

How low can they go? Aerobic respiration by microorganisms under apparent anoxia

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One sentence summary: The discovery of microbial oxygen respiration at and below the oxygen detection limit is changing our understanding of biogeochemical cycling in oxygen-limited environments, from the early Earth to present-day expanding hypoxic zones.

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Abstract

Oxygen (O₂) is the ultimate oxidant on Earth and its respiration confers such an energetic advantage that microorganisms have evolved the capacity to scavenge O₂ down to nanomolar concentrations. The respiration of O₂ at extremely low levels is proving to be common to diverse microbial taxa, including organisms formerly considered strict anaerobes. Motivated by recent advances in O₂ sensing and DNA/RNA sequencing technologies, we performed a systematic review of environmental metatranscriptomes revealing that microbial respiration of O₂ at nanomolar concentrations is ubiquitous and drives microbial activity in seemingly anoxic aquatic habitats. These habitats were key to the early evolution of life and are projected to become more prevalent in the near future due to anthropogenic-driven environmental change. Here, we summarize our current understanding of aerobic microbial respiration under apparent anoxia, including novel processes, their underlying biochemical pathways, the involved microorganisms, and their environmental importance and evolutionary origin.

Keywords: aerobic respiration, evolution, anoxia, terminal oxidases, transport processes, biogeochemical cycling

Introduction

Molecular oxygen (O₂) is the most favorable electron acceptor in terms of free energy yield, and the capacity to respire it enabled the development and proliferation of multicellular life on Earth (Hedges *et al.* 2004; Falkowski 2006; Thannickal 2009). O₂ has existed on Earth at least since the evolution of photosynthesis 2.7–2.5 billion years ago (Buick 2008; Farquhar, Zerkle and Bekker 2011), although O₂ likely did not begin to accumulate in the atmosphere until 2.45–2.32 billion years ago (Bekker *et al.* 2004). The deep ocean remained predominantly anoxic (Shen, Canfield and Knoll 2002; Anbar *et al.* 2007) until a second major rise of atmospheric O₂ levels 0.8–0.55 billion years ago (Canfield and Teske 1996). Considering that the generation time of most microorganisms is on the order of hours to days, prokaryotic life on Earth has had a lot of time to evolve a tolerance to, and eventually exploit, O₂ even at extremely low levels.

Aerobic respiration today is estimated to account for up to 90% of organic matter remineralization in the world's ocean waters and sediments (Reimers and Suess 1983), thus playing a key role in the global carbon cycle and climate system. According to the microbial redox cascade model, the most favorable electron acceptor in nature (O₂) will be preferentially respired over other, anaerobic electron acceptors, although the energy yield may vary under

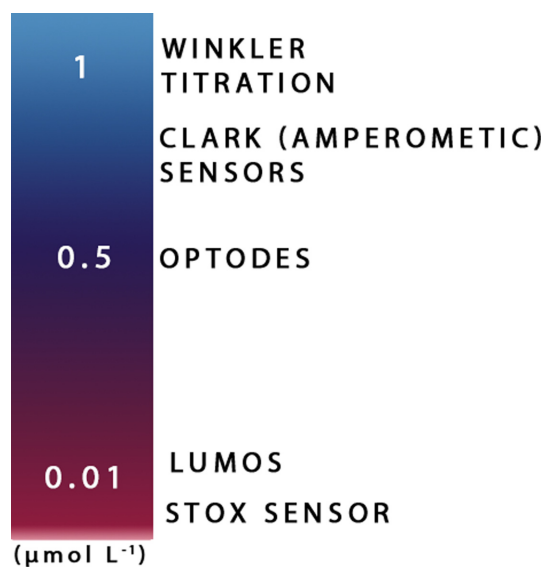
in situ conditions. Owing to this and the inhibition of specialized anaerobic enzymes by O₂, obligately anaerobic life in Earth's surface environments is restricted to the few environments where O₂ diffusion is limited, such as marine oxygen minimum zones (OMZs), stratified lakes, wetland soils, leguminous root nodules, sediments, wastewater, anoxic microniches within marine snow particles and animal guts. With the advent of new O₂-sensing technologies (see Box 1), however, the detection limits for O₂ are being pushed ever lower, revealing that less and less of Earth's surface environments are truly anoxic. The earliest method for measuring dissolved O₂ was the Winkler titration method, which in 1922 enabled the discovery of deep waters off the Pacific coast of Panama 'contain[ing] practically no oxygen at all' (Winkler 1888). Since then, dissolved O₂ concentrations in the Eastern North Pacific and other OMZs have been reassessed using STOX (switchable trace oxygen) sensors, revealing low but highly fluctuating O₂ concentrations in OMZs (Revsbech *et al.* 2009; Thamdrup, Dalsgaard and Revsbech 2012). These studies highlight that absolute anoxia, or zero O₂ concentration, cannot be measured directly in the environment because any assay will have some—even if very low—detection limit, currently at 3–10 nmol·L⁻¹.

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Box 1: Anoxia has been redefined by evolving technologies.

The Winkler titration method developed in 1888 for dissolved O₂ quantification is highly accurate and is still used to calibrate automated instruments. Improvements to the detection of O₂ in fluids were later made in the biomedical field with the first amperometric electrodes used to measure oxygen tension in blood (Clark *et al.* 1953). Oxygen optode technology has actually been around for ~80 years (Kautsky 1939) but was only recently introduced to aquatic research (Tengberg *et al.* 2006). Optodes combine robust measurements and low O₂ detection limits and are the sensors of choice for ocean monitoring over large scales of space and time. The lowest detection limits (in the 0.5–10 nmol/L range) can be achieved by the luminescence measuring oxygen sensor and STOX sensors (Revsbech *et al.* 2009; Lehner *et al.* 2015). These new O₂-sensing methods have revealed trace amounts of O₂ where we previously thought there was none, highlighting the fact that anoxia is defined by the detection limits of our current technologies.



There is no universally accepted terminology for low-O₂ conditions across the fields of microbiology, ecology, geology and oceanography (reviewed in Tyson and Pearson 1991; Algeo and Li 2020). The multitude of similar terms defined by poorly constrained or seemingly arbitrary O₂ thresholds is the result of numerous challenges arising from the attempt to define O₂-deficient regimes based on geochemical criteria (which change alongside improving O₂-sensing technologies) or biological thresholds (which differ for each organism). Keeping these limitations in mind, we have summarized existing terminology and their upper O₂ thresholds, adding the new distinction ‘nanoxic’ due to the emerging biological importance of ‘nanaerobic’ respiration (Table 1).

Nanaerobic is a term that was coined almost two decades ago to describe the ability of some prokaryotes to grow aerobically at nanomolar O₂ concentrations (Baughn and Malamy 2004), but the geochemical importance of nanoxia as well as the environmental prevalence of this phenomenon is thus far largely unknown. Research on aerobic respiration at the O₂ detection limit has been

Table 1. Redox classification modified from Tyson and Pearson (1991) and Algeo and Li (2020). Hypoxia is the limit at which O₂ is considered insufficient to support living animals, but O₂ demand is of course specific to each organism. Suboxic conditions are characterized by the onset of denitrification (Breck 1974). Functional anoxia is defined by limit at which O₂ is no longer significant in biological metabolisms or other major biogeochemical processes (Thamdrup, Dalsgaard and Revsbech 2012), whereas apparent anoxia is defined by the detection limit of our current sensors. We presume ultralow amounts of O₂ may still be present because absolute anoxia cannot technically be measured.

