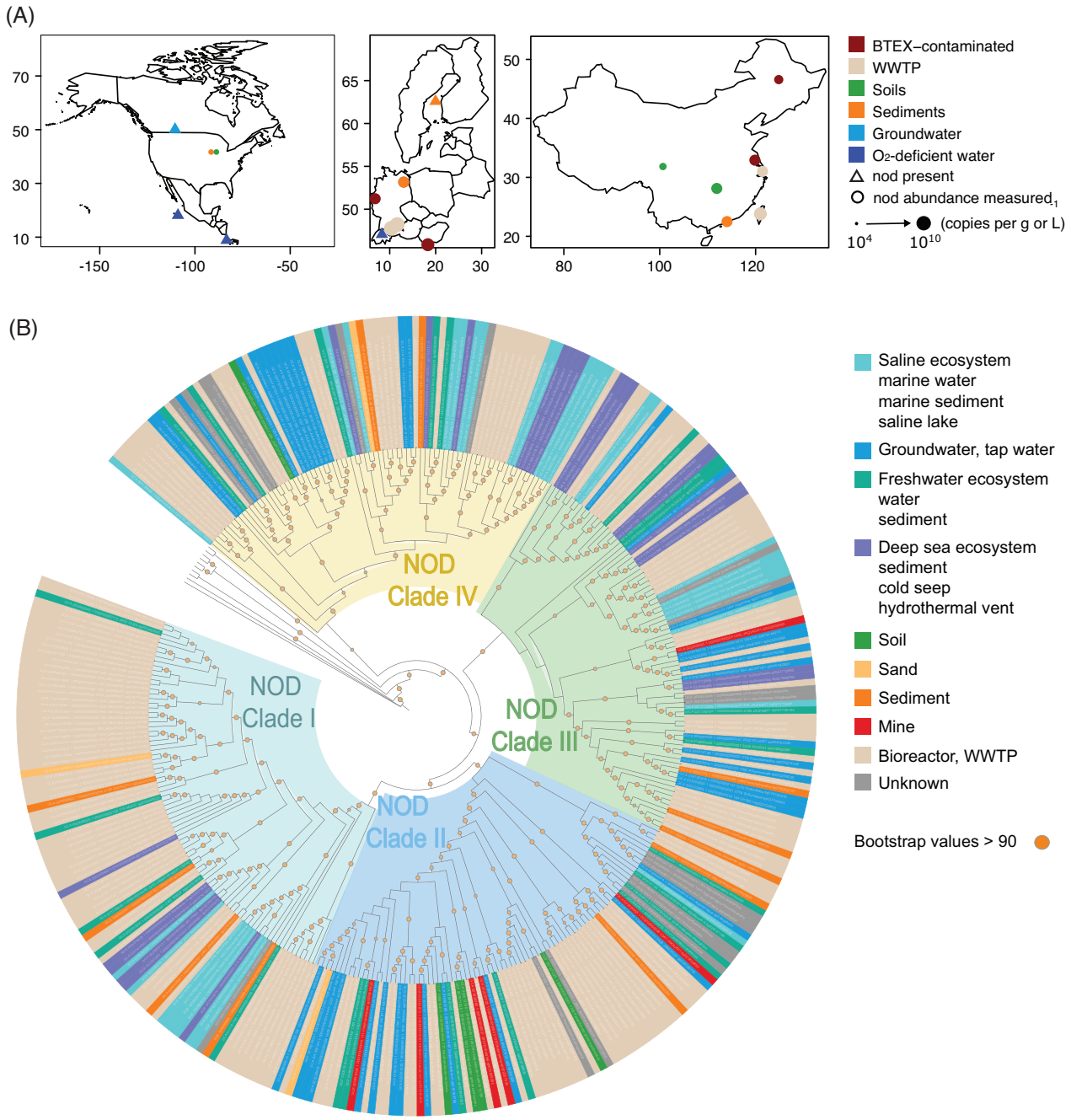

Figure 4. Environmental distribution of NOD. (A) Map of NOD presence and abundance reported in the literature (Supplementary Table 2). (B) Phylogenetic tree showing NOD sequences colored by the environments in which they were identified as described in the metadata accompanying the BioSample of each MAG from which NOD was recovered (Supplementary Table 2). The four clades identified in the NOD phylogenetic tree in Fig. 3 were colored in the same shades to correlate the distribution of Nod clades in different environments. BTEX: benzene, toluene, and xylenes; WWTP: wastewater treatment plant.

