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Review paper

Protozoa and Oxygen

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Abstract. Aerobic protozoa can maintain fully aerobic metabolic rates even at very low O_2-tensions; this is related to their small sizes. Many – or perhaps all – protozoa show particular preferences for a given range of O_2-tensions. The reasons for this and the role for their distribution in nature are discussed and examples of protozoan biota in O_2-gradients in aquatic systems are presented. Facultative anaerobes capable of both aerobic and anaerobic growth are probably common within several protozoan taxa. It is concluded that further progress in this area is contingent on physiological studies of phenotypes.

Key words: Protozoa, chemosensory behavior, oxygen, oxygen toxicity, microaerobic protozoa, facultative anaerobes, microaerobic and anaerobic habitats.

INTRODUCTION

Increasing evidence suggests that the last common ancestor of extant eukaryotes was mitochondriate and had an aerobic energy metabolism. While representatives of different protist taxa have secondarily adapted to an anaerobic life style, all known protists possess either mitochondria capable of oxidative phosphorylation or – in anaerobic species – have modified mitochondria that play a different role in energy metabolism or at least maintain some other functions of mitochondria; these modified mitochondria are termed hydrogenosomes and mitosomes, respectively (Van der Giezen 2011). Most anaerobic protozoa depend on different types of fermentative metabolism producing various low molecular weight organics and in some cases H_2 as metabolic end products. Some ciliates and foraminifera use nitrate as a terminal electron acceptor in a respiratory process (for a review on anaerobic protozoa, see Fenchel 2011).

The great majority of protozoan species, however, depend on aerobic energy metabolism. Among protists with an aerobic metabolism many – or perhaps all – show preferences for particular levels of oxygen tension below atmospheric saturation. This represents an important niche component and an important determinant for the spatial distribution of microorganisms in nature. There are several reasons for this including oxygen toxicity, correlation between oxygen tension and the distribution of preferred prey items, and oxygen requirements of symbionts (Fenchel and Finlay 2008).

In the tradition of bacteriologists we can distinguish between obligate anaerobes that only possess an anaerobic energy metabolism and that are to a variable

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degree sensitive to exposure to oxygen (Fenchel and Finlay 1990), microaerophiles, and aerobes. Here I arbitrarily define microaerophiles as species that grow best and show chemosensory preference for O_2 tensions somewhere within the range 0–10% atmospheric oxygen saturation. In at least some cases these show diminished growth rates or other signs of decreased fitness at higher oxygen tensions. Finally I refer to aerobes as forms that normally occur at higher O_2-tensions. Facultative anaerobes are species that can grow aerobically, but are also capable of sustained balanced growth under strict anaerobic conditions, albeit with correspondingly lower growth rates and cell yields (Bernard and Fenchel 1996). In all studied cases, protozoa show chemosensory motile responses to O_2-tension, something that may be a universal trait of motile microbes (Fenchel and Finlay 2008).

The present paper discusses the physiological and behavioral responses to pO_2 and habitats characterized by O_2-gradients in nature. It is emphasized that responses to O_2-tension is a significant aspect of protozoan ecology.

PHYSIOLOGICAL ASPECTS

Respiration rate as function of ambient O_2 concentration

The uptake rate of a solute by a spherical cell R is given by $R = 4D\pi r'[C(\infty) - C']$ where D is the diffusion coefficient of the solute (here for O_2 in water), r' is the radius of the cell, and $C(\infty)$ and C' are the O_2 concentration far away from the cell (bulk O_2 concentration) and the O_2 concentration at the cell surface, respectively (Berg 1983). Obviously, the maximum uptake is achieved by minimizing C'. There will, however, always be a maximum potential rate of O_2 uptake, R_m (dimension T^{-1}) that is realized under otherwise optimal conditions and when an ambient O_2 concentration is not limiting. If the maximum uptake rate, R_m, is sufficiently high, the cells could in principle be able to reduce C' to zero. However, R_m is finite and we can then assume that the oxygen uptake as a function of ambient O_2 is given by $R = 4D\pi r'R_mC(\infty)[1 - R/R_m]$. Solving for R we find that $R = R_mC(\infty)/[K_m + C(\infty)]$ where $K_m = R_m/(4D\pi r')$ and $[C(\infty) - C'] = C(\infty)K_m/[K_m + C(\infty)]$. This is Monod kinetics and K_m is the half saturation constant; that is, the bulk O_2 concentration that allows for an uptake that is half that of R_m. Assuming that R_m scales as (cell volume)$^{3/4}$ or $(r')^{9/4}$ (Fenchel and Finlay 1983), K_m will be proportional to $(r')^{1.25}$. Thus K_m decreases with decreasing cell size and small aerobic organisms can thus cope with very low oxygen concentrations. Especially larger cells typically diverge from a spherical shape, but then some linear dimension instead of r' will still approximately apply.