Biological regime	Geochemical regime	Oxygen threshold
Aerobic	Oxic	260–61 μmol·L ⁻¹
Hypoaerobic/dysaerobic	Hypoxic/dysoxic	<60 μmol·L ⁻¹
Microaerobic	Microoxic	<10 μmol·L ⁻¹
Subaerobic	Suboxic	<5 μmol·L ⁻¹
Nanaerobic	Nanoxic	<1 μmol·L ⁻¹
Functional anoxia	Apparently anoxic	<3 nmol·L ⁻¹
Anaerobic	Anoxic	0

almost exclusively restricted to human gut microbiota (Leclerc *et al.* 2015) and N₂-fixing bacteria in legume root nodules (Bergersen and Turner 1990; Kuzma, Hunt and Layzell 1993). In a major breakthrough, the ability to respire O₂ at concentrations below our current detection limits was demonstrated for the classical aerobic *Escherichia coli* (Stolper, Revsbech and Canfield 2010). More recently, the potential for nanaerobic respiration has been expanded by the discovery that microorganisms typically considered strict anaerobes can respire aerobically (Cypionka 2000; Berg *et al.* 2019; Lee *et al.* 2019). A glimpse into the metabolic capacities of the vast numbers of uncultured environmental microorganisms through metagenomics has revealed that the potential for aerobic respiration is phylogenetically more widespread (70% of species surveyed in one study possessed high-affinity oxidases; Morris and Schmidt 2013) and pervasive (present in so-called anoxic environments; Ulloa *et al.* 2012; Kalvelage *et al.* 2015; Milucka *et al.* 2015; Bristow *et al.* 2016; Garcia-Robledo *et al.* 2017; Berg *et al.* 2019) than previously thought.

Only very recently has it been shown that the respiration of O₂ in apparently anoxic waters significantly contributes to aquatic ecosystem processes such as ammonium and nitrite oxidation (Kalvelage *et al.* 2011, 2015; Beman, Lilei Shih and Popp 2013; Bristow *et al.* 2016), methane oxidation (Milucka *et al.* 2015; Oswald *et al.* 2015) and sulfide oxidation (Callbeck *et al.* 2018; Berg *et al.* 2019). Still surprisingly, little is known about the prevalence of nanaerobic respiration and its contribution to global respiration processes, the ecophysiology of nanaerobes in the environment, and the true limits to aerobic growth on modern and ancient Earth.

The recent explosion of research on expanding OMZs and lake hypoxia along with continuous development of new sequencing technologies have added a wealth of new data on (nan)aerobic microbiology. Here, we will explore these new insights into nanaerobic life and their implications for past and present biogeochemical cycling. Although we focus on aquatic environments due to their importance as a habitat for life on early Earth to the present day, the following questions apply to all microbiology-related disciplines:

- Where do nanoxic environments occur and how do they form?

- How prevalent is the capacity for nanaerobic respiration in the environment and how common is it across microbial taxa?
- Where is the boundary between aerobes/anaerobes and are our definitions adequate to accommodate new metabolic pathways (hybrid metabolisms, intra-aerobic respiration)?
- What role has nanaerobic respiration played in global biogeochemical cycling from early Earth to the modern day?

Oxygen production and transport sustain nanaerobic respiration in anoxic waters

The low solubility of O₂ in water (284 and 225 μmol L⁻¹ in freshwater and seawater, respectively, at 20°C) and its slow diffusion is a fundamental constraint on aquatic life. Wherever the respiration of O₂ exceeds replenishment by oxygenic photosynthesis or by advective or diffusive transport processes, anoxic aquatic environments can develop over spatial scales of a few micrometers to hundreds of kilometers. Although these environments might appear O₂ depleted, they are subject to dynamic O₂ intrusions driven by biological, chemical and physical processes or a combination of these (Fig. 1).

In the water column, O₂ is mixed in from the atmosphere or produced via photosynthesis if sufficient light and nutrients trigger the activity of phytoplankton (oxygenic photosynthetic cyanobacteria and eukaryotes), which form the basis of complex aquatic food networks. Exopolymers excreted by or leaking from phytoplankton lead to coagulation and formation of organic matter particles known as suspended flocs or marine snow. These particles are hot spots of microbial activity in the water column, and O₂ consumption by heterotrophs can generate anoxic microniches at their inner core (Alldredge and Cohen 1987; Smriga, Ciccacese and Babbin 2021). At the same time, sinking of aggregates through the water column reduces the surrounding diffusive boundary layer, thus enhancing the oxygenation of the particle interior (Kjørboe, Ploug and Thygesen 2001; Ploug 2001). The water volume entrained by the sinking particle along with water trapped in the particle interior might vertically transport substantial amounts of O₂, but the extent of this remains to be quantified.

In situ production is one of the most easily overlooked sources of O₂ to anoxic waters because the transfer of O₂ can occur by physical association between photosynthetic algae and microorganisms within the phycosphere or diffusional transfer over short length-scales without release of measurable O₂ to the surrounding water. It is not surprising, therefore, that O₂ produced *in situ* via photosynthesis has only recently been recognized as an important oxidant in stratified systems, accounting for up to 90–100% of methane oxidation and 10–50% of sulfide oxidation in anoxic lakes (Milucka et al. 2015; Oswald et al. 2015; Berg et al. 2019). In the oceans, deep chlorophyll maxima have been observed in association with and within OMZs (Cepeda-Morales et al. 2009; Márquez-Artavia et al. 2019) supporting aerobic respiration rates that are even higher than in over- and underlying oxic waters (Tiano et al. 2014). However, the impact of deep photosynthesis on biogeochemical cycles in seemingly anoxic waters has yet to be quantified.

Muddy aquatic sediments are typically diffusion limited and therefore some bacteria have evolved a combination of motility and storage to bridge the distance between O₂ and the supply of substrates/electron donors. The large sulfur bacteria *Thioploca* spp. and *Beggiatoa* spp. store reduced sulfur compounds intracellularly and migrate vertically between reducing and oxidizing sed-

iment environments (Schulz and Jørgensen 2001). So-called ‘cable bacteria’, filamentous bacteria belonging to the family Desulfobulbaceae, exhibit yet another fascinating adaptation to exploit valuable O₂, forming conductive filaments that can conduct electrons over centimeter distances resulting in spatially separated sulfide oxidation and O₂ reduction reactions (Pfeffer et al. 2012; Liu et al. 2021). Bioturbating and bioirrigating animals can also increase the flux of O₂ into surrounding sediments three- to 7-fold (Meysman et al. 2006; Volkenborn et al. 2007; Pischedda et al. 2008). Likewise, partially or fully submerged aquatic plants such as seagrasses transport O₂ down to the subsurface through vascular tissue, leaking O₂ into the rhizosphere and generating microoxic environments with high spatial and temporal dynamics following day–night cycles (Frederiksen and Glud 2006). Sandy sediments, which cover >60% of the coastal ocean, are permeable to water flow and can thus be ventilated by bottom-water currents and waves (Huetzel, Berg and Kostka 2014). These forces may also trigger sediment transport (migration of sand ripples on the sea or lake floor) (Ahmerkamp et al. 2017; Waska et al. 2019) leading to the formation of variable redox zones, which harbor opportunistic microorganisms that can switch from aerobic to anaerobic respiration (Bühning, Elvert and Witte 2005; Marchant et al. 2017; Ahmerkamp et al. 2020).

