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KARST SPRING MICROBIAL DIVERSITY DIFFERS ACROSS AN OXYGEN-SULPHIDE ECOCLINE AND REVEALS POTENTIAL FOR NOVEL TAXA DISCOVERY

PESTRA MIKROBNA DIVERZITETA VZDOLŽ EKOKLINE KISIK-SULFID KRAŠKEGA IZVIRA ODKRIVA POTENCIAL ZA OKRITJE NOVIH TAKSONOV

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Abstract UDC 551.44:549.3(497.4), 551.44:579.8(497.4)

Janez Mulec & Annette Summers Engel: Karst spring microbial diversity differs across an oxygen-sulphide ecocline and reveals potential for novel taxa discovery

Strong geochemical gradients of dissolved oxygen and sulphide establish habitats where specialized bacterial and archaeal taxonomic groups occupy specific redox-sensitive niches, primarily based on metabolic and ecological requirements. In contrast, knowledge of microeukaryote diversity and their ecology in redox-stratified habitats is poor, as species-specific occupation of such geochemical gradients has not been well established. Here we assessed total microbial diversity from rRNA genes retrieved from two morphologically distinct microbial mats formed along an oxygen-sulphide gradient in the outflow channel from the Žveplenica sulphidic karst spring, Slovenia. Microbial mats contained diverse bacteria and archaea associated with chemolithoautotrophic and primary productivity, and overall microeukaryotic diversity was higher under oxygenated conditions. The oxygenated mats were comprised of undescribed and undifferentiated fungi, Annelida, Nematoda, Apicomplexa, and Gastrotricha, some being represented by novel lineages. Under anoxic conditions, diversity was dominated by Ciliophora, Nematoda, and Fungi-Ascomycota, also affiliated with novel lineages. Colonization of the distinct mat types related to ecological tolerance of specific geochemical conditions, and the associations between bacterial and archaeal diversity with distinct microeukaryotes may be related to grazing options and food web structure within the karst system.

Key words: karst, spring, sulphide, geochemical gradient, diversity, microeukaryotes.

Izvleček UDK 551.44:549.3(497.4), 551.44:579.8(497.4)

Janez Mulec & Annette Summers: Pestra mikrobna diverziteta vzdolž ekokline kisik-sulfid kraškega izvira odkriva potencial za okritje novih taksonov

Izraziti geokemijski gradienti raztopljenega kisika in sulfida so osnova za habitate, kjer v odvisnosti od redoks potenciala, predvsem pa glede na metabolne in ekološke zahteve, specializirane bakterijske in arhejske taksonomske skupine zasedajo občutljive ekološke niše. Nasprotno pa je poznavanje diverzitete mikroevkariontov in njihove ekologije v habitatih, stratificiranih glede na redoks potencial, pomanjkljivo, saj vrstno specifične kolonizacije takšnih gradientnih okolij še nismo dobro proučili. Celotno mikrobno diverziteto smo ovrednotili na podlagi zaporedij rRNA genov, ki so bila pridobljena iz dveh morfološko različnih mikrobnih biofilmov, ki nastajata vzdolž gradienta kisik-sulfid v žveplenem kraškem izviru Žveplenica, Slovenija. Mikrobni biofilmi so vsebovali pestro združbo bakterij in arhej, ki jim pripisujemo kemolitoavtotrofen metabolizem s primarno produkcijo, celotna mikroevkariontska diverziteta pa je bila v oksigeniranih okoljskih razmerah višja. Oksigeniran biofilm so večinoma sestavljale še neopisane in nepoznane glive ter predstavniki skupin Annelida, Nematoda, Apicomplexa in Gastrotricha; nekatere izmed njih pripadajo celo novim linijam. V anoksičnih razmerah so prevladovali predstavniki skupin Ciliophora, Nematoda in Glive-Ascomycota, ki tudi pripadajo novim genetskim linijam. Kolonizacija različnih tipov biofilmov glede na ekološko toleranco specifičnih geokemijskih razmer ter povezava med bakterijsko in arhejsko diverziteto z značilnimi mikroevkariontskimi predstavniki sta lahko povezana z različnimi možnostmi prehranjevanja in s strukturo prehranjevalne verige v kraškem sistemu.