Figure 1 shows an example of data on the respiratory rate of a ciliated protozoan, *Euplotes* sp. (Fig. 2b) as function of ambient O_2 tension and Fig. 3 shows empirically determined values of K_m for different sized unicellular organisms and for isolated mitochondria as function of their linear dimensions. The conclusion of this is that small aerobic protozoa can approach their maximum O_2 uptake rate even at very low ambient O_2 concentrations.

Why are so many protozoa microaerophiles?

That oxygen toxicity for microaerophilic prokaryotes is caused by the formation of oxygen radicals combined with a limited capacity for detoxifying them is well established in the case of prokaryotes (e.g. Krieg and Hoffman 1986), but evidence is more limited in the case of eukaryotic microbes. It was found that while the O_2 uptake of the *Euplotes* sp. (Fig. 1) increased with increasing ambient O_2 concentration up to 100% atm. sat., the growth rate and cell yield were maximized at an ambient O_2 tension of 4–5% atm. sat., and cell yield as well as the growth rate constant decreased by about 30% when grown under atmospheric saturation (Fenchel *et al.* 1989). This perhaps represents the energetic costs of detoxification of oxygen radicals. The response to O_2 tension of the freshwater ciliate *Loxodes* is light dependent. In darkness the ciliate has a preference for an O_2 tension of 5–10%, but at a sufficiently high light intensity it prefers anoxia (Fenchel and Finlay 1984). This ciliate is a facultative anaerobe that can use nitrate reduction under anoxia (Finlay *et al.* 1983). The reason for the increased oxygen sensitivity when exposed to light may relate to the fact that the pigments of *Loxodes* causes photochemical generation of superoxide when illuminated in the presence of oxygen (Finlay *et al.* 1986). Whether this explanation also applies to photophobic responses of other pigmented ciliates such as species of *Blepharisma* and *Stentor* (e.g. Matsuoka 1983) remains to be studied. It has been shown that some anaerobic protozoa in the presence of oxygen have an O_2 uptake that is not coupled to energy conservation and this has been interpreted as a protection mechanism (Fenchel and Finlay 1990; Lloyd *et al.*

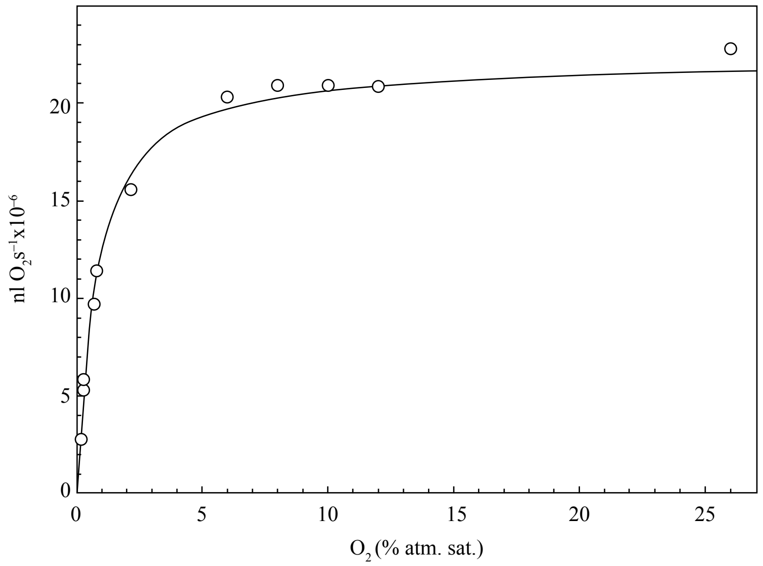

Fig. 1. Respiration rate of a *Euplotes* sp. as function of bulk O_2 tension fitted to a Monod function (R_m = 2.2×10^{-5} nl s.$^{-1}$; K_m = 0.8% atm. sat.). Data from Fenchel *et al.* (1989).

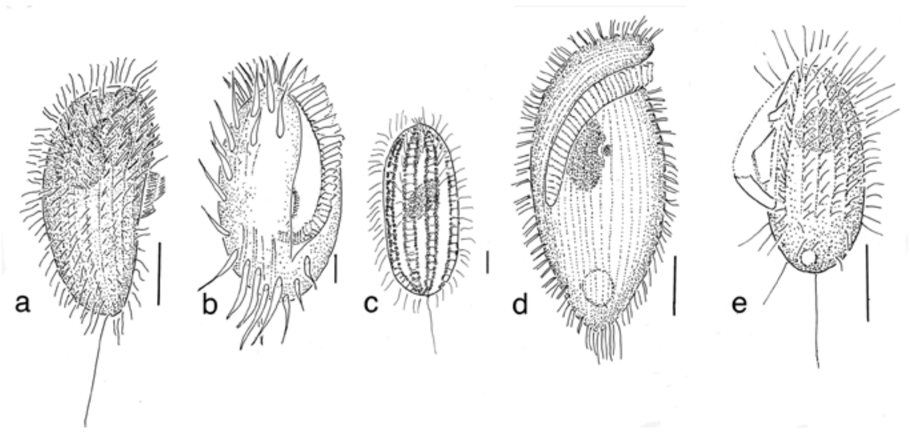

Fig. 2. Some microaerobic and anaerobic marine ciliates. **a** – *Uronema filificum* that prefers an O_2-tension of 1–2% atm. sat.; **b** – *Euplotes* sp. (Fenchel *et al.* 1989, Bernard and Fenchel 1996) that prefers an O_2-tension of 4–5% atm sat.; **c** – *Plagiopogon loricatus* that occurs at low O_2-tensions in both the stratified water column and in sediments; **d** – *Metopus contortus* is an obligate anaerobe with hydogenosomes; it occurs in nearly all marine anaerobic habitats; **e** – *Cyclidium* cf. *flagellatum* is a microaerophile that prefers an O_2-tension around 2%, but is also capable of sustained growth under anaerobic conditions (Bernard and Fenchel 1996). All scale bars: 10 µm.

1982). The effect of oxygen toxicity in protozoa merits further investigations.

In many cases O_2 preferences of protozoa correlate with those of their prey. Thus many ciliates feed preferentially on colorless sulfur bacteria that are typically found in the chemocline in the narrow zone where the presence of sulfide overlap with the presence of oxygen in stratified water columns and in sediments. In general, the chemocline represents a region of high bacterial productivity due to chemolithotrophic bacteria that oxidize reduced compounds diffusing upwards from the anoxic zone (Fenchel 1969, Fenchel *et al.* 1995). The ciliate *Kentrophoros* carries extracellular symbiotic sulfur bacteria on parts of its cell surface; the bacteria serve as food for the host and in accordance with the requirements of sulfur bacteria for the simultaneous presence of low concentrations of sulfide and oxygen the ciliate prefers microaerobic conditions (Fenchel and Finlay 1989).

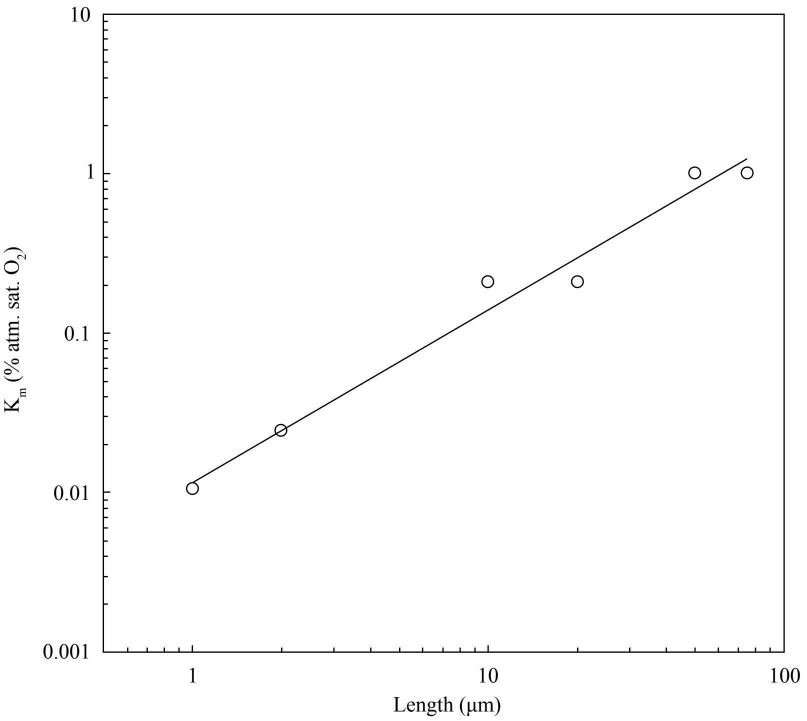

Fig. 3. K_m for different cells as function of the largest linear dimension: from left to right: *Escherichia coli*, mitochondria (from an annelid worm), yeast cells, *Acanthamoeba*, *Tetrahymena* and *Euplotes* sp. Data from Stolper *et al.* (2010), Degn and Kristensen (1981), Lloyd *et al.* (1980), Fenchel *et al.* (1989).

Motile behavior in oxygen gradients

The observation that some aerobic protozoa avoid oxygen tensions above a certain level goes back to Jennings (1906). When sufficiently dense cell suspension is placed in glass capillaries that are open in one end, the respiration of the cells creates an oxygen gradient; at the open end the meniscus is in equilibrium with the surrounding atmosphere. The cells will then form a distinct band within a limited range of O_2-tension and inserting an O_2 microelectrode then allows for determination of the O_2 gradient. An example is shown in Fig. 4. Alternatively it is possible to enclose the capillary in an airtight and transparent container and successively inject known amounts of atmospheric air into the initially pure N_2 headspace gas and then to determine the point at which the cells retreat from the meniscus (Fenchel *et al.* 1989, Fenchel and Bernard 1996).

All known mechanisms of microbial chemosensory motile behavior play a role in the orientation in oxygen gradients – often they are all applied simultaneously within a single species. A kinetic response means that the cells slow down swimming velocity or even attach to solid surfaces under optimal conditions, a mechanism that tends to accumulate the cells there. Phobic responses mean that the cells reverse the direction of their swimming or creeping path or they make a more or less semicircular turn when entering less favorable conditions. A series of such reversals – so called temporal gradient sensing – results in a drift of cells along a chemical gradient to more favorable conditions. A variation of this is helical klinotaxis: that is, cells swim in helical paths and when encountering less favorable conditions cells can bend the axis of the helical swimming path. The width of the bands of cells formed in oxygen gradients is a function of the steepness of the gradients (Fenchel and Bernard 1996). The ciliate *Loxodes* (and presumably the related marine *Remanella*) applies geotaxis: when exposed to high O_2 concentrations (or intense light) they swim downwards and exposed to anoxia they tend to swim upwards (Fenchel and Finlay 1984).

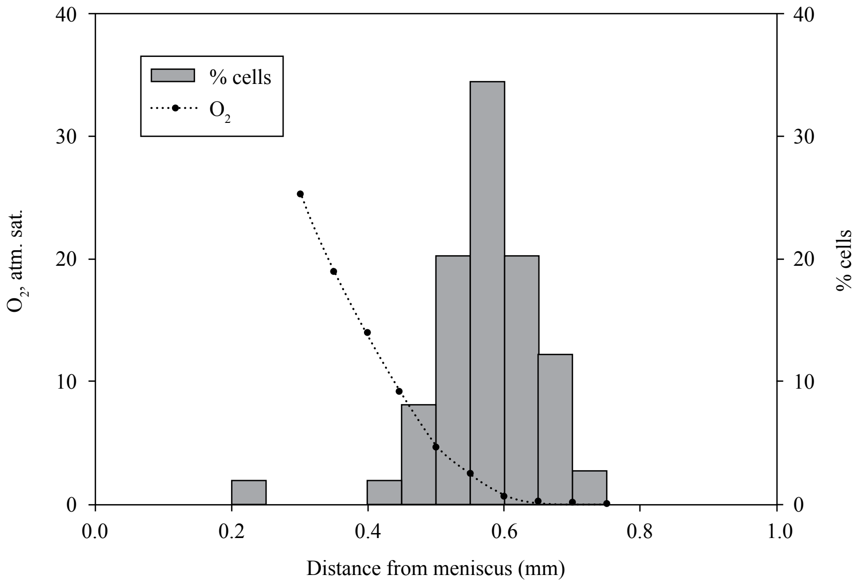

Fig. 4. Formation of a band by the ciliate *Uronema filificum* (Fig. 2a) in an oxygen gradient in a glass capillary. Data from Fenchel and Bernard (1996).

Knowledge on responses to oxygen gradients among other groups of protists is very limited. However, it has been shown that benthic foraminifera show motile responses to oxygen tension (Alve and Bernhard 1995).

Where O_2-gradients are less steep such as in the water column it is likely that chemosensory behavior plays a smaller or no role in the distribution of the protozoa; rather the zonation patterns reflect the zone with positive growth that exceeds the loss of cells to the surrounding due to random motility or slow turbulent mixing in the water column.

MICROAEROBIC HABITATS IN NATURE

Types of O_2 gradients: spatial and temporal scales

Oxygen gradients are widespread in aquatic habitats at all spatial and temporal scales. The fundamental reason for this is that oxygen consumption and production rates exceed transport rates. At sufficiently small spatial scales such transport depends exclusively on molecular diffusion. In sediments, accumulating detritus and biofilms, dissolved O_2 transport is exclusively by molecular diffusion with diffusion coefficients around 2×10^{-5} cm^2 s^{-1}. Combined with high reaction rates this creates gradients that may span from super-saturation to complete anoxia over a mm scale. Aquatic sediments are always anoxic at some depth. In productive shallow water sediments the anoxic zone may reach or even rise above the surface; in deep-sea sediments underlying oligotrophic waters the depth of the anoxic zone can exceed a meter. In detritus and in biofilms a complex pattern of O_2-concentrations arise due to "hotspots" of O_2-production or consumption. Due to the development of oxygen microelectrodes or "optodes" such gradients can now be measured with a spatial resolution of about 10 µm. In the light, exposed biofilms and shallow water sediments with a high photosynthetic activity vertical migrations of the O_2-gradients of several mm occur on a diurnal basis (e.g. Glud 2008, Kühl *et al.* 2007; Fig. 5). Deviations from simple vertical gradients in sediments are caused by animal activity such as the ventilation of burrows that causes seepage of O_2 to the surrounding anoxic sediment (e.g. Fenchel 1996a).

Oxygen gradients and anoxic deep water develop in the water column of stratified lakes seasonally due to thermal stratification or permanently in some deep lakes or due to the presence of a halocline. Anoxic deep waters are also characteristic of some fjords with

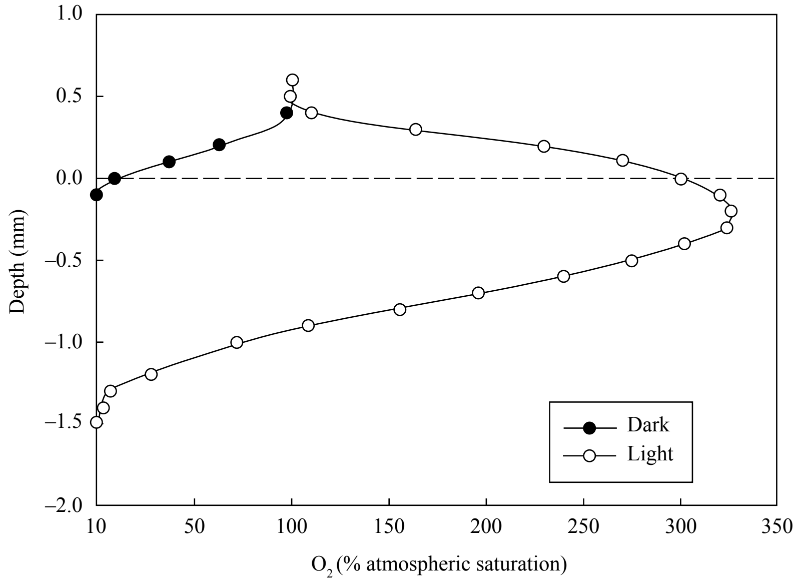

Fig. 5. Oxygen gradient in the light and in the dark above and in the surface layers of a marine shallow water sediment with a high photosynthetic activity. In the light oxygen tension spans from 3 × atmospheric saturation to anoxia over a little more than 1 mm.