The contribution of biological mixing to O₂ transport, measured as the amount of mechanical power invested in the global oceans by the locomotion of animals, has been estimated to be a nontrivial 0.63 TW, which is on the same scale as wind and tidal inputs (Dewar et al. 2006). Though it was assumed for a long time that smaller organisms having a small Reynolds number could not overcome viscous forces and that kinetic energy would dissipate as heat, bacteria can collectively entrain O₂ into anoxic waters by swarming, as has been observed for *Thiovulum majus* (Petroff and Libchaber 2014) and *Chromatium okenii* (Sommer et al. 2017). On millimeter to centimeter scales, mixing has been demonstrated by different sizes of jellyfish (Katija and Dabiri 2009). Larger-scale mixing can be driven by collective swimming, such as by schools of fish or shrimp, when the flow in the wakes of individual organisms coalesces to form a large-scale downward jet (Kunze et al. 2006; Houghton et al. 2018).

Physical processes such as wind-induced internal waves and circular water currents called eddies also bring oxygenated water masses in contact with anoxic waters. Internal waves are generated by tides and surface winds and break in contact with terrestrial slopes generating turbulence and hence, mixing across interfaces with contrasting densities (temperature, salinity, dissolved gases) (Lamb and Xiao 2014). This type of mixing can transport substantial amounts of O₂ into coastal OMZs, covering up to one-third of the total oxygen demand (Fischer et al. 2013). Mesoscale eddies (on the order of 100 km in diameter), formed along coastal or topographical features, have been shown to ventilate the upper part of the ocean down to the oxycline (Thomsen et al. 2016b) and even the interior of the OMZ itself (Thomsen et al. 2016a) on temporal scales of days. However, estimates of the ventilation efficiency of these processes generally average out temporal dynamics, so eddy transport has often been neglected from global ocean models. It can be inferred from large eddies that smaller eddies of a few centimeters to several meters likely transport O₂ deeply into O₂-depleted waters as well but studies on these are lacking. Intrusions of oxygenated water can also be caused by diffuse groundwater seepage or underwater springs below the water surface (submarine or sublacustrine discharge). In coastal areas such as brackish estuaries, submarine groundwater discharge can be volumetrically important, accounting for an estimated 6–10%

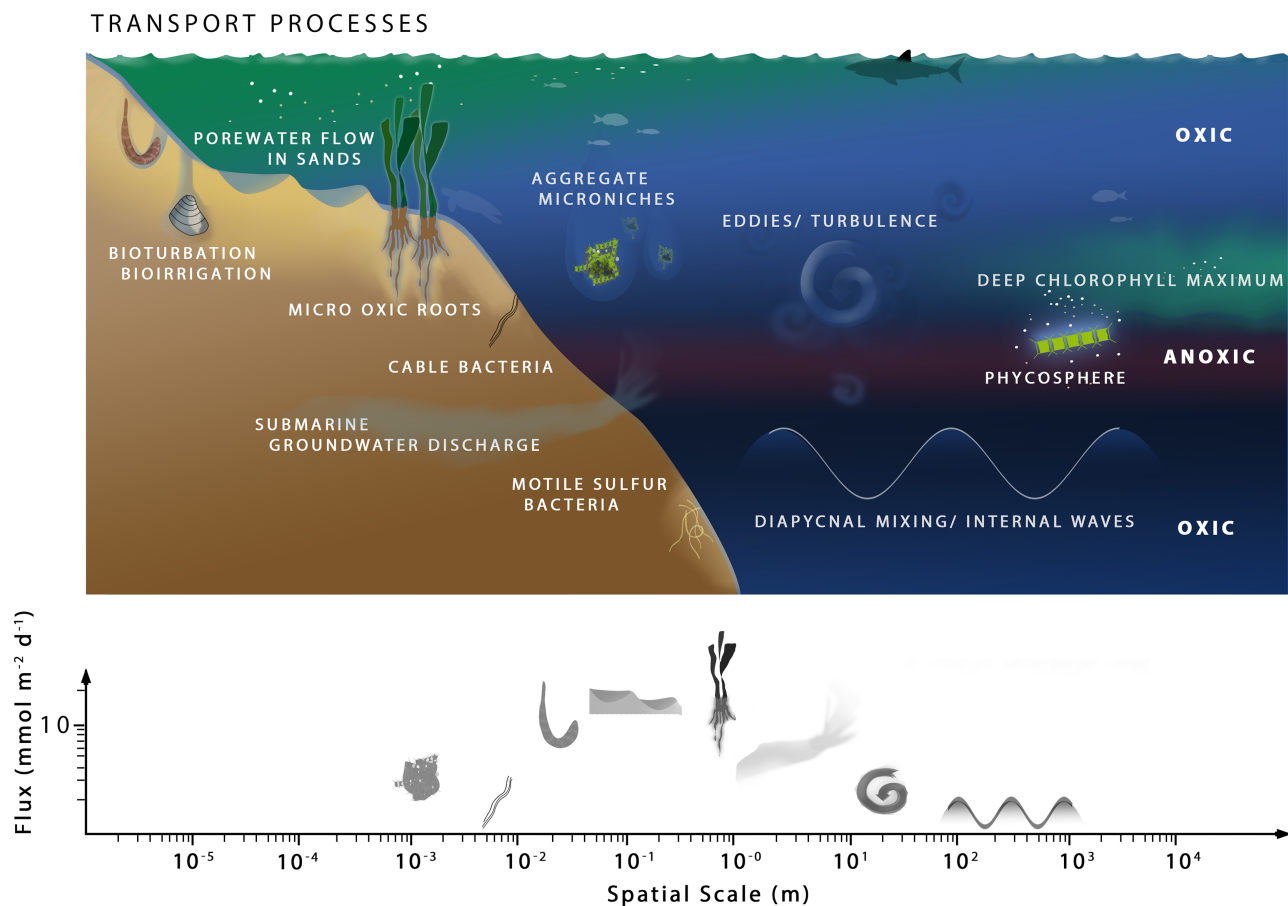


Figure 1. Chemical, physical and biological transport mechanisms of O_2 to 'anoxic' environments. Symbols in the lower panel represent the relative importance of processes shown in the upper panel. Estimates were made based on available literature for cable bacteria (Liu et al. 2021; Scilipoti et al. 2021), sinking aggregates (Ploug 2001), bioturbation (Volkenborn et al. 2007; Pischedda et al. 2008; Santos, Eyre and Huettel 2012), porewater flow in sands (Ahmerkamp et al. 2015), aquatic plant roots (Pedersen et al. 1998; Jensen et al. 2005), groundwater discharge (Taniguchi et al. 2002; Moore 2010), eddies/turbulence (Fiedler et al. 2016; Czeschel et al. 2018) and diapycnal mixing/internal waves (Fischer et al. 2013). Although these studies are mostly from the marine biome, these processes are relevant to lakes, wetlands and soils with the exception of large eddies. Approximations should be taken with a grain of salt due to the limited number of quantitative estimates for some processes, and fluxes of some processes were too uncertain to be scaled.

of surface discharge to the oceans globally, and up to 85% locally (Taniguchi et al. 2002). Groundwater further stimulates aerobic respiration via concomitant supply of dissolved organic carbon, methane, and nutrients such as ammonium, nitrate and phosphate alongside O_2 (Moore 2010).