Ključne besede: kras, izvir, sulfid, geokemijski gradient, diverziteta, mikroevkarionti.

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INTRODUCTION

Geochemical gradients established by dissolved oxygen and reduced sulphur compounds, such as hydrogen sulphide, create an ecotonal transition between adjacent aquatic ecological systems where communities dependent on oxygenated conditions meet and interact with communities associated with anoxia (Kolasa & Zalewski 1995; Behnke *et al*. 2006; Mulec *et al*. 2015). Diverse bacterial and archaeal communities commonly colonize oxygen-sulphide ecotones, with chemolithoautotrophic metabolism sustaining the ecosystems (Skirnisdottir *et al*. 2000; Takai *et al*. 2004; Mattes *et al*. 2013; Offre *et al*. 2013; Anderson *et al*. 2015; Hamilton *et al*. 2015). Higher eukaryotes thrive in these ecotone habitats, from deep-sea hydrothermal vents and other marine settings (Lopez-Garcia *et al*. 2001; Edgcomb *et al*. 2002; Lopez-Garcia *et al*. 2003; Behnke *et al*. 2010; Orsi *et al*. 2011; Holder *et al*. 2013) to continental karst aquifers and caves (Engel 2007; Por *et al*. 2013). But, microeukaryote diversity and ecology in these oxygen-sulphide ecotones are less understood, particularly those living in anoxic conditions (Fenchel & Finlay 1995; Dawson & Pace 2002; Edgcomb *et al*. 2002; Stoeck *et al*. 2003; Luo *et al*. 2005; Behnke *et al*. 2006; Epstein & Lopez-Garcia 2008; Behnke *et al*. 2010; Creer & Sinniger 2012; Parris *et al*. 2014; Wang *et al*. 2014; Oikonomou *et al*. 2015). In contrast, protist diversity in freshwater, continental habitats with oxygen gradients has been shown to be greater than previously considered (Oikonomou *et al*. 2015). The expectation is that microeukaryote diversity from these habitats is undersampled and novel diversity has yet to be discovered.

Aquatic cave and karst habitats have been investigated previously to uncover microeukaryotes diversity (Gittleson & Hoover 1969; Walochnik & Mulec 2009; Sigala-Regalado *et al*. 2011), with some efforts to characterize microeukaryotes communities from karst systems with sulphidic to oxic ecotones (Thompson & Olson 1988; Sarbu 1990; Latella *et al*. 1999; Maggi *et al*. 2002; Nold *et al*. 2010a). In regions where caves are not accessible, or where there are limited wells and boreholes to sample subsurface water, continental sulphidic karst springs that discharge at the surface from fissured carbonate bedrock are ideal habitats to investigate microeukaryotes living in oxygen-sulphide ecotones in karst. This is because spring water contains nutrients and geochemical energy sourced from the subsurface (e.g., hydrogen sulphide), and the water also has the potential to flush out and carry subsurface microeukaryotes to the surface. Essentially, subsurface microeukaryotes can be sampled without needing to depend on wells, boreholes, or even caves to enter. Previous karst spring studies identify autochthonous microbial endokarst communities (AMEC) comprised of specific bacterial groups, such as members of Acidobacteria, Nitrospira, Gammaproteobacteria, and Deltaproteobacteria (Farnleitner *et al*. 2005; Pronk *et al*. 2009), where microeukaryotes are not been included. One of the distinguishing factors for AMEC is water residence time; longer residence times depend on the connectivity to the surface and flowpath tortuosity (Ford & Williams 2007). Travel time can range from months to years (Griebler & Lueders 2009), with longer times favouring the development of AMEC (Farnleitner *et al*. 2005; Brannen-Donnelly & Engel 2015).