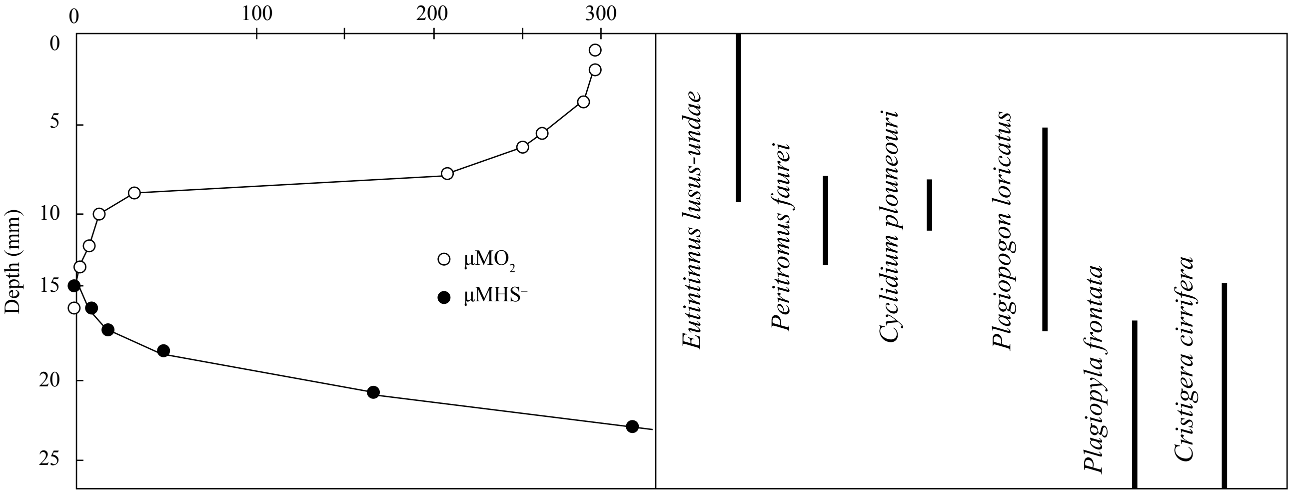

Fig. 6. Oxygen and sulfide gradients in the stratified Mariager Fjord and examples of the vertical distribution of six ciliate species. Data from Fenchel *et al*. (1995).

a sill and the stability of the stratification of the water column is often enforced by a halocline (Behnke *et al*. 2010, Fenchel *et al*. 1995, Orsi *et al*. 2012; Fig. 6). In the open sea deep basins may be permanently or periodically anoxic below a certain depth such as in the Black Sea, the Cariaco Trench, and some basins in the Baltic Sea. The East Pacific and Indian Oceans are characterized by an oxygen minimum zone which is typically found in the water column below around 200 m depth and with a thickness of several hundred meters and in which the oxygen tension may fall to almost zero (Riley and Skirrow 1975). Even in stratified water columns there will always be some turbulent mixing so that vertical transport exceeds what is possible solely due to molecular diffusion; also biological reaction rates are typically lower than what is found in shallow water sediments and biofilms. Consequently the oxygen gradients in the water column oxygen gradients typically span several meters.

THE PROTOZOAN BIOTA OF MICROAEROBIC HABITATS

General considerations

Evidence of microaerophilic preferences of protozoa derive in part of experimental evidence and in part from the distribution of the different species in oxygen gradients in nature. Experimental evidence also shows that some aerobic species are capable of growth at oxygen tensions from zero to atmospheric saturation, albeit at different growth rates. Thus it has been found that a *Strombidium* sp., *Euplotes aberans*, *Euplotes* sp., and *Cyclidium* cf. *flagellatum* (Fig. 2b, e) maintain balanced growth under complete anoxia or in the presence of up to 1 mM cyanide or up to 5 mM sulfide – an ability that may be widespread among microaerobic protozoa. Presumably their energy metabolism is then based on some sort of fermentative process or nitrate reduction. A number of other species cannot grow in absolute anoxia, but they remain viable for long periods of time (Bernard and Fenchel 1996). This means that the occurrence of particular species is not necessarily restricted to what appears to be the optimal O_2-tension as revealed in experimental steep gradients: this applies in particular to the stratified water column that does not display steep gradients and is also characterized by some turbulent mixing that will affect the efficiency of chemosensory motile behavior.

Nevertheless, in all natural systems characterized by oxygen-gradients it is possible to distinguish three relative distinct groups of protozoa: fully aerobic, microaerophilic, and obligate anaerobes. Evidence is so far best in the case of ciliated protozoa and to some extent for various flagellate groups.

With respect to the protozoan distribution in stratified marine water columns two approaches have been used: a traditional survey of observed morphospecies including cultivation of some of the species; alternatively environmental DNA and sequencing rRNA-genes produce a number of "operational taxonomic units" (OTUs). While the latter approach in one sense apparently reflects more unambiguous data, it is also cruder in that it often provides evidence only for the presence of representatives of some higher taxonomical levels. Also, some of the sequences found may derive from non-living DNA. Finally there is evidence of considerable genetic divergence among similar phenotypes so that diversity of OTUs not necessarily reflects functional diversity such as the *Tetrahymena* complex or that constituted by the scuticociliate *Cyclidium glaucoma* (Nanney *et al.* 1998, Fenchel and Finlay 2006). Diversity estimates based on OTUs have sometimes shown a lower species diversity than what was obtained by a more traditional approach. Thus the studies on the protozoan diversity in the stratified Mariager Fjord based on a traditional approach (Fenchel *et al.* 1990, 1995) revealed a larger number of nominal species than the number of rRNA-genotypes recorded in a subsequent study based on environmental DNA (Zuendorf *et al.* 2006). With respect to ciliates the number of OTUs recorded beneath the oxycline (total: 12) was relatively similar to those obtained by microscopic observations (total: 14); with respect to flagellates beneath the oxycline, however, a considerable larger number of microscopically identified species (19) compared to flagellate OTUs (5) and the molecular methods did not detect representatives of the characteristic anaerobic flagellates such as diplomonads, retortamonads, excavates, heterolobosea, and *Mastigamoeba.*

The marine water column

Mariager Fjord on the east coast of Jutland, Denmark is a fjord with a sill and a deeper basin some 20 km from the entrance. The stratification is stabilized by a halocline at a depth of about 15–16 m and below that the water is typically anoxic and sulfidic; around 10 m depth the oxygen content is about 10% atm. sat. Among ciliates found in surface waters (12 species) nearly all also appeared down to about 16 m depth. But between 10 and 16 m depth a new set of ciliate species also occurred, belonging to e.g. representatives of the genera *Cyclidium*, *Plagiopogon* (Fig. 2c), *Cardiostomella*, *Prorodon*, *Coleps, Strombidium*, *Peritromus*, *Euplotes*, and *Litonotus*. Beneath 16 m depth a change in the composition occurred including anaerobic genera such as *Metopus* (Fig. 2d), *Plagiopyla*, *Caenomorpha*, *Saprodinium* – and also some species belonging to *Cyclidium* and *Cristigera* among which some are obligate anaerobes (Fig. 6). The total ciliate biomass as well as the number of species peak in the microaerobic zone between 10 and 16 m depth. A similar pattern of three groups of flagellates was found (including a number of then undescribed species from the microaerobic and anaerobic zone). A few species were found throughout the water column from surface to the sediment (*Telonema subtile*, *Diaphanoeca grandis*, and *Goniomonas* sp.). As in the case of ciliates, the total biomass of heterotrophic flagellates peaked in the oxycline. Amoebae or other unicellular eukaryotes were not studied in any detail (Fenchel *et al*. 1990, 1995).

Other similar systems have been studied by molecular methods including the Gotland Deep in the Baltic Sea (Stock *et al*. 2009), the Framvaren Fjord in Norway (Behnke *et al*. 2010), and Saanich Inlet in British Columbia (Orsi *et al*. 2012). Others have only looked at the anoxic and sulfidic part of the water column such as in the Cariaco Basin off Venezuela (Edgcomb *et al*. 2011) and in an anoxic basin in the Mediterranean Sea (Stock *et al*. 2012). These studies are not inconsistent with direct microscopic studies; they are interesting in that they reveal the presence of some groups previously neglected in anaerobic habitats such as several cercozoans as well as representatives of various fungal groups although it cannot be ruled out that some of these may represent non-living DNA.

Regarding the oceanic oxygen minimum zone there have been several studies on the prokaryote biota and prokaryotic metabolic processes (e.g. Ulloa *et al*. 2012), but the biota of eukaryotic microorganisms have so far been neglected.

Water column of stratified lakes

A similar distinction between fully aerobic, microaerobic and anaerobic biota has been recorded from the water column of stratified lakes. Again there is a group of obligate anaerobic ciliates (largely belonging to the same genera as found in the marine anoxic habitats), microaerobes and forms characteristic for the fully aerobic surface waters while other protist groups have attracted less attention in this respect. In monomictic lakes (that is, lakes that become stratified with anoxic deep water during summer, but with a fully aerobic water column during winter) microaerophiles and anaerobes retreat to the sediment when the water column turns over in autumn and return to the water column as oxygen tension decreases in the deeper parts of the lake during spring. Large microaerophilic ciliates include e.g. species of *Loxodes*, *Spirostomum* and *Frontonia* (Finlay *et al*. 1997, Finlay and Esteban 1998).

A special feature of freshwater ciliates is that some microaerophilic species with endosymbiotic *Chlorella* cells (such as *Euplotes daidaleos* and *Halteria viridis*) are capable of occupying the anaerobic zone of the stratified water column based on the oxygen supply from the symbionts in the light (Finlay *et al*. 1996).

The distribution of other protozoan groups in freshwater in relation to oxygen has so far not received much attention.

Aquatic sediments

The protozoan biota in aquatic sediments is to a large extent determined by the mechanical properties of the sediment: porous sediments (well sorted sand) and also loose detritus accumulating in stagnant water harbor a great variety of protozoa, not least among ciliates. In more fine grained sediments only smaller forms like amoebae and microflagellates occur, and in compact sediments (clay) protozoan life is restricted to the surface (Fenchel 1969).

All sediments display vertical oxygen gradients and they become anaerobic at some depth beneath the surface. In sandy shallow water sediments a vertical zonation of ciliate species was early recognized and correlated with redox potentials (Fenchel 1969). Redox potentials in natural waters are not easily interpreted in terms of the chemical environment; however, negative values indicate the presence of dissolved sulfides and a values below ~ +100 to +200 mV probably indicate anoxia. Anaerobes (including species of e.g. *Metopus*, *Plagiopyla*, *Caenomorpha*, *Parablepharisma*, *Sonderia*, and some *Cyclidium* and *Cristigera* spp.) occur in the anoxic and sulfidic zone; in part these species are identical to species that occur in the anaerobic water column. A large number of species occurs above the sulfidic zone, but below the oxygen rich surface layers including e.g. *Remanella* spp., *Geleia* spp. *Cardiostomella vermiformis*, *Kentrophoros* spp., *Tracheloraphis* spp., and several others. As in the case of the water column, ciliate species diversity as well as biomass peak in the oxycline. More recently it has been found that this zonation is controlled by oxygen tension and that ciliates migrate following changes in the oxygen gradients in sediments with photosynthic activity when exposed to light (Fenchel and Bernard 1996). A similar zonation pattern of ciliate distribution occurs within 5 mm from ventilated worm burrows (Fenchel 1996b).

A study on the micro-distribution of amoebae in the sandy shallow water sediment in relation to the distribution of oxygen concentration did not reveal any distinct relation between O_2-tension (including the absence of O_2) and species composition; only species diversity and density of cells were highest in the oxycline (Smirnov and Thar 2004); also two species of amoebae have been isolated that grow under anoxia (Smirnov and Fenchel 1996). The distribution of flagellates in sediments in relation to oxygen has not been carried out. A study on the flagellates recovered from anoxic sediments revealed a large number of species that showed sustained growth under anaerobic conditions; these included known anaerobes such as diplomonads, retortamonads, trichomonads and the flagellated amoeba *Mastigamoeba*, but also a number of species otherwise known from aerobic habitats (Bernard *et al*. 2000). These studies suggest that facultative anaerobes are common within many protozoan groups.

CONCLUDING REMARKS

It is clear that adaptation to life at different oxygen tensions plays a significant role in protozoan ecology and that oxygen tension to a large extent controls the distribution of different species within many types of aquatic habitats. It is also established that the oxycline harbors diverse biota as well as a high biomass of protozoa. However, several aspects remain to be studied.

With respect to representatives of several protozoan groups, the relationship to oxygen is not clear. This in particular applies to the amoebae and to different groups of flagellates in that some morphospecies seem capable of sustained growth at oxygen tensions spanning from atmospheric saturation to anoxia. The nature of their energy metabolism under anaerobic conditions is understudied; it is possible that nitrate respiration is a much more widespread among protozoa than hitherto known; so far it has been recorded only for a ciliate and for some benthic foraminifera (Fenchel 2011). The exact nature of the adverse effects of high oxygen tensions on microaerophilic protozoa also requires further studies.

Finally, the cited studies based on rRNA-gene sequences indicate the presence in anoxic habitats of some protist groups that have not hitherto been considered as components of anaerobic habitats and some that have apparently not been formally described. Further progress in understanding the relation between protists and oxygen will require descriptions of the phenotypes that are present and also physiological studies on the adaptive traits in terms of energy metabolism, oxygen preferences, and oxygen toxicity beyond the mere listing taxa based on rRNA-gene sequencing.

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