Kalvelage et al. 2015). The presence of O_2 in oxygen minimum zones (OMZs) is often attributed to laterally or vertically advected "whiffs," which is certainly a possible explanation for the prevalence of aerobic microbes. However, the presence of oxygenic phototrophs (Garcia-Robledo et al. 2017) and of microbes expressing *nod* genes (Padilla et al. 2016, Elbon et al. 2024) in OMZs also indicates the potential for *in situ* production—with or without light—as an O_2 source. Despite this possible importance, such production is difficult to detect directly because trace levels of produced O_2 in OMZs would not be expected to accumulate to detectable levels but would likely be consumed immediately (Canfield and Kraft 2022).

Evidence for the activity of aerobes was also found several meters below the seafloor of the permanently anoxic Arabian Sea OMZ (Bhattacharya et al. 2020). These sediments contained obligate aerobes that, upon isolation, only grew with oxygen. Also, *Nitrosopumilus* sp. and *nod* genes were present, and oxidase genes were transcribed (Sarkar et al. 2024). In marine seeps and mud volcanoes, aerobic methanotrophs are abundant in methane-rich sediments well below the measurable oxygen penetration depth

(Lo¨sekann et al. 2007, Ruff et al. 2013, 2015, 2019). It was speculated that these sediments may be actively oxygenated, e.g. by bioturbation due to seep-associated fauna. *Nitrosopumilaceae* as well as *Methylomirabilota* are also widespread and often abundant in deep subseafloor sediments indicating a genetic potential for DOP in environments that have been disconnected from surface processes on geologic time scales (Ruff et al. 2024).

Lake and wetland environments

Aerobic methanotrophs have been detected in anoxic sediments of Lake Constance, Germany (Pester et al. 2004), and of thermokarst Lake Vault, Alaska (Martinez-Cruz et al. 2017), as well as in sediments (up to 70 cm depth) at an active methane seep in Lake Qalluuraq, Alaska (He et al. 2022). Anoxic mesocosms using sediments from these Alaskan sites contained aerobic gammaproteobacterial methanotrophs that dominated methane assimilation, as shown by DNA-based stable isotope probing (Martinez-Cruz et al. 2017, He et al. 2022). In the seep site, the authors speculate that methanotrophy was coupled to iron reduction, yet it is unlikely that the first step of methane oxidation, catalyzed by methane monooxygenases (encoded by e.g. *pmoA* gene) could occur without O_2. Evidence of aerobic methanotrophy has also been detected in the suboxic/upper anoxic zones of Lake Untersee, Antarctica (Brady et al. 2023). Similar observations were made in Lake Zug, Switzerland, where aerobic alpha- and gammaproteobacterial methanotrophs were found to be more abundant in anoxic waters than in the oxycline and oxic waters (Oswald et al. 2016). In a follow-up study, the methane-oxidizing *Methylococcales* were shown to consume up to 0.2 µM methane per day, under both hypoxic and anoxic conditions (Schorn et al. 2024). These findings are supported by incubation experiments using methanogenic sediments of Lake Kinneret, Israel (Almog et al. 2024). Here, the authors found that *Methylococcales* made up one-third of the microbial community under hypoxic conditions. Both studies describe potential adaptations of *Methylococcales* to hypoxia or apparent anoxia, e.g. the metabolic potential for fermentation-based methanotrophy to overcome oxygen limitation (Kalyuzhnaya et al. 2013). However, the initial step in methane oxidation still requires O_2 even in this process. In Lake Zug, the anoxic layer also comprises denitrifying methanotrophs affiliating with *Methylomirabilota* (Graf et al. 2018, Schorn et al. 2024). These organisms that are capable of producing O_2, yet have not been shown to leak dissolved oxygen into the environment.

The purple sulfur bacteria *Chromatium okenii* thriving in Lake Cadagno, Switzerland, aerobically oxidize sulfide in dark and apparently anoxic waters. It was hypothesized that in this case, O_2 could derive from transport and convection processes occurring in the lake, allowing microbial populations at the oxic/anoxic interface to be active (Berg et al. 2019). In addition to convection of O_2, the occurrence of local microbial DOP could be contemplated. In Lake Lugano, Switzerland, *pmoA* genes were found in the anoxic, but not in the oxic part of the water column. Here, most of the detected bacterial populations affiliated with *Methylobacter* sp. and showed maximum activity at the suboxic–anoxic interface and in the deeper anoxic layer (Blees et al. 2014). In the boreal lake Alinen-Mustajärvi, Finland, aerobic methanotrophic *Methylobacter* and newly described *Ca. Methyloumidiphilus alinesnsis* were active, and transcripts of *pmoA* were present in the anoxic hypolimnion and in dark and anaerobic incubations (Rissanen et al. 2018). The addition of nitrate to the incubations stimulated methane oxidation, and the authors speculated that trace amounts of O_2 could have diffused through or out of the septa allowing the microaerobic methane activation and subsequent coupling to denitrification (Rissanen et al. 2018).

Similar to lakes, *Methylobacter* sequences have been found to be active in an Arctic wetland, with the highest activity detected in the anoxic and the transition zones (Graef et al. 2011). Further, *Methylobacter* and *Methylococcus* sequences were detected in the Zoige wetland, China, both in oxic and anoxic zones. While the abundance of aerobic methanotrophs was highest in the oxic zone, their diversity was highest in the anoxic zone, which indicates diverse aerobic niches that can support distinct populations of aerobes (Yun et al. 2010). Anaerobic continuous cultures inoculated with wetland sediments and fed with methane and nitrous oxide were dominated by aerobic methanotrophic *Methylocaldum* after 500 days; stable isotope analyses and an increase of methane monooxygenase suggested that aerobic methane oxidation was driven by nitrous oxide consumption (Cheng et al. 2021). The widespread occurrence, activity, and persistence of aerobic methanotrophs in anoxic freshwater habitats were suggested to be caused by diverse mechanisms, including direct electron transfer to metal oxides, alternative electron acceptors, and the presence of intracellular gas vesicles (Reis et al. 2024). This review adds DOP to the list of possible processes that can explain aerobic niches in anoxic wetland habitats.

Groundwater and subsurface environments

AOA, which require O_2 for the oxidation of ammonia to hydroxylamine, have been found together with *Methylomirabilota* in dysoxic aquifers (Mosley et al. 2022). In that study, it was speculated that O_2 is provided to AOA by *Methylomirabilota* via microbial cooperation. Such a "leakage" of dark O_2 into the surrounding environment has indeed been observed in a laboratory setup (Kraft et al. 2022). However, the AOA *N. maritimus* itself was shown to produce and leak O_2 in dark and anoxic conditions (Kraft et al. 2022), thus obviating the need to invoke *Methylomirabilota* as an O_2 source. Microbial O_2 leakage from chlorite or NO dismutation could also explain the presence of O_2 and the observed co-occurrence of strictly aerobic methanotrophic *Methylobacter* with *nod* and *cld* gene-containing organisms in old groundwaters (Ruff et al. 2023). A laboratory experiment using sand-packed microcosms and groundwater as inoculum showed that methane oxidation in the *Methylobacter*-dominated microcosms was strictly dependent on O_2, despite the presence of nitrate, which underlines that O_2 is needed for the activation of methane even if the oxidation of methanol can be coupled to denitrification (Kuloyo et al. 2020). Deep aquifer systems can also contain viable aerobic heterotrophs that are indicative of relatively oxidizing environments (Hicks and Fredrickson 1989).

Groundwater environments are often contaminated with hydrocarbons from industrial or military facilities. Interestingly, benzene-contaminated aquifers have been shown to comprise aerobic and denitrifying communities at low- to below-detection-limit O_2 concentrations (Aburto et al. 2009). The characterization of benzene-oxidizing and chlorate-reducing enrichment cultures indicated that anaerobic benzene degradation could be bypassed by DOP from chlorate-reducing communities (Weelink et al. 2007). In another study, O_2 production was measured during benzene-degradation under nitrate-reducing conditions (Atashgahi et al. 2018). For the toluene-degrading microbe *Georgfuchsia toluolica*, it was shown that traces of air introduced during sampling are sufficient to supply O_2 for the O_2-dependent enzymatic steps of aerobic toluene degradation, while the main electron flux and energy generation occurred simultaneously via nitrate reduction (Atash-

gahi et al. 2021). This shows that very small amounts of O_2, e.g. supplied by DOP, can suffice for the initial activation of hydrocarbons, and establish aerobic niches in anoxic environments.

Strictly aerobic methanotrophs have also been found in lignite and coal formations (Mills et al. 2010, Stępniewska et al. 2013, 2014, Pytlak et al. 2014). Similarly, cores from oil sands and coal beds in Alberta have been shown to contain unexpectedly high proportions of aerobic hydrocarbon-degrading bacteria as well as metagenomes with high proportions of genes for enzymes involved in aerobic hydrocarbon metabolisms (An et al. 2013, Ridley and Voordouw 2018). Deep crystalline bedrock was shown to contain strictly aerobic methanotrophs (Kalyuzhnaya et al. 1999, Hirayama et al. 2011, Kietäväinen and Purkamo 2015, Rajala et al. 2015) and water samples from rock fractures of the Deep Mine Microbial Observatory comprised methane monooxygenases and enzymes for oxygen respiration (Momper et al. 2023). Even ancient fluids of Canadian shield bedrock contained viable heterotrophic aerobes that apparently belonged to an autochthonous community (Song et al. 2024). The radiolysis of water is a source of O_2 in certain subsurface systems and could explain, e.g. the presence of strict aerobes in sediment and rock layers near a uranium mine (Mills et al. 2010). However, the widespread occurrence of O_2-dependent pathways and of dissolved O_2 at detectable concentrations in deep subsurface ecosystems merits a closer investigation of other possible processes, e.g. using stable-isotope methods.

Detecting and quantifying DOP

Dissolved oxygen anomalies have been detected in several subsurface ecosystems, including shallow (Ronen et al. 1987) and deep oxygenated groundwater (Winograd and Robertson 1982, Ruff et al. 2023) and fluids trapped within ancient bedrock (Nisson et al. 2023). Despite the detection of O_2 in subsurface ecosystems and the widespread occurrence of putative O_2-producing enzymes and metabolisms in apparently anoxic environments, confidently quantifying the amount of O_2 produced *in situ* remains challenging, particularly using traditional microbiological tools. Stable oxygen isotope analysis remains the most promising method to date to distinguish atmospherically derived from *in situ* produced dissolved oxygen, and to quantify their relative proportions. The utility of this approach largely relies on the so-called "Dole effect" (Dole 1936), in which isotope fractionation during oxic respiration leads to atmospheric O_2 being enriched in ^{18}O by several tens of permil relative to water (i.e. $\delta^{18}O = 24‰$ for atmospheric O_2; $\delta^{18}O$ ranging from ~ -25 to 0‰ for typical meteoric waters and seawater (Sharp et al. 2018, Wostbrock et al. 2020). The major stable-oxygen isotope composition of any oxygen-bearing material is written as

$$\delta^{18}O = \left(\frac{^{18}R_{sample}}{^{18}R_{VSMOW}} - 1\right) \quad (1)$$

where ^{18}R is the $^{18}O/^{16}O$ ratio and VSMOW is the Vienna Standard Mean Ocean Water international standard. Results are often reported in units of "permil" (‰) by multiplying Equation 1 by 1000. In contrast to respiration, photosynthesis—and possibly DOP—generates O_2 with an isotopic composition lighter than that of the atmosphere. Specifically, the $\delta^{18}O$ value of photosynthesis-derived O_2 resembles that of the parent water from which it formed (Helman et al. 2005). The isotopic composition of DOP-derived O_2 is currently unknown because DOP fractionation factors remain unconstrained. However, the detection of dissolved oxygen that is depleted in ^{18}O relative to atmospheric O_2 likely implies *in situ* production, the amount of which is quantitatively related to the difference in $\delta^{18}O$ value between atmospheric O_2 and measured dissolved oxygen.

Following these principles, several studies have analyzed $\delta^{18}O$ values of dissolved oxygen in groundwaters to constrain O_2 cycling dynamics (Aggarwal and Dillon 1998, Révész et al. 1999, Wassenaar and Hendry 2007, Smith et al. 2011, Parker et al. 2012, 2014, Ruff et al. 2023); we compiled these results here (Fig. 5A). Authors typically assume that groundwater initially contains dissolved O_2 in equilibrium with the atmosphere. If this dissolved O_2 subsequently undergoes closed-system consumption either by microbial respiration or by abiotic processes (e.g. Fe(II) or H_2S oxidation; Oba and Poulson 2009a, 2009b), then residual dissolved oxygen $\delta^{18}O$ values will become progressively higher due to isotopic fractionation (so-called "Rayleigh fractionation"). Such mechanisms successfully explain ≈35% of all groundwater observations compiled here (i.e. values that plot close to the green and blue/orange shaded regions in Fig. 5A).

However, many studies also observe ^{18}O-depleted dissolved oxygen in groundwater. This cannot be explained by consumption mechanisms alone, all of which are expected to lead to ^{18}O enrichment (Mader et al. 2017). Rather, these authors have mostly invoked the downward diffusion of atmospheric O_2 through the vadose zone to explain ^{18}O-depleted values in shallow aquifers (Smith et al. 2011, Parker et al. 2012, 2014). Diffusion is known to induce isotopic fractionation due to differences in isotopologue-specific diffusion coefficients. Diffusion can thus lead to ^{18}O-depleted dissolved oxygen despite being derived from atmospheric O_2, but only in low concentrations (Knox et al. 1992, Li et al. 2019, Cao 2022). As diffusion-derived dissolved oxygen concentrations increase, isotopic compositions approach their equilibrium value of $\delta^{18}O \approx 24‰$ (i.e. following the green shaded regions in Fig. 5). By considering diffusion in addition to consumption, the percentage of groundwater observations compiled here that can be explained by traditional mechanisms increases to ≈53%. This analysis thus suggests that nearly half of all groundwater dissolved oxygen $\delta^{18}O$ values compiled here can only be explained by invoking an additional source of ^{18}O-depleted O_2, e.g. by *in situ* DOP. Such processes—regardless of specific biotic versus abiotic mechanism—will drive groundwater dissolved oxygen to higher concentrations and lower $\delta^{18}O$ values (red arrows in Fig. 5).

In addition to canonical ^{18}O measurements, recent analytical advancements have led to increased use of so-called "triple-oxygen isotopes" (written as $\Delta'^{17}O$) to track O_2 cycling. The triple-oxygen isotope composition of any oxygen-bearing material is typically written as

$$\Delta'^{17}O = \ln\left(\frac{^{17}R_{sample}}{^{17}R_{VSMOW}}\right) - \theta_{RL} \times \ln\left(\frac{^{18}R_{sample}}{^{18}R_{VSMOW}}\right) \quad (2)$$

where ^{17}R is the $^{17}O/^{16}O$ ratio and θ_{RL} is a reference line slope. Here, we let $\theta_{RL} = 0.5305$ (Bao et al. 2016). Results are often reported in units of "parts per million" (ppm) by multiplying Equation 2 by 10^6. Of relevance here is the fact that atmospheric O_2 is anomalously depleted in ^{17}O due to mass-independent isotope exchange processes in the stratosphere (Hemingway and Claire 2025). This leads to atmospheric O_2 with a $\Delta'^{17}O$ value of ≈ -500 ppm. In contrast, seawater and all meteoric fluids fall along a mass-dependent meteoric water line, with $\Delta'^{17}O \geq 0$ ppm (Sharp et al. 2018). Like in $\delta^{18}O$ versus dissolved oxygen concentration space, several processes of interest here will lead to unique fractionation trajectories in a triple-oxygen isotope plot (Fig. 5B). Importantly, only by combining all three measurements—concentration, $\delta^{18}O$, and $\Delta'^{17}O$—can the source and cycling of dissolved oxygen be

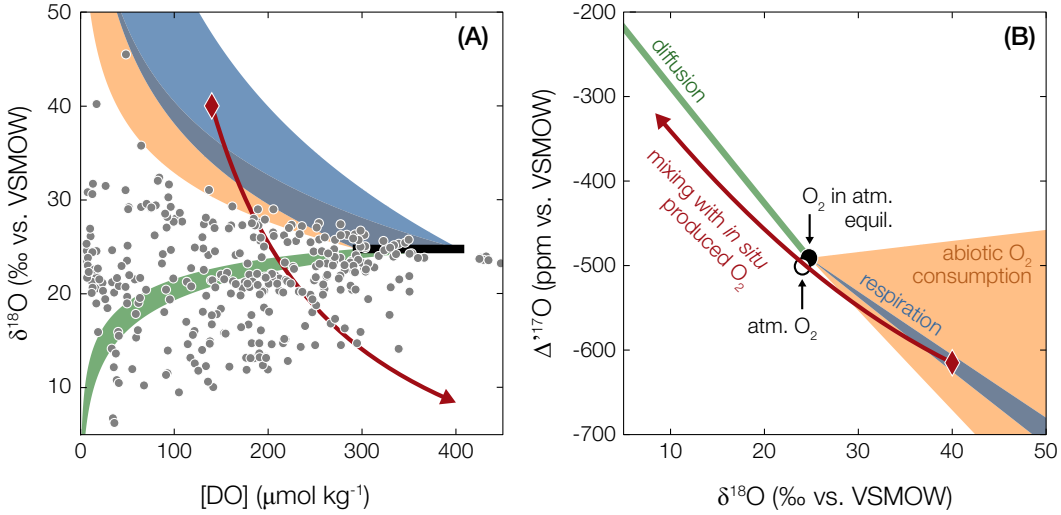

Figure 5. Groundwater dissolved oxygen isotope plots showing (A) $\delta^{18}O$ as a function of dissolved oxygen concentration [DO] and (B) $\Delta'^{17}O$ as a function of $\delta^{18}O$ (so-called "triple-oxygen isotope plot"). Modern-day atmospheric O_2 isotopic composition (Wostbrock et al. 2020) is shown in panel B as an open white circle. Dissolved O_2 is assumed to begin in equilibrium with the atmosphere at standard temperature and pressure in fluids between 5°C and 15°C with salinity between 0 and 10 psu (thick black line in panel A; black circle in panel B; Garcia and Gordon 1992, Li et al. 2019). Groundwater dissolved O_2 can then decrease in concentration and become isotopically fractionated via consumption by microbial respiration (blue shaded region) or abiotic processes (e.g. Fe(II) or H_2S oxidation; orange shaded region). Mixing between inward-diffusing O_2 and water that has undergone dissolved O_2 consumption will result in isotope values between the green and blue/orange shaded regions. In contrast, mixing with *in situ*-produced O_2 shifts isotopic compositions toward an end member resembling source water (modified by fractionation during the O_2 production process). For example, the red arrow indicates *in situ* production starting from an arbitrary initial point within the respiration array (red diamond) assuming that *in situ* O_2 forms from groundwater with $\delta^{18}O = -17.5‰$ and $\Delta'^{17}O = 84$ ppm, i.e. the average of all measured values compiled here, assuming they fall on the meteoric water line (Sharp et al. 2018) and assuming that O_2/H_2O fractionation is described by the temperature-dependent equilibrium fractionation factor (Hemingway et al. 2022). Regardless of the exact fractionation factors, *in situ* production is the only process within this framework that can explain high concentrations of ^{18}O-depleted dissolved O_2 (i.e. to the lower-right of the diffusion array). Gray circles in panel A represent 338 measured values from globally distributed groundwaters (Aggarwal and Dillon 1998, Révész et al. 1999, Wassenaar and Hendry 2007, Smith et al. 2011, Parker et al. 2012, 2014, Ruff et al. 2023). Nearly half of all measurements are best explained by *in situ* production of isotopically light O_2 (Supplementary Table 3).

uniquely constrained. For example, partial closed-system respiration followed by DOP could be falsely interpreted as reflecting diffusion in a concentration versus $\delta^{18}O$ plot (intersection of red arrow and green shaded region in Fig. 5A), but these processes can be uniquely separated when including $\Delta'^{17}O$ (no intersection of red arrow and green shaded region in Fig. 5B).

Astrobiological relevance of DOP

Atmospheric production of (per)chlorate occurs in Earth's stratosphere from oxidation of Cl^-, ClO^-_x, or ClO_x through ozone or light (Sturchio et al. 2009, Jackson et al. 2010), whereas nitrate is formed through oxidation of nitrogen dioxide gas to nitric acid (Smith et al. 2014). Accumulation of these compounds after deposition on the Earth's surface correlates with aridity, thus explaining the elevated levels of (per)chlorate in dry conditions such as those of the Atacama Desert (Catling et al. 2010). Evidence exists that both nitrate and (per)chlorate also occur on the lunar surface, on Mars, and within Martian meteorites (Kounaves et al. 2014, Stern et al. 2015, Martin et al. 2020), with detected nitrate levels in Martian sediments (up to 1000 ppm; Stern et al. 2015) reaching those found in the Atacama Desert (Walvoord et al. 2003). Microbial enrichment cultures from this nitrate-rich arid environment have shown microbial growth possibility (Shen et al. 2019), and NO_3^-/ClO_4^- reducing microbial populations were identified from desert soil samples that serve as sites analogous to the Martian environment (Cortés et al. 2024). Due to highly conserved ratio of NO_3^-/ClO_4^- in non-biologically active areas on Earth, it may be possible to use alterations of this ratio as a biomarker of past biological activity on Mars (Jackson et al. 2015).

Subsurface nitrates on Mars could potentially provide a source of available nitrogen and O_2 to support past or present life, which is most likely found in the planet's subsurface ecosystems (Michalski et al. 2018). Aerobic niches caused by DOP could in theory be present also in dark environments on ocean worlds, as biologically available nitrogen was detected in Enceladus' plume. Furthermore, Enceladus' global ocean is suspected to harbor hydrothermal activity (Hsu et al. 2015, Waite et al. 2017). On Earth, hydrothermal systems present available forms of nitrogen and are colonized by a variety of microorganisms involved in the nitrogen cycle (Zeng et al. 2021). Hydrothermal systems additionally drive abiotic O_2 generation via the surface-bound radical mechanism (Stone et al. 2022). DOP—whether abiotic or biotic—could therefore be a source of O_2 in hydrothermally active, nitrate-rich ocean worlds even if light is not available.

Concluding remarks and outlook

Following a quote from the 1982 paper "Deep Oxygenated Groundwater: Anomaly or Common Occurrence?" (Winograd and Robertson 1982) in which the authors write: "We hope that this report will stimulate a systematic appraisal of DO [dissolved oxygen] in future geochemical studies of shallow and deep ground water," we would like to conclude this review on similar hopes. Oxygen anomalies have been reported from numerous ecosystems, aerobic organisms are widespread in anoxic environments, and the metabolic capabilities to produce O_2 via dismutation of chlorite or NO are nearly ubiquitous (Fig. 6). In contrast to the early 1980s, we now have advanced molecular tools to detect putative *nod* genes, e.g. through specific oligonucleotide primers for gene

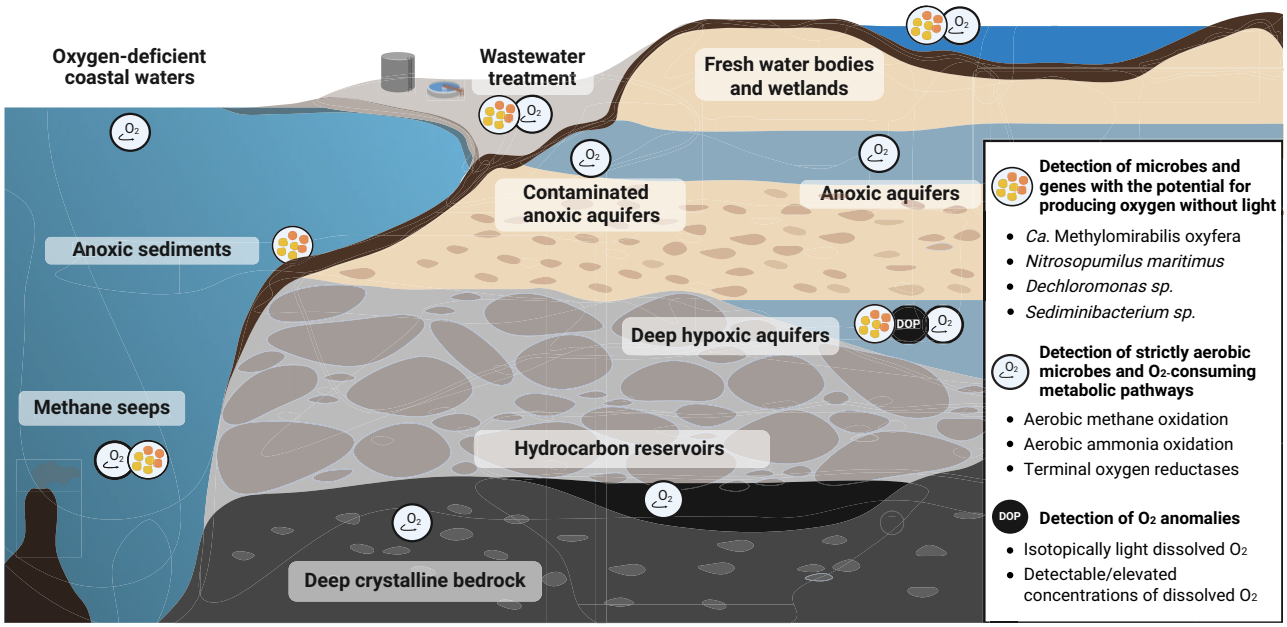

Figure 6. Overview of hypoxic or apparently anoxic ecosystems that have been reported to contain isotopically light dissolved oxygen, comprise strictly aerobic microorganisms, or O_2-producing or -consuming metabolic genes and pathways. Traces of O_2 were found in groundwaters (e.g. Winograd and Robertson 1982, Ruff et al. 2023) and fracture fluids (e.g. Nisson et al. 2023), while aerobic microorganisms and O_2-dependent enzymes were reported from a variety of anoxic systems, such as bedrock, groundwater, marine and freshwater sediments, and hydrocarbon reservoirs. These microbes were associated with oxidation of methane, other hydrocarbons, and ammonia (e.g. Hayashi et al. 2007, Lo¨sekann et al. 2007, Aburto et al. 2009, Mills et al. 2010, Ruff et al. 2013, 2019, Stępniewska et al. 2013, 2014, Tavormina et al. 2013, Pytlak et al. 2014, Tiano et al. 2014, Kalvelage et al. 2015, Martinez-Cruz et al. 2017, Padilla et al. 2017, Bhattacharya et al. 2020, He et al. 2022, Mosley et al. 2022, Almog et al. 2024, Schorn et al. 2024). Expression of *nod* and *cld* genes was detected in O_2-deficient waters, sediments, groundwater, and wastewater treatment plants (e.g. Bhattacharjee et al. 2016, Padilla et al. 2016, Cheng et al. 2021, Ruff et al. 2023, 2024, Elbon et al. 2024, Sarkar et al. 2024), and DOP was confirmed in *P. aeruginosa*, *Ca.* M. oxyfera, and *N. maritimus* using stable-isotope labeling approaches (Ettwig et al. 2010, Lichtenberg et al. 2021, Kraft et al. 2022). The figure and legend features select examples, details are provided in the respective section of this review.

amplification (Bhattacharjee et al. 2016, Zhu et al. 2017, Hu et al. 2019), and achieve high-quality long-read whole genome sequencing data even from low-biomass samples and environments (Simon et al. 2023). The widespread availability and utilization of improved sequence-similarity tools that include curated HMMs to detect target proteins will improve the characterization of genes and pathways involved in DOP and allow us to understand their global diversity, abundance, and evolution. Indeed, our analysis of published sequencing data demonstrates the unexpected and striking occurrence of *nod* genes in the phylum *Bacteroidota* and suggests a role for *Bacteroidota* in NOD-associated DOP. Putting together the environmental co-occurrence between hydrocarbon degradation and DOP and the genomic potential in many *Bacteroidota* to perform hydrocarbon oxidation, we could infer that aerobic hydrocarbon degradation in anoxic environments is one of the major driving forces for the evolutionary selection of DOP well beyond the phylum *Methylomirabilota*. Future studies looking at the co-expression of DOP and hydrocarbon degradation pathways within apparently anoxic environments by gene transcript sequencing, and protein mass spectrometry will provide insight into potential fluxes of O_2 from DOP into various aerobic pathways.

New observational and experimental tools are becoming routinely available to further constrain DOP. For example, the detection limit for bulk dissolved oxygen concentrations has decreased by several orders of magnitude in the last decade (Tiano et al. 2014, Lehner et al. 2015, Larsen et al. 2016). The measurement of dissolved O_2—including its (triple)-oxygen isotopic composition—should become a standard procedure when measuring and monitoring environmental parameters in subsurface ecosystems, along with nitrate, nitrite, (per)chlorate, and chlorite concentration measurements. If shown to be isotopically unique and utilized in biosynthetic pathways, it may even be possible to track DOP-derived oxygen incorporation into biomolecules such as long-chain alcohols (Johnson and Galy 2022). Furthermore, experiments and incubations using isotopically labeled substrates, e.g. $^{15}N^{18}O$, can yield valuable insights into dismutation processes, the involved organisms, and metabolic rates. Finally, AI-based protein structure prediction tools (Varadi et al. 2024) and heterologous expression of *nod* genes and pathways into a recipient cell such as *Escherichia coli* may shed additional light on the diversity and biochemistry of the enzymes of interest. Such experiments—including those targeting the responsible enzymes—will additionally benefit from continued efforts to culture and isolate the organisms capable of DOP.

Despite the promise of stable oxygen isotopes as a method to track dark-oxygen production, several key uncertainties remain. In particular, the (triple-)oxygen isotope fractionation factors for many processes of interest are unknown. First, only ^{18}O fractionation for abiotic O_2 consumption has been determined to date (Oba and Poulson 2009a, 2009b), thus leading to large uncertainty in the triple-oxygen isotope effect of this process. Second, to our knowledge, no fractionation factor measurements currently exist for any *in situ* DOP mechanism, neither biological metabolisms nor abiotic processes such as radiolysis. Third, O_2 produced via DOP may not accumulate to high enough levels to assess isotope fractionation due to consumption by nanaerobic microbes. Future work should aim to directly quantify these fractionation

factors using laboratory experiments and culturing studies. Once the fractionation factors have been accurately quantified, then (triple-)oxygen isotope analysis of dissolved oxygen will provide a powerful tool to quantitatively track O_2 cycling, including the effect of DOP.

Overall, in this review, we show that DOP is likely an overlooked process of global relevance not only in subsurface ecosystems, but also in many apparently anoxic ecosystems on Earth's surface. DOP can explain many of the O_2-related anomalies and enigmatic occurrences of strict aerobes that have been reported for decades. The production of molecular oxygen in the dark may be crucial for the biogeochemistry, ecology, and evolution of many globally distributed ecosystems.

Acknowledgments

We would like to thank the participants of the 2nd Joint Symposium of the International. Societies for Environmental Biogeochemistry & Subsurface Microbiology 2023 for their lively discussions and feedback.

Author contributions

S. Emil Ruff (Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing), Laura Schwab (Investigation, Visualization, Writing – original draft, Writing – review & editing), Emeline Vidal (Investigation, Visualization, Writing – original draft, Writing – review & editing), Jordon D. Hemingway (Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing), Beate Kraft (Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing), and Ranjani Murali (Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing)

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: None declared.

Funding

S.E.R., J.D.H., and B.K. received funds from the Human Frontier Science Program (RGEC34/2023). This work was supported by a grant from the Simons Foundation [824763, S.E.R.]. This work is part of a project that has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program [946150, J.D.H.].

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Received 15 May 2024; **revised** 23 July 2024; **accepted** 25 September 2024