At the Žveplenica sulphidic karst spring in Slovenia, the discharging water is anoxic to suboxic and sulphidic, and abundant white, filamentous microbial mats occur at the spring orifice, previously identified as Epsilonproteobacteria (Rossmassler *et al*. 2012). Epigean copepods in different developmental stages, with many of them being alive (Mulec *et al*. 2015), are washed out from the spring orifice (*Bryocamptus echinatus luenensis*, *Bryocamptus zschokkei,* and *Paracyclops fimbriatus*). Wider representation of other eukaryotes would be expected for the whole karst system. Therefore, the objectives of our new study were to estimate the diversity of microeukaryotes from 18S rRNA gene sequences, evaluate the spatial composition of microeukaryotes in anoxic and oxygenated subhabitats in the spring microbial mats, and discuss their ecology related to bacterial and archaeal taxonomy determined from 16S rRNA gene sequences. To our knowledge, this is one of the first assessments of microeukaryotes from sulphidic karst habitats by using molecular genetics methods, the other being of benthic microbial mats from a limestone sinkhole formed in Lake Huron, USA (Nold *et al*. 2010a; Nold *et al*. 2010b).

MATERIALS AND METHODS

SITE DESCRIPTION, WATER GEOCHEMISTRY, SAMPLING AND MICROBIAL MAT MORPHOLOGY

The Žveplenica sulphidic karst spring (46°03′59·46″N, 13°49′37·81″E; 253 m a.s.l.) from coarse-grained, massive Upper Triassic dolomite (Mulec *et al.* 2015) discharged at ~ 1.5 to 2.0 litres per min from June 2008 to February 2011. A 1.5 m long channel is formed downstream of a 4-cm diameter orifice of the spring before discharging into the Trebušica River. The groundwater geochemical composition indicates dissolution of dolomite (Zega et al. 2015).

From May 2009 to February 2011, the spring water temperature was monitored every 15 min. Temperature varied seasonally, with the average being 10.5 °C and ranging from 6.3 °C to 13.9 °C. From August 2009 until February 2011, as reported in Mulec *et al.* (2015), average specific conductance was 418 µS/cm and pH was 7.58. Water at the spring orifice was actually anoxic (0.13 ±0.11 mg/ml of dissolved oxygen), with 8.5 mg/l dissolved sulphide. Alkalinity was 223.2 mg/l (expressed as $CaCO_3$), and there were no NO_3^- and low concentrations of PO_4^{3-} (0.02 mg/l), SO_4^{2-} (9.00 mg/l) and Cl^- (10.00 mg/l).

Sparse white filaments were attached to sediment in the orifice, and filaments of different lengths extended downstream where they coalesced to form a grey microbial mat in the stream channel. Long filaments detached, floated downstream, then sank in the Trebušica River. After heavy rain precipitation, especially in spring and autumn, the spring orifice would be flooded by the river, and filaments would be completely stripped from the orifice and stream channel. Downstream, dissolved oxygen concentrations increased and dissolved sulphide concentrations decreased to undetectable concentrations beyond the sampled grey mat (Mulec *et al.* 2015). Approximately 5 g of white microbial filaments were collected from the orifice and about the same quantity of grey mats were collected 50 cm downstream from the orifice in June 2008, in conjunction with another study (Rossmassler *et al.* 2012).

DNA EXTRACTION, PCR AMPLIFICATION, CLONING, AND SEQUENCING

Total environmental DNA was extracted in triplicate using methods as previously described (Engel *et al.* 2003). Approximately 0.5 g of microbial mat was aseptically collected and transferred into DNA extraction buffer (10 mM Tris-HCl, 100 mM EDTA, 2% sodium dodecyl sulphate). The extraction protocol was similar to the commercially available Purgene DNA extraction kits (Gentra Systems, USA), with some modifications. 9 µl of proteinase K (20 mg/ml) was added to DNA extraction buffer prior to digestion. A freeze-thaw series (three times at −80°C to 65°C) was used to disrupt the mat structure. Samples were further incubated at 55°C overnight to digest cellular material and RNase was added and incubated at 37°C for up to 1 h. Proteins were precipitated in 10 M ammonium acetate. Nucleic acids were precipitated in isopropanol overnight at −20°C and finally washed in 70% ethanol. DNA yield was assessed from TBE gel electrophoresis with ethidium bromide staining and spectrophotometrically using standard absorbance at 260 nm and 280 nm on a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA).

All PCR amplification reactions were done with 5PRIME Perfect Taq polymerase and a MJ Research thermal cycler. Bacterial 16S rRNA gene sequences were amplified using the forward primer 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1510R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane 1991). PCR conditions for bacterial-specific primers were: initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 47°C for white mat or 50°C for grey mat and elongation at 72°C for 1.5 min; final extension at 72°C for 20 min. Annealing temperatures for each sample type were optimized from temperature-gradient tests to obtain the highest amplification product yield and from electrophoresis gel verification of no extra banding. Archaeal 16S rRNA gene sequences were amplified by using the forward primer UA571F (5′-GCY TAA AGS RIC CGT AGC-3′) and reverse primer UA1204R (5′-TTM GGG GCA TRC IKA CCT -3′) (Baker & Cowan 2004). PCR conditions for both mat types using archaeal-specific primers were from Baker *et al.* (2003). For amplification of eukaryotic 18S rRNA, we used protocols and primer pairing from Zuendorf *et al.* (2006) to obtain gene fragments ranging up to 1800 bp in length. The J1 primer set used the forward primer EukA (5′-AAC CTG GTT GAT CCT GCC AGT-3′) (Diez *et al.* 2001) and reverse primer U1517R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The M2 primer set used the EukA forward primer and U1391R reverse primer (5′-GGG CGG TGT GTA CAA RGR-3′) (Zuendorf *et al.* 2006). PCR amplification for both mat types was done with an initial hot start (15 min at 95°C) followed by 30 cycles of denaturing at 95°C for 45 sec, annealing for 1 min at 55°C for the J1 primer set, or 56°C for primer set M2, and extension at 72°C for 2.5

min. A final extension was done at 72°C for 7 min. Each reaction contained ~20 ng of template DNA, 0.8 μM of each primer, 1.6 mM dNTP, 1.6 mM MgCl2, 1×PCR buffer, 0.16 mg/ml BSA, and 1 unit of Taq polymerase for the J1 primers and 2 units for the M2 primers.

Amplified PCR products using the different primer sets were purified using a TAE low-melt agarose gel and a Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, Wisconsin, USA). PCR products were cloned separately for each primer set using Invitrogen TA Cloning kits (Carlsbad, California, USA), following manufacturer instructions. Clones having the correct size insert for each library were confirmed by M13-primer PCR amplification, as described previously (Porter *et al.* 2009), and screened using TBE electrophoresis gels and ethidium bromide staining. Clones were sequenced at the University of Washington, High-throughput Genomics Unit, using capillary sequencers (Sanger sequencing) and M13 primers and the internal primer 907R for 16S rRNA genes (5'-CCG TCA ATT CMT TTR AGT TT-3') and internal primer 582F (5'-CGGTAATTCCAGCTC-3') for 18S rRNA genes (Elwood *et al.* 1985).

CLONE LIBRARIES AND PHYLOGENETIC ANALYSES

rRNA gene sequences were assembled using ContigExpress from Vector NTI Advance® (Invitrogen Corp., USA), then screened for the presence of chimera by using the computer programs UCHIME (Edgar *et al.* 2011) and DECIPHER (http://DECIPHER.cee.wisc.edu) (Wright *et al.* 2012). Chimeras were also checked manually and ~10% of all the 16S rRNA gene sequences were removed. Six 18S rRNA gene sequences were removed due to poor alignment. Together, 131 bacterial, 186 archaeal, and 156 eukaryotic sequences were analysed. Retained 16S and 18S rRNA sequences were analysed by using NCBI BLAST (Camacho *et al.* 2009), the RDP (version 11) Classifier (Cole *et al.* 2014) and the SILVANGS analysis pipeline (SILVA SSU and LSU databases 119.1) (Quast *et al.* 2013) to determine sequence taxonomic affiliations and to obtain the closest gene sequence matches. Eukaryotic taxonomy was done according to Adl *et al.* (2005). For sequences, single phylotype clades were made when sequences had 97% or greater sequence identity. For 16S rRNA genes for both bacterial and archaeal clone libraries, multiple sequence alignments were performed using Greengenes workbench (DeSantis *et al.* 2006). Multiple sequence alignments of 18S rDNA libraries were performed using SILVA for major known eukaryotic phyla (Pruesse *et al.* 2007; Pruesse *et al.* 2012). Only conserved and unambiguously aligned positions were used in the subsequent phylogenetic analyses. All sequences were compiled and evolutionary analyses were conducted in MEGA 6.0 (Tamura *et al.* 2013). Rarefaction analyses and diversity indices were calculated with the Mothur computer software (Schloss *et al.* 2009).

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

rRNA gene sequences reported in this study were deposited in the GenBank database, for Bacteria and Archaea under the accession numbers KT072259 to KT072575, and for the eukaryotic sequences under the accession numbers KT072097 to KT072258.

RESULTS

16S rRNA GENE SEQUENCE DIVERSITY AT THE SULPHIDIC TO OXIC ECOCLINE

Retrieved bacterial sequences from both microbial mat types were affiliated with putative sulphur-metabolizing or chemolithoautotrophic taxa within the Proteobacteria, notably belonging to the Gamma-, Beta-, and Epsilonproteobacteria (Figs. 1 and 2). Gammaproteobacteria belonging to the genus *Thiothrix* represented ~23.6% of the relative abundances for both white and grey mat communities, and Betaproteobacteria dominated the grey mat sample in the aerobic portion of the stream (29.1% relative abundance). Genera within the Betaproteobacteria included the *Azospira*, *Iodobacter*, *Georgfuchsia*, *Pelomonas*, *Rhodoferax*, *Undibacterium*, and *Thiobacillus*. Epsilonproteobacteria were more prevalent in the white mat (15.8% relative abundance in the community) compared to the grey mat (12.7% relative abundance), with genera belonging to the *Dehalospirillum*, *Sulfuricurvum*, and *Sulfurovum*. The white mat in the anoxic portion of the stream also had sequences affiliated with the Chloroflexi, within the Anaerolineaceae (7.9% of the community), and the Deltaproteobacteria (5.3%) affiliated with the genus *Desulfocapsa* and *Geobacter*. Bacteroidetes, affiliated with the genera *Flavobacterium*, *Haliscomenobacter*, *Gilvibacter*, and *Paludibacter*, comprised 26.3% of the white mat community and 20.0% of the grey mat com-

munity. Representation from other taxonomic groups included the Alphaproteobacteria, Firmicutes, Planctomycetes, Lentisphaerae, and the Candidate Division SR1. Members of the Candidate Division SR1 comprised 11.8% of the white mat community and 5.5% of the grey mat community and were closely related to an environmental clone retrieved from a meromictic lake in France [FJ482219] (Borrel *et al.* 2010).

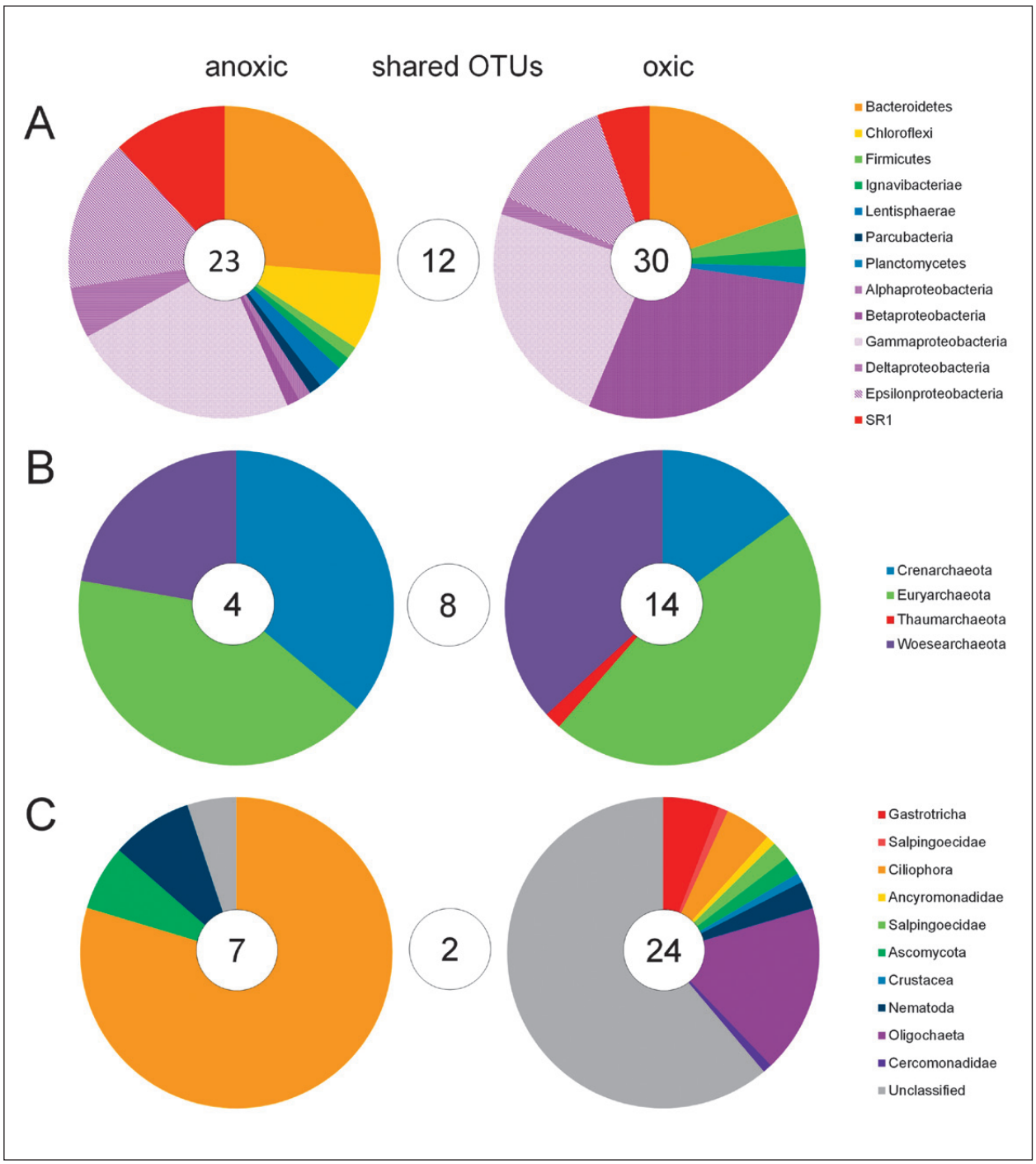

Fig. 1: Taxonomic distribution of 16S rDNA OTU-based phylotypes of (A) bacteria, (B) archaea, and (C) eukaryotic 18S rDNA retrieved from upstream anoxic and downstream oxygenated microbial mats. Taxonomic assignments were based on a minimum of 97% sequence identities. The number of unique OTUs per sample are noted, and the number of shared OTUs between the mat types are presented (distance 0.03).

Among the Archaea, diversity was lower from the white mats in the anoxic part of the channel (Figs. 1 and 3). And the majority of clones were affiliated with Euryarchaeota, with 41.7% in the white mat and 46.5% in the grey mat community. The relative proportions of clones belonging to the orders Methanomicrobiales, Halobacteriales, and Thermoplasmatales were similar for both mats. The relative abