The ‘unseen’ microbial diversity of a Spanish solution lake: ecological and other implications

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ABSTRACT

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The ‘unseen’ microbial diversity of a small solution lake in central Spain is investigated using electron microscopy. The water has an elevated concentration of dissolved sulphate that supports significant abundance of sulphate reducing bacteria. Water samples were collected from the 13m stratum, fixed *in situ* and prepared for posterior electron microscopy examination. The results show high diversity of prokaryotes and microbial associations, particularly predatory bacteria, thriving in the anoxic layers of the water body. This high abundance and biodiversity, including multi-infestation of individual cells, increase the number of lateral links in the otherwise impoverished anaerobic food chain. The wider potential applications of predatory bacteria are discussed.

Key words: predatory bacteria, sulphide, anaerobic ciliates, *Chromatium*, carbon cycls

RESUMEN

Biodiversidad microbiana ‘oculta’ en un lago cárstico

La diversidad microbiana "oculta" de un pequeño lago cárstico situado en el centro de España se investiga mediante microscopía electrónica. El agua tiene una concentración elevada de sulfato disuelto que sostiene una alta abundancia de bacterias reductoras de sulfato. Se recogieron muestras de agua del estrato situado a 13 m de profundidad, las cuales se fijaron ‘in situ’ *y se prepararon para su posterior observación con microscopía electrónica. Los resultados muestran una gran diversidad de procariotas y de asociaciones microbianas, particularmente de bacterias depredadoras, que prosperan en las capas anóxicas del cuerpo de agua. Esta gran abundancia y biodiversidad, incluida la infestación múltiple por bacterias depredadoras, tiene como consecuencia el aumento del número de enlaces colaterales de la cadena alimentaria anaeróbica, que de otro modo es una cadena muy empobrecida. Estas observaciones permiten deliberar sobre las posibles aplicaciones de bacterias depredadoras.*

Palabras clave: *bacterias depredadoras, sulfato, ciliados anaerobios,* Chromatium*, ciclo del carbono*

INTRODUCTION

Our collaborative research over the years mostly focussed on aquatic microbial eukaryotes, particularly the biodiversity of anaerobic ciliates in solution lakes in central Spain. These investigations yielded the discovery of new ciliate species (Esteban *et al.*, 1993), first accounts of ciliates' associations with endosymbiotic (methanogenic and other) archaea for these habitats (Finlay *et al.*, 1991), and the description of difficult-to-document prokaryotic life cycles (Clarke *et al.*, 1993). Despite this wealth of discoveries there still remained a significant amount of microbiological material collected during the intense sampling summer campaigns that, although it was examined, it was never published, and it remained 'unseen'. This is precisely the aim of this *'in memoriam'* article – to bring to light some of the interesting prokaryote associations, some (probably) predator/prey related, which were observed and documented from these remarkable anoxic aquatic habitats. We also discuss the ecological and other potential implications of these microbial consortia.

MATERIAL AND METHODS

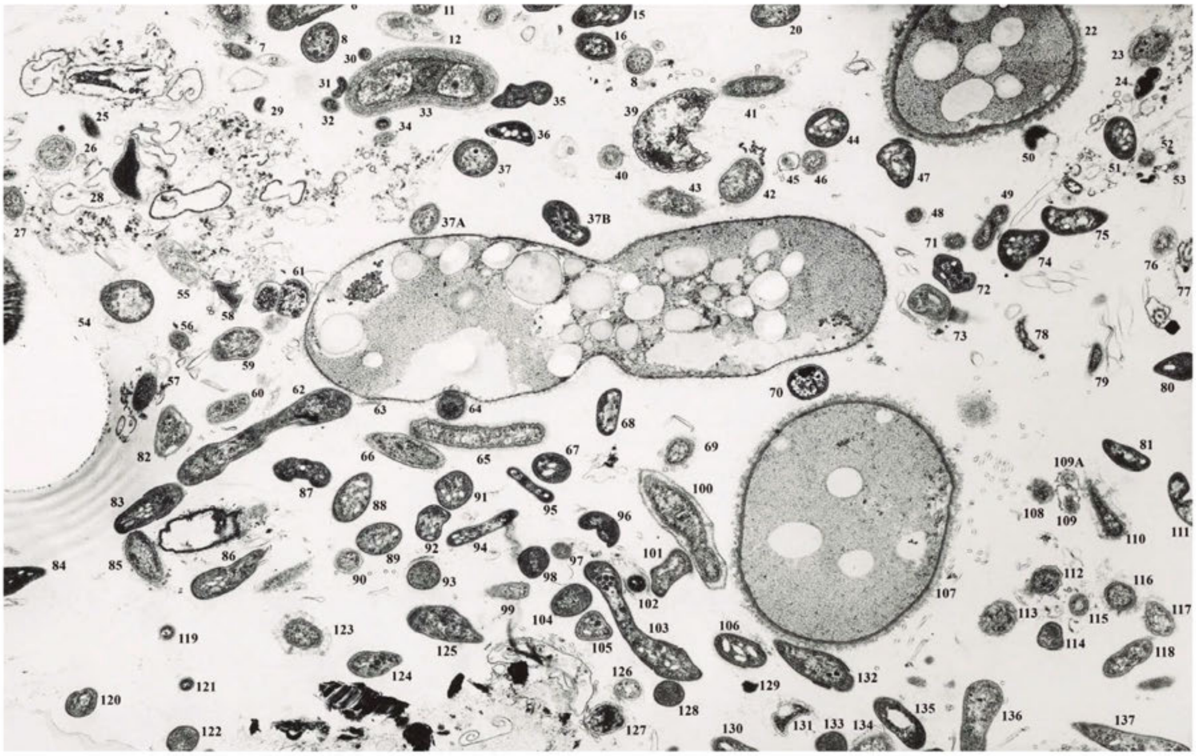
Study site, sampling and electron microscopy

Samples were collected within the 13-metre stratum in Arcas-2, a small solution lake (formed by dissolution of gypsum-rich marls) in Cuenca, central Spain. The lake has a relatively constant depth of 14 m, with the bottom 7 m being anoxic during the summer months (Finlay *et al.*, 1991). The water has an elevated concentration of dissolved sulphate that supports significant bacterial sulphate reduction (Camacho *et al.*, 2000; Rodrigo *et al.*, 2000). Water samples from within the 13 m deep anoxic layer (approximately 4.5 m below the oxic-anoxic boundary) were collected using a pump sampler; samples were kept in an air-free, air-tight bottle in the dark until their immediate processing (see below). Detailed sampling methods have been described previously (Finlay *et al.*, 1991). Prof. Miracle was involved in the sample collection for recording data, and also in the *'in situ'* examination of the living and fixed samples. On the banks of Arcas-2, each sample was immediately given a range of preparation procedures for Electron Microscopy, based on the standard fixation of glutaraldehyde followed by osmium tetroxide (Clarke *et al.*, 1993). Back at the Ferry House on the Windermere shores (England, UK) the embedded and cut sections were stained with our usual triple stain lead citrate/uranyl acetate/lead citrate, before examination with Transmission Electron Microscopy. All the samples were collected in 1992, and photographed in early and mid-1993.

RESULTS AND DISCUSSION

High abundance and biodiversity of prokaryote 'morphotypes' were observed in both, the environmental water samples and inside food vacuoles of anaerobic ciliates. Figure 1 shows a snap-shot of the morphological diversity of prokaryotes in one single sample and one single Electron-Microscop-ultra-thin section photograph: 140 cells counted belonging to 24 morphotypes. One of the most common prokaryotes observed is a 'winged' bacterium found in the water and in food vacuoles of ciliates inhabiting the anoxic layers of Arcas-2 (Fig. 2; see also Finlay *et al.*, 1991), demonstrating the active role of anaerobic ciliates in predating upon bacteria, and their contribution to the carbon-sulphur cycles in this lake. The 'winged' appearance of the bacteria shown in Figs. 1 and 2 corresponds to filaments of overlapping cells into one another – hence the 'wings'. These short bacterial filaments are probably sulphate reducers, and the inclusions seen inside them (Figs 1, 2) are probably either sulphur or gas (Finlay *et al.*, 1991).

The high productivity of Arcas-2 is stimulated by the significant supply of sulphate (from ground water percolating through gypsum), which sustains the growth of sulphate-reducing bacteria in the anoxic layers of the lake (Clarke *et al.*, 1993; Vicente *et al.*, 1991). The sulphide diffuses to upper layers of the water column and up to the metalimnion, being consumed along its way by phototrophic bacteria like *Chromatium*, providing an interesting link between the carbon and sulphur cycles (Finlay *et al.*, 1991) in the anoxic worlds. *Chromatium* is a significant



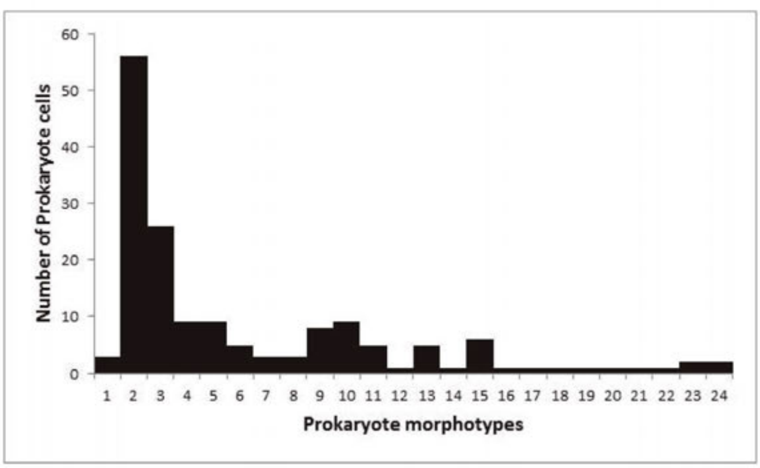


Figure 1. Transmission Electron micrograph (top) showing diversity of prokaryote morphotypes in one single ultrathin-section from one single sample from 13 depth in Arcas-2 (Spain). Total number of cells: 140, belonging to 24 different morphotypes. The 'winged' bacteria is one of the most abundant prokaryotes, see, e.g. 6, 14, 15, 93, 103, 132; note also the abundance of prokaryote associations, e.g. 31 with 33, 63 with 64, 100 with 101, 103 with 128. The black rectangle at the top right of the image corresponds to the TEM film negative number. Bottom image shows the individual abundance of each of the 24 morphotypes. Scale bar = 1 µm. *Micrografía electrónica de transmisión (imagen superior) mostrando la diversidad de morfotipos procariotas en una sola sección ultrafina de una sola muestra de 13m de profundidad en Arcas-2 (España). Número total de células: 140, pertenecientes a 24 morfotipos diferentes. La bacteria "alada" es uno de los procariotas más abundantes, ver, p. 6, 14, 15, 93, 103, 132; nótese también la abundancia de asociaciones procariotas, p. 31 con 33, 63 con 64, 100 con 101, 103 con 128. El rectángulo negro en la parte superior derecha de la imagen corresponde al número de negativo de la película TEM. La imagen inferior muestra la abundancia individual de cada uno de los 24 morfotipos. Barra de escala = 1 µm.*

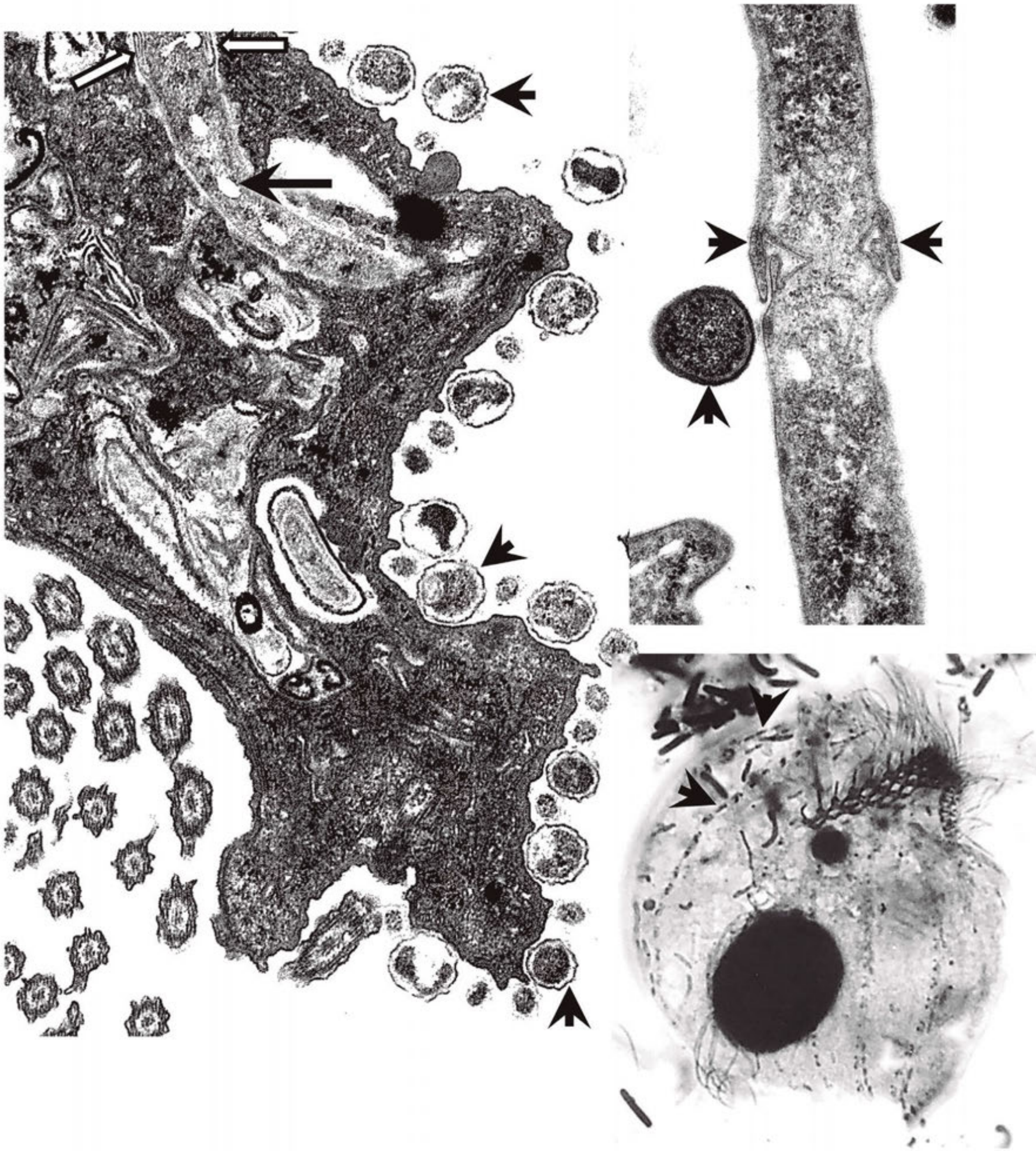


Figure 2. Transmission Electron micrographs of a ciliate (left, probably *Caenomorpha* sp.) showing the ectosymbiotic bacteria (arrows) on the ciliate surface, and the 'winged' bacteria inside the food vacuole (white arrows, top of image). The 'winged' bacterium (right) were also observed bearing prokaryote attached to their surface. The bottom image shows the ectosymbiotic bacteria aligned on the surface of the ciliate *Saprodinium difficile*, scale bar for this image = 5µm. *Micrografías electrónicas de transmisión de un ciliado anaerobio (izquierda, probablemente* Caenomorpha *sp.) que muestra las bacterias ectosimbióticas (flechas) en la superficie del ciliado y las bacterias "aladas" dentro de la vacuola alimentaria (flechas blancas, parte superior de la imagen). La bacteria "alada" (derecha) también se observó con procariotas unidos a su superficie. Barras de escala = 0.5 µm. La imagen inferior muestra una micrografía (microscopía óptica) de las bacterias ectosimbióticas alineadas en la superficie del ciliado anaerobio* Saprodinium difficile*; barra de escala para esta imagen = 5 µm.*

Figure 3. Transmission Electron micrographs showing associations between prokaryotes from 13m depth in Arcas-2, Spain. Some bacteria were associated with more than one prokaryote, probably predatory bacteria. Scale bars = 0.5µm. *Micrografías electrónicas de transmisión que muestran asociaciones entre procariotas encontradas a 13 m de profundidad en Arcas-2, España. Algunas bacterias se asociaron con más de un procariota, y son probablemente bacterias depredadoras. Barras de escala = 0.5 µm.*

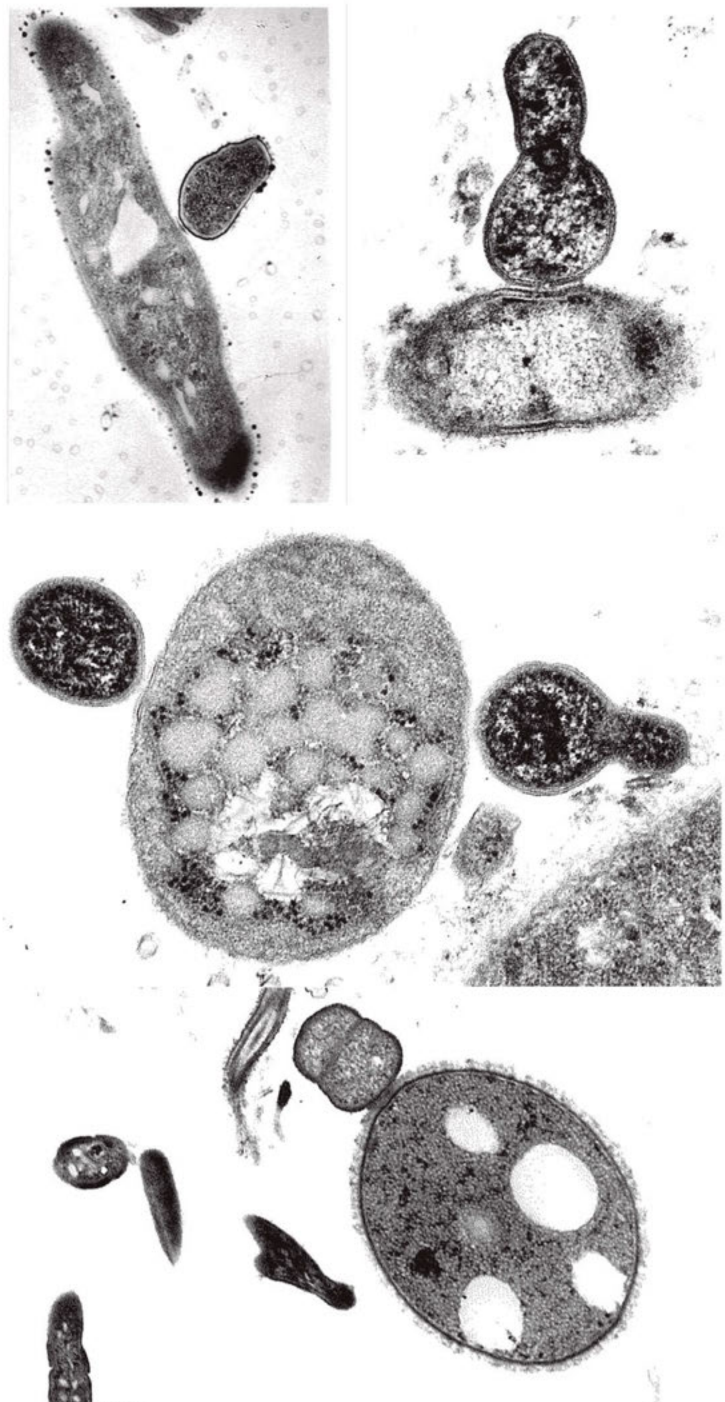


Figure 4. Transmission Electron micrographs of further microbial associations found at 13m depth in Arcas-2 (Spain). Some bacteria were associated with more than one prokaryote, probably predatory bacteria. Bottom image shows the prokaryote epibiont of the photosynthetic bacterium *Chromatium weissei*. Scale bars = 0.5µm. *Micrografías electrónicas de transmisión de otras asociaciones microbianas encontradas a 13 m de profundidad en Arcas-2 (España). Algunas bacterias se asociaron con más de un procariota, probablemente bacterias depredadoras. La imagen inferior muestra el epibionte procariota de la bacteria fotosintética* Chromatium weissei. *Barras de escala = 0.5 µm.*

source of food for ciliates in these environments (Finlay *et al.*, 1991); carbon fixed by these phototrophic bacteria that are ingested and subsequently digested by anaerobic ciliates is incorporated into ciliate biomass. Furthermore, since most anaerobic ciliates in Arcas-2 harbour endosymbiotic methanogenic archaea (Finlay *et al.*, 1991) that require hydrogen for their growth, the carbon fixed from the digested phototrophic bacteria will also be used in archaeal methane production that is eventually released into the surrounding water and then into the atmosphere. The sulphur ingested by the ciliates will probably be eliminated via the usual cytoproct's waste removal process.

The external surface of some of the anaerobic ciliates found in Arcas-2 has rod-shaped ectosymbiotic prokaryotes attached to it (Fig 2; Esteban *et al.*, 1993), adding another layer of complexity to the microbial food web found in the lake's anoxic water. Further examples of microbial consortia in this habitat are delivered by the high abundance of other types of prokaryote associations; in some instances, bacteria have two or more prokaryotes fastened to their surfaces (Fig. 1), like *Chromatium* (Fig. 4); others seem to be predators (Figs. 2, 3), with some bacteria being simultaneously infested by several bacterial predators as well as epibionts (Fig. 3).