Overall, the persistence of O_2 -depleted environments despite the numerous physical and biological inputs of O_2 (which are not limited to those described here) highlights the importance of the O_2 -consuming processes there. It is thus important to recognize that fluxes of O_2 on various temporal and spatial scales, rather than absolute concentrations, are important for maintaining anaerobic life. Yet, the occurrence of aerobic respiration is still too often dismissed in environments where O_2 concentrations are below detection levels.

Terminal oxidases are transcribed by microorganisms in environments considered functionally anoxic

The prevalence of persistent O_2 fluxes into ostensibly anoxic waters is reflected by the number of facultatively and obligately aerobic microorganisms present in these habitats. Based on the absence of measurable O_2 , however, these obligate aerobes de-

tected in the anoxic core of marine OMZs (Thamdrup et al. 2019) or anoxic lake sediments (Jiang et al. 2009) have been assumed to be metabolically inactive, but perhaps unjustly so. In fact, the capacity to respire O_2 appears to confer an obvious ecological advantage to microorganisms in environments classified as anoxic and is proving phylogenetically more widespread than previously recognized. Perhaps the most telling evidence is that virtually all microorganisms capable of anaerobic denitrification also retain the capacity to respire O_2 with very few known exceptions (Kuypers, Marchant and Kartal 2018; Graf et al. 2021). Not only do the majority of sequenced microbial genomes encode the capacity for microaerobic respiration (Morris and Schmidt 2013), but also some organisms typically considered strict anaerobes such as sulfate-reducing bacteria, anoxygenic phototrophic bacteria (Chromatiaceae) and intestinal *Bacteroides* have been shown to couple O_2 reduction to growth (Kampf and Pfennig 1980; Baughn and Malamy 2004; Berg et al. 2019; Schoeffler et al. 2019). Even some members of the green sulfur bacteria (Chlorobiaceae), until now considered strict anaerobes, encode genes for O_2 reduction (Ducluzeau, Ouchane and Nitschke 2008), but whether these are utilized for energy conservation has not been demonstrated.

Most of biological O_2 reduction to water is catalyzed by the heme-copper oxygen reductases (HCORs) that directly conserve energy through the generation of a proton motive force (Wikström

and Verkhovsky 2007). Based on the structure of their substrate binding sites and proton translocating channels, HCOs can be classified into three groups: A-, B- and C-type enzymes (Sousa et al. 2012). The three HCO types display different affinities toward O_2 : A-type HCOs are commonly referred to as low-affinity terminal oxygenases (LATO) with K_m values for O_2 that are above 200 nM (reviewed in Degli Esposti et al. 2019), while B- and C-type HCOs are so-called high-affinity terminal oxygenases (HATO) with K_m values as low as 3–8 nM (D'mello, Hill and Poole 1996). The catalytic subunit (subunit I) is the only protein common to all HCO types. Subunit I also exhibits structural similarity with the Nitric-Oxide reductases (NORs), which catalyze the reduction of NO to N_2O , but which do not all necessarily drive proton translocation (Al-Attar and de Vries 2015; Vázquez-Torres and Bäumlner 2016). While these enzymes appear to share common ancestry, whether they primordially functioned as an O_2 or NO reductase is still a matter of debate (Vries and Schröder 2002; Brochier-Armanet, Talla and Gribaldo 2009; Ducluzeau et al. 2009). Besides by HCOs, O_2 reduction in prokaryotes is often catalyzed by a second group of unrelated, respiratory oxidases: the cytochrome *bd* quinol oxidases that, like B- and C-type HCOs, are considered to be HATOs (Borisov et al. 2011). However, the presence and expression of cytochrome *bd* quinol oxidases is not a robust indicator for O_2 respiration, as they have also been shown to be involved in oxidative and nitrosative stress defense in various bacterial species (Giuffrè et al. 2014).

Additionally, there are actually a plethora (~720 identified so far) of other enzymes such as monooxygenases and dioxygenases utilizing O_2 as an electron acceptor, and it was recently found that the presence of genes encoding these proteins in microbial genomes are actually a good indicator of aerobic versus anaerobic lifestyle (Jabłońska and Tawfik 2019). Nevertheless, HCOs remain the most commonly employed indicators of aerobic respiration and were therefore the focus of our metasurvey of environmental transcriptomes evaluating the ecological prevalence of this metabolism.

It has been shown that different HCOs are not confined to a specific phylogenetic group of microorganisms, or a defined environmental niche (Sousa et al. 2012; Morris and Schmidt 2013) (see also Fig. 2A). The subgroup of C-type HCOs, cytochrome *cbb3* oxidase, for example, is distributed across all bacterial phyla with the exception of Thermotogales, Deinococcales and Firmicutes suggesting that the capacity for O_2 respiration at extremely low concentrations is a primordial and valuable function for survival (Ducluzeau, Ouchane and Nitschke 2008). Based on genetic information alone, it cannot be inferred whether terminal oxidases are utilized, and whether O_2 reduction is linked to energy conservation. Nevertheless, there is emerging evidence from metatranscriptomic data (Karsenti et al. 2011; Kalvelage et al. 2015; Kitlinger et al. 2019) as well as direct activity measurements of microorganisms in pure culture (Cypionka 2000; Schoeffler et al. 2019) or in single cells of environmental bacteria (Berg et al. 2019) that both aerobic and anaerobic organisms can respire O_2 in environments typically considered anoxic and link this metabolism to growth. Our survey of publicly available metatranscriptomes from oxic ($>10 \mu\text{mol } O_2 \text{ L}^{-1}$), microoxic (6–10 $\mu\text{mol } O_2 \text{ L}^{-1}$), sub-oxic (1–5 $\mu\text{mol } O_2 \text{ L}^{-1}$) and nanoxic ($<1 \mu\text{mol } O_2 \text{ L}^{-1}$) environmental water samples shows that the genetic capacity for nanaerobic respiration is actually transcribed in ostensibly anoxic aquatic habitats (Fig. 2B). In low- O_2 environments, the relative transcription of high-affinity HCOs appears higher than that of low-affinity HCOs in the dataset presented here. However, transcripts of low-affinity HCOs were also detected in low- O_2 environments, even

in nanoxic ones. These low-affinity HCOs could be constitutively transcribed, or they may also be active in low- O_2 environments. In fact, a recent study experimentally demonstrated that low-affinity HCOs actually can enable aerobic respiration by some *Acidobacteria* at O_2 levels as low as 1 nmol L^{-1} , suggesting that they do play a broader environmental role than currently recognized (Trojan et al. 2021). It is important to note that despite the large number of publicly available environmental metatranscriptomic datasets, only a limited number of metatranscriptomes could be included in this analysis ($n = 38$), due to the lack of linked geochemical data, namely O_2 concentration measurements, which are essential for establishing a clear connection between microbial activity (metatranscriptomes) and environmental conditions.

Of course, some caution must be taken in interpreting these transcriptomic datasets that are sensitive to physicochemical variations induced by sample handling. Our metasurvey reveals that it is possible to observe transcriptional differences in HCOs that appear to correlate with the oxygenation level of investigated environments (Fig. 2B). Nonetheless, changes in aquatic medium during retrieval of environmental samples has been shown to induce physiological responses that alter the transcription profile of labile mRNA even within 30 min (Feike et al. 2012; Edgcomb et al. 2016). The ubiquitous presence of O_2 in our surroundings makes it difficult to prevent the exposure of anoxic samples to O_2 during sampling (García-Robledo et al. 2021), and even the diffusion of O_2 from the rubber seal of stoppers and lids can be a potential source of contamination (De Brabandere et al. 2012). Fortunately, promising *in situ* sample fixation technologies (Feike et al. 2012) may help us generate more accurate gene transcription and expression profiles for investigation of nanaerobic respiration at the O_2 detection limit. Spatial transcriptome imaging also promises to help visually capture microscale metabolic responses to O_2 availability *in situ* (Dar et al. 2021) but has yet to be combined with an O_2 -visualization technique. The careful recording and depositing of *in situ* physicochemical parameters and sampling procedures together with genetic data would greatly benefit future studies by providing a context for interpreting transcriptomic datasets.

Nanaerobic respiration takes place in the metabolic gray zone

We know that the capacity for O_2 respiration is globally and phylogenetically widespread but identifying where and when aerobic respiration does occur appears to be the greater challenge. Recent research is revealing that aerobic and anaerobic processes can co-occur over a broad range of O_2 concentrations in the environment, thus contradicting the concept of the microbial redox cascade. For example, anaerobic NO_3^- reduction can remain fully active in the presence of up to at least 25 $\mu\text{mol } O_2 \text{ L}^{-1}$ and, under microoxic conditions, simultaneous sulfate and O_2 reduction are also possible (Cypionka 2000; Kalvelage et al. 2011; Schoeffler et al. 2019). These findings demonstrate that while the redox cascade concept is often interpreted to mean the literal exclusion of anaerobic respiration by the presence of O_2 , the ordering of respiration processes in nature is much more nuanced, being influenced by factors other than just energetics.

From a thermodynamic standpoint, O_2 is the most favorable electron acceptor, although it yields only slightly more energy (–2870 kJ/mol glucose) than nitrate reduction to N_2 (–2670 kJ/mol glucose) under standard conditions (Strohm et al. 2007). From a bioenergetics perspective, however, the enzyme systems involved in proton translocation during aerobic respiration confer a much

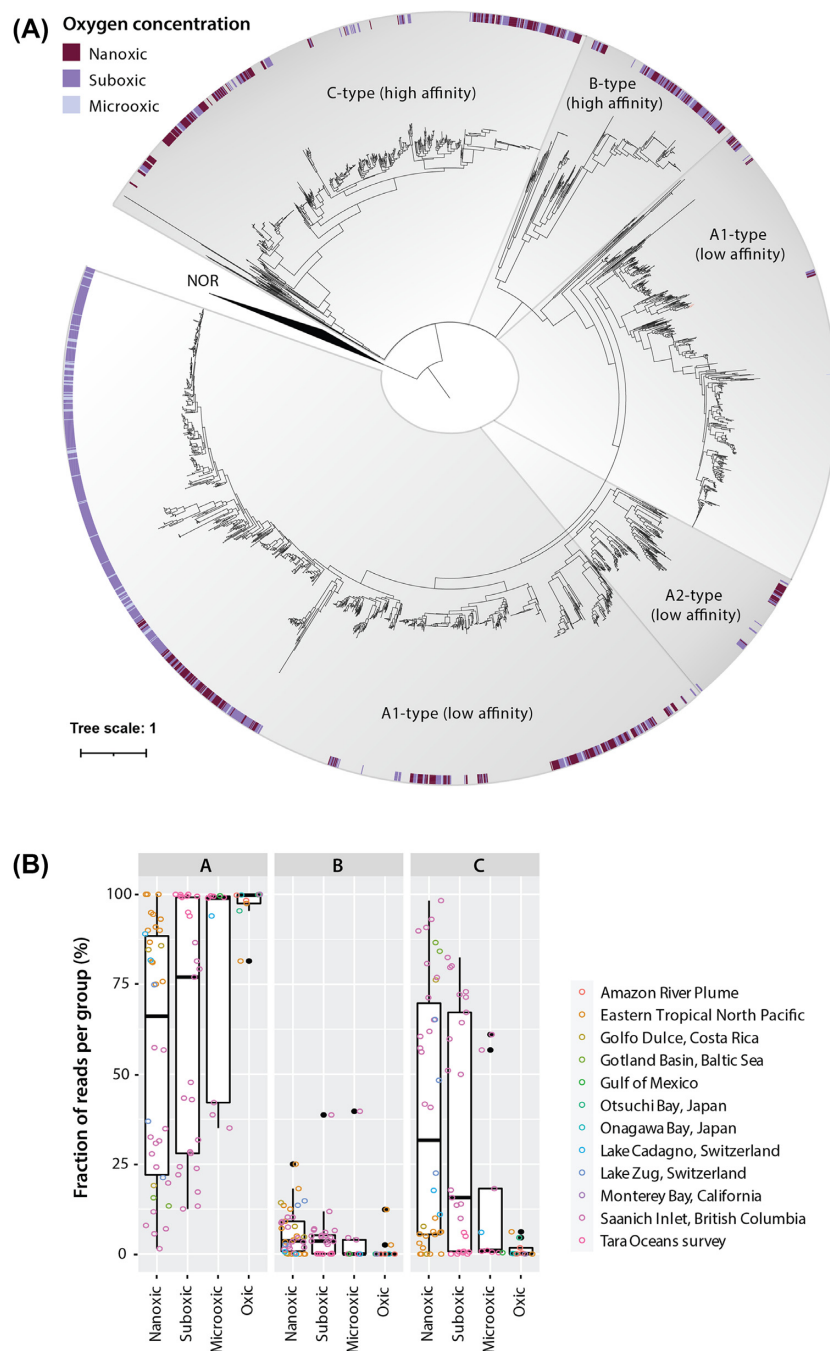


Figure 2. (A) The phylogenetic tree represents HCO amino acid sequences from the Tara Oceans and Saanich Inlet environmental metatranscriptome datasets and classified reference sequences obtained from the reference database of the HCO classifier tool (<http://www.evocell.org/hco>). This maximum likelihood approximation is based on FastTree with the LG model using NORs as the outgroup. The shading represents the HCO sequence type classification, propagated from the reference HCO dataset to classified environmental metatranscriptome sequences. The color in the outer circle (see legend) represents the degree of oxygenation of the source environment from which environmental sequences were derived and reference sequences remain uncolored. An interactive searchable version of the tree is available online at <https://itol.embl.de/tree/1311303419282761574438152>.

(B) The relative expression of terminal oxidases HCO types A, B and C in publicly available metatranscriptomes from oxygen-limited and selected O_2 nonlimited, aqueous environments for which background O_2 concentration data have been recorded. High-affinity oxidases are more highly expressed in low- O_2 environments classified as follows: oxic ($>11 \mu\text{mol } O_2 \text{ L}^{-1}$), suboxic ($6\text{--}10 \mu\text{mol } O_2 \text{ L}^{-1}$), microoxic ($1\text{--}5 \mu\text{mol } O_2 \text{ L}^{-1}$) and nanoxic ($<1 \mu\text{mol } O_2 \text{ L}^{-1}$). Individual metatranscriptomic datasets are represented as colored dots and outliers are indicated by adjacent black dots. The Y axis represents the number of HCO reads for each category divided by the number of HCO reads in all categories, calculated separately for each selected metatranscriptome (more details in the Supporting Information). Locations of the sampled environments are indicated in Fig. 4.

greater advantage (50% of energy is conserved) over denitrification (30%) (Chen and Strous 2013). Moreover, aerobic respiration is very efficient, requiring only one enzyme in addition to the core electron transport chain. Maintaining the cellular capacity for aerobic respiration thus requires a lower energy investment than for nitrate respiration and occupies very little of the cytoplasmic membrane surface area, not counting the defense machinery present in most microorganisms (even strict anaerobes) to protect against reactive oxygen species (Lu and Imlay 2021). The benefits of manufacturing the enzymes to reduce O_2 even under O_2 -limiting conditions certainly outweigh the costs given that unprotected exposure to such an oxidizing molecule would otherwise result in the inhibition of anaerobic enzymes with redox-sensitive metal centers, or in cellular damage by reactive oxygen species (Lu and Imlay 2021).

Because energy yield depends on the concentration of reactants, decreasing O_2 concentration at some point tips the balance so that another electron acceptor becomes more favorable. What, then, is the threshold O_2 level triggering a switch in metabolism and what is the minimum O_2 concentration microorganisms can respire? The theoretical limit at which aerobic respiration can occur has been calculated in the range of 0.1 to a few hundred nanomolar and depends on cell size (diffusive surface area), growth efficiency (moles of biomass synthesized per mole of oxygen) and temperature (diffusion coefficient) (Zakem and Follows 2017). Below this dynamic O_2 limit for aerobic microbial growth, aerobic and anaerobic respiration can nonetheless coexist to varying degrees.

In practice, the lower limit to aerobic respiration has been observed to vary from organism to organism depending on the types of enzymes (high or low O_2 affinity) and alternative respiratory pathways they possess. Below a certain threshold concentration, O_2 reduction alone may be insufficient to provide the energy required for maintenance and microbial growth triggering a switch in metabolism. However, it is not known whether this switch occurs at the microbial community, population or single-cell level. Microbial communities have been observed to perform aerobic and anaerobic respiration simultaneously (Marchant et al. 2017), and this metabolic differentiation is at least partly determined by species adaptations to different O_2 concentrations. Metabolic heterogeneity also exists within microbial populations (Koch et al. 2015; Schreiber et al. 2016) and it is possible that even a single cell can simultaneously utilize multiple metabolic pathways (Chen and Strous 2013). However, it is extremely difficult to demonstrate such metabolic diversification at the cellular level since O_2 and other terminal electron acceptors are dissimilated during respiration.

The co-occurrence of aerobic and anaerobic respiration and the widespread phylogenetic distribution of hybrid electron transport chains accommodating more than one type of electron acceptor certainly cast doubt on the validity of our concepts of distinct aerobic and anaerobic lifestyles. Moreover, variations of classical aerobic respiration are constantly being discovered. External electron transfer, for example, allows bacteria to bridge a spatial gap between a cell and an external oxidant. Filamentous cable bacteria are composed of individual, interconnected cells, of which only the uppermost <10% are exposed to O_2 and exhibit highest biomass-specific O_2 consumption rates known to date for a prokaryote (Scilipoti et al. 2021). This enables the cells in the lower part of the filament to subsist on aerobic respiration in the absence of O_2 . However, these organisms do not possess any canonical terminal oxidases. Instead, a unique cytochrome-truncated hemoglobin fusion protein has been hypothesized to catalyze

periplasmic O_2 reduction (Trojan et al. 2016). Another interesting newly discovered mechanism of external electron transfer has been demonstrated in *Pseudomonas aeruginosa* biofilms (Saunders et al. 2020). It was found that redox-active shuttles called phenazines are bound to extracellular DNA (originating from dead cells) and mediate efficient electron transfer from *P. aeruginosa* cells within the anoxic biofilm interior to surrounding oxygenated medium. This adds to our inventory of external electron transfer mechanisms known mostly from mineral-reducing bacteria such as *Geobacter* and *Shewanella* (Richter, Schicklberger and Gescher 2012).

Yet another intriguing respiration mechanism is the intra-aerobic pathway of nitrite-dependent methane oxidation of *Candidatus Methyloirabilis oxyfera* (Ettwig et al. 2010). This bacterium inhabits anoxic habitats and dismutates NO intracellularly to form N_2 and O_2 , the latter of which is used mainly for methane activation and oxidation, in addition to respiration (Ettwig et al. 2010; Wu et al. 2011). Interestingly, *Ca. M. oxyfera* encodes at least four terminal oxidases, but exposure to microoxic conditions has a detrimental effect on growth (Luesken et al. 2012). Since the discovery of *Ca. M. oxyfera*, NO dismutation genes have been found to be phylogenetically and environmentally widespread (Zhu et al. 2017). Recent results, indicate that an ammonia-oxidizing archaeon might also produce its own molecular O_2 from nitrite (Kraft et al. 2022). These intriguing metabolic strategies highlight that the classification of aerobe/anaerobe is nonbinary and yet has profoundly shaped the vocabulary we use to describe microbial adaptations to the environments around us.

Nanaerobic respiration on early earth to the present

Since the emergence of oxygenic photosynthesis 2.7–2.5 billion years ago, the subsequent evolution of life on Earth has involved continuous adaptation to more O_2 . However, alternative O_2 sources may have enabled the evolution of aerobic respiration even prior to this. The radiolysis and photolysis of water, for example, could have generated peroxides and other radicals that decomposed to O_2 (Chen and Taylor 1957; Pastina and LaVerne 1999). Perchlorates and NO present in the primordial atmosphere (Dasgupta et al. 2005; Martin, Mather and Pyle 2007) could also have been converted to O_2 via respective microbial dismutation pathways (Ettwig et al. 2012). While these sources of O_2 were likely very minor, especially considering the abiotic reductants abounding in the reducing Archean atmosphere and oceans, the extremely low amounts of O_2 may have been sufficient for nanaerobic microbial respiration. After the emergence of oxygenic photosynthesis but prior to the great oxygenation event, there is geochemical evidence for whiffs of O_2 to the Earth's atmosphere (Anbar et al. 2007; Koehler et al. 2018), which may have stimulated the early proliferation of nanaerobic life. While these whiffs of O_2 remain contested (e.g. Ward, Kirschvink and Fischer 2016), nanaerobes could have thrived in microenvironments in close proximity of O_2 -producing cyanobacteria, similar to observations from modern ancient ocean analogues (Milucka et al. 2015).

It is difficult to identify conclusive fossil evidence for aerobic microbial metabolism on early Earth, but gene histories tell an interesting story. The birth of most redox-sensitive and O_2 -utilizing protein families, such as superoxide dismutase, nonspecific peroxidases and the NAD(P)H: oxygen oxidoreductase protein family (Raymond and Segrè 2006), has been dated to the time period referred to as the Archean Expansion ~3.33–2.85 billion years ago (David and Alm 2011; Wang et al. 2011; Kim et al. 2012; Jabłońska

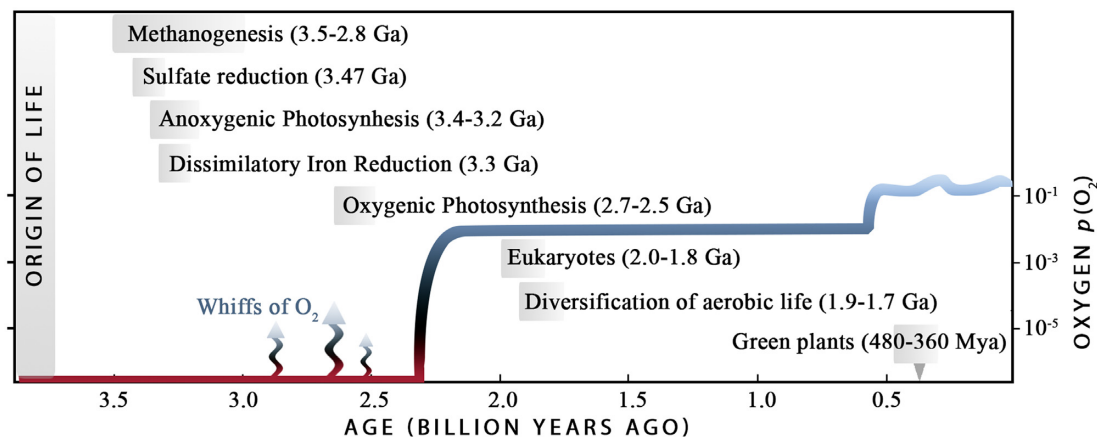


Figure 3. Biogeological timeline showing the evolution of microbial metabolisms in parallel with the rise in atmospheric oxygen concentrations. Modified from Lyons et al. (2014). Dates for the emergence of life (Arndt and Nisbet 2012), methanogenesis (Ueno et al. 2006; Wolfe and Fournier 2018), anoxygenic photosynthesis (Tice and Lowe 2004; Butterfield 2015), sulfate reduction (Shen and Buick 2004), iron reduction (Lovley 2004), oxygenic photosynthesis (Buick 2008; Farquhar, Zerkle and Bekker 2011), eukaryotes (Eme et al. 2014; Betts et al. 2018), diversification of aerobes (Wang et al. 2011; Blank 2013) and green plants (Kenrick and Crane 1997) were obtained from the literature available at the time of writing. Gray boxes represent the uncertainties surrounding these dates.

and Tawfik 2021), long before the great oxygenation event and even before the first conclusive evidence of oxygenic photosynthesis. It was shown that the four major families of O_2 -reductases evolved separately and followed different evolutionary histories. Most notably, the low affinity A-type HCOs were already present prior to the divergence of major present-day bacterial and archaeal phyla, thus predating the emergence of cyanobacteria and oxygenic photosynthesis (Brochier-Armanet, Talla and Gribaldo 2009). While the presence of a single O_2 -reductase does not imply an obligately aerobic lifestyle and an alternative function of these primitive enzymes be ruled out, their early evolution could suggest an O_2 -scavenging response to minute concentrations of free O_2 (Planavsky and Mills 2021).

The first oxidoreductases may have evolved as detoxification mechanisms rather than energy conserving mechanisms but coupling O_2 reduction to proton translocation appears to be a surprisingly small evolutionary step. In fact, this type of evolution has been observed in pure cultures of *Desulfovibrio vulgaris* Hildenborough, which after 114 generations of growth under O_2 stress, were able to grow at O_2 concentrations 32 times higher than the ancestral strain and conserve energy from this process (Schoeffler et al. 2019). Energy conservation evolved separately in two of the *Desulfovibrio* daughter strains, one via a single point mutation and one via gene deletion. Thus, on a genetic level, very few changes are required for the transition from O_2 detoxification to proton translocation. Detoxification via O_2 reduction is energetically costly, e.g. it has been shown that under normal O_2 concentrations, most of the energetic costs of N_2 fixation (~60%) are derived from the protection of O_2 -sensitive nitrogenase enzymes by removal of intracellular O_2 or by the reversal of oxidative damage (Großkopf and LaRoche 2012). It therefore makes evolutionary sense to harness the oxidative power of O_2 with proton pumping.

In the modern oxidized surface world, protection against oxidative stress is a requirement for survival and as a consequence, O_2 -reducing enzymes are phylogenetically widespread. The phenomenon of O_2 respiration at ultralow concentrations suggests not only that O_2 reduction is more common among anaerobes than currently recognized, but also that it played an important role in the evolution of life on early Earth. Although it is currently impossible to resolve the evolutionary order and history

of microbial metabolisms, sulfate reduction, denitrification and manganese/iron reduction are postulated to be some of the earliest metabolisms to have evolved on Earth (Fig. 3). These anaerobic metabolisms rely on terminal electron acceptors such as sulfate, oxidized nitrogen species, iron- and manganese oxides, which may have been formed to a small extent by lightning, photochemical reactions and anoxygenic photosynthesis prior to the great oxygenation event (Canfield and Raiswell 1999; Wong et al. 2017; Schad et al. 2019; Lyons, Diamond and Konhauser 2020). Somewhat ironically, it was the rise of free O_2 that induced rapid changes in ocean-atmosphere chemistry, producing large amounts of these alternative electron acceptors that enabled the proliferation of anaerobic life as well. For example, it may have been the enhanced oxidative weathering of sulfides on land and the onset of marine sulfur redox cycling following the great oxygenation event that stimulated anaerobic methane oxidation, removing a main source of greenhouse gas to the atmosphere and resulting in the large-scale climate cooling at the end of the Proterozoic Eon (~2.4 Ga) (Fakraee et al. 2019).

While nanoxic environments were likely more prevalent on early Earth, nanoxic conditions can still be found in today'soxic world as microscale niches in marine snow to mesoscale zones in stratified lakes and OMZs in the ocean. OMZs have been the focus of intense study as hot spots of fixed nitrogen loss from the world's oceans via anaerobic respiration, accounting for 30–50% of global oceanic N loss (Codispoti et al. 2001). Respiration of O_2 in OMZs also significantly contributes to biogeochemical processes such as ammonium, nitrite, methane and sulfide oxidation (Füssel et al. 2012; Beman, Leilei Shih and Popp 2013; Kalvelage et al. 2015; Bristow et al. 2016). Ocean models have long used a threshold of 1–10 $\mu\text{mol L}^{-1}$ (Paulmier and Ruiz-Pino 2009; Deutsch et al. 2011; Gutknecht et al. 2013; Lima, Lam and Doney 2014) to define anoxia even though the O_2 concentration limit for nanaerobic respiration is clearly several orders of magnitude lower. Recently, Zakem and colleagues proposed a new model based on resource ratio theory, or the ratio of O_2 to electron donor supply rather than an O_2 -based threshold (Zakem et al. 2020). This model, which takes into account the coexistence of aerobic and anaerobic metabolisms at nanomolar O_2 concentrations, has already proven to be an accurate predictor of anaerobic activity and nitrogen loss in OMZs

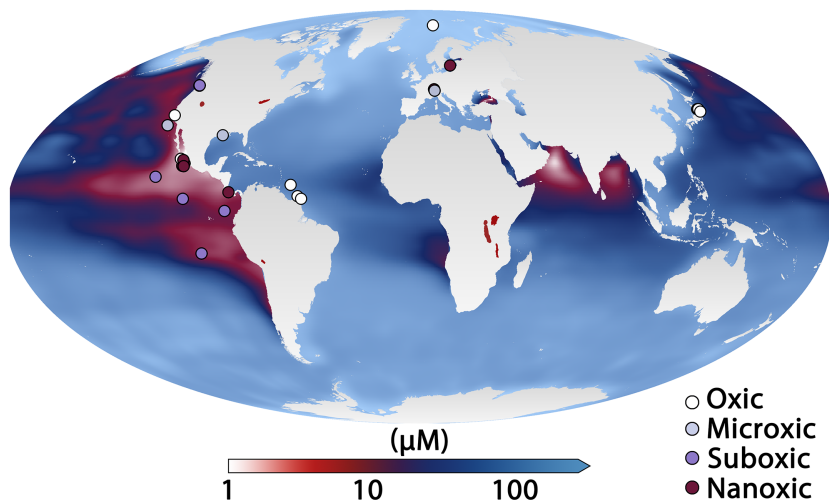
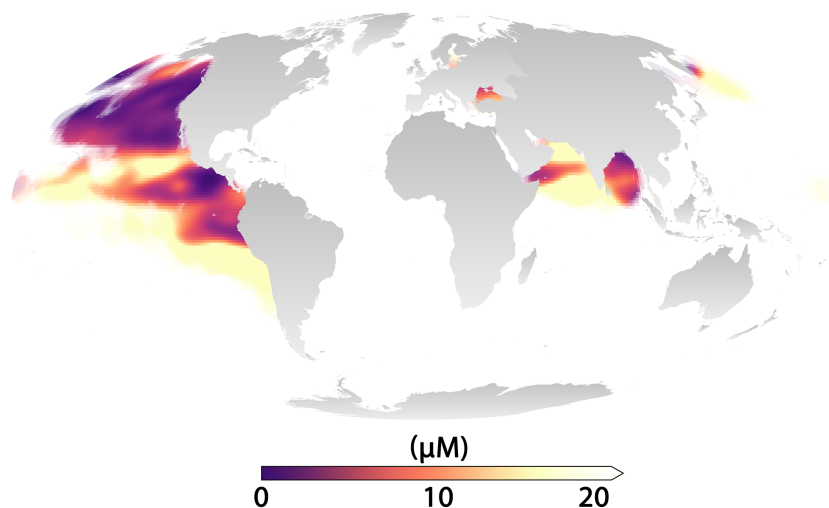
(A) OXYGEN CONCENTRATION**(B) OXYGEN VARIABILITY**

Figure 4. (A) World map of minimum O_2 concentrations in aquatic environments derived from the World Ocean Atlas. For each region, the minimum value from the entire water column depth is shown. Dots depict the sampling locations included in our metatranscriptomic survey. (B) Deviations from the minimum O_2 concentrations in panel (A) over time (data on lake O_2 was not available). Essentially, the huge seasonal variability of dissolved oxygen concentrations emphasizes that microorganisms have had to adapt to huge variations in O_2 over both space and time.

(Zakem et al. 2021). In fact, anthropogenic activity together with global climate change are leading to an expansion of these anoxic zones worldwide (Fig. 4) (Stramma et al. 2008; Jenny et al. 2016). Nanoxic aquatic habitats are expected to increase disproportionately in volume as according to models, a reduction of the mean upper ocean O_2 content by only 1% would mean a doubling of water masses with $O_2 \leq 5 \mu\text{mol L}^{-1}$ due to the lower solubility of O_2 in warmer waters (Deutsch et al. 2011). Microbial activity in low- O_2 waters will thus play an increasingly important role in biogeochemical cycling, making it ever more important to revise our models to correctly estimate the contribution of nanaerobic respiration to ocean carbon turnover and its link to the global climate system.

Future directions of nanaerobic research

In the environment, nanaerobic respiration remains a largely overlooked process with an unknown role in ecosystem- and

global-level biogeochemical cycling. For example, the importance of nanaerobic respiration relative to abiotic O_2 -scavenging reactions at ultralow O_2 concentrations in the environment remains unconstrained. Since the vast majority of microorganisms remains uncultivated, we strongly depend on culture-independent methods such as gene transcription and expression analyses to elucidate the *in situ* activity and identity of nanaerobes. However, it is imperative that geochemical data, specifically O_2 concentrations, be recorded simultaneously and linked to this genetic information to better constrain the role of nanaerobic respiration in the environment. We can indirectly link nanaerobic respiration to specific microbial taxa via single-cell activity measurements (i.e. nanoSIMS) combined with bulk O_2 -sensing technologies (Berg et al. 2019). Nonetheless, further methodological innovations are necessary to directly quantify nanaerobic respiration at the single-cell level, which is essential to disentangle the contribution of microbial versus abiotic O_2 -consuming reactions.

There is ample evidence for aerobic and anaerobic processes co-occurring at the microbial community level in environments ranging from OMZs (Lam and Kuypers 2011; Zakem et al. 2020) to wastewater (Rajta et al. 2020) to human dental plaque (Schreiber et al. 2010). New experimental approaches using microfluidic devices have great potential to study the impact of microscale heterogeneity in O_2 concentrations and fluxes on the co-occurrence of aerobic and anaerobic microbial respiration (e.g. Smriga, Ciccarese and Babbini 2021). Aerobic and anaerobic respiration might, however, also occur simultaneously within a single microbial cell but direct evidence for this is so far missing. Moreover, for many environmentally relevant microbial taxa, the biochemical mechanisms regulating the switching point from aerobic to anaerobic respiration is still unknown. A better mechanistic understanding could serve practical bioengineering purposes of maintaining simultaneous aerobic and anaerobic processes that are favorable for wastewater treatment and soil remediation (Yang et al. 2020).

As a result of rapid, anthropogenic-driven change, hypoxic waters are expanding globally with drastic consequences for aquatic animals, such as fish, which cannot survive at low O_2 levels (Diaz 2001). Likely, nanaerobic respiration will gain importance for carbon turnover, nitrogen loss and phosphorus remineralization. So far, anaerobic processes are generally presumed to be largely responsible for biogeochemical element cycling in anoxic waters, such as the core of OMZs where O_2 is below detection limits (Bertagnolli and Stewart 2018). Besides abundant NO_x species, a number of other less common alternative electron acceptors such as iodate, perchlorate and phosphite have been proposed to drive microbial metabolisms in such environments (Coates and Achenbach 2004; Ewens et al. 2021; Reyes-Umana et al. 2021). However, nanaerobic microorganisms appear well adapted to and thrive under ultralow O_2 conditions, even at concentrations below the detection limits of our sensors. This nanaerobic respiration is fueled by the continuous transport of O_2 into O_2 -depleted environments where it is consumed faster than it can accumulate. As such, the flux of O_2 , rather than its absolute concentration, is important for sustaining microbial respiration. Innovative methods are needed to better constrain O_2 fluxes into functionally anoxic zones.

The knowledge that prokaryotic microorganisms can respire O_2 where none can be detected also raises interesting questions about eukaryotic life in such habitats and the physiological mechanisms that they employ to thrive under nanoxia. Several such (unicellular) eukaryotic adaptations to O_2 -limited environments are already known and involve physiological (e.g. expression of alternative quinones; Anand et al. 2015) or structural changes (e.g. localization of mitochondria close to the outer cell membrane; Fenchel 2014) or symbioses with anaerobic bacteria (Graf et al. 2021). Recognizing the prevalence of these mechanisms among free-living eukaryotes can help us to better constrain the impact of nanaerobic metabolism on the ecology and evolutionary history of eukaryotes.

Supplementary data

Supplementary data are available at [FEMSRE](https://femsre.onlinelibrary.wiley.com/doi/10.1111/femsre.12500) online.

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