Anaerobic food chains are very short due to the much lower energy yield of anaerobic metabolism (Fenchel & Finlay, 1995), where protozoa are the only top predators able to complete the life cycle in total absence of oxygen. The string of diverse microbial associations found in the anoxic water of Arcas-2 increases the number of lateral links in these short anaerobic food chains, e.g. the heterotrophic prokaryote on the surface of the phototrophic *Chromatium* (Fig. 4) probably depends exclusively on the DOC produced and excreted by the latter (Clarke *et al.*, 1993), and the predatory bacteria in anoxic water contribute to the 'recycling' of carbon.

Predatory prokaryotes have long been known in the scientific literature, e.g. *Vampirococcus*, *Bdelovibrium*, *Daptobacter* (Guerrero *et al.*, 1986). This predatory behaviour provides a window into early evolution of eukaryotes, showing how bacteria-bacteria predation can enlighten the origin of intracellular organelles (see e.g. Fig 3, and Guerrero *et al.*, 1986, 1987; Jurkevitch & Davidov, 2007). Furthermore, research on 'predatory bacteria' is currently a promising line of enquiry into the fight against antibiotic-resistant bacteria. It has successfully been experimented with the predatory *Bdellovibrium* and *Micavibrio* – both bacteria have the ability to prey and reduce many multidrug-resistant pathogens associated with human infection (Dashiff *et al.*, 2011). The use of predatory bacteria (also known as 'living antibiotics') has been proposed as potential solution to the rise of multidrug-resistant bacterial infections (Kadouri *et al.*, 2013; Shatzkes *et al.*, 2016; Dwidar & Yokobayashi, 2017; Madhusoodanan, 2019).

The 'unseen' microbial diversity of Arcas-2 has allowed us to reveal a variety of prokaryotes and microbial associations that thrive in the absence of oxygen in sulphate-rich solution lakes, their ecological importance in increasing the number of trophic interactions in the low energy-yield short anaerobic food chains, and how bacteria-bacteria predation may hold the key to a post-antibiotic future. Most importantly, it reveals how the intricacies and potentials of microbes remain largely unknown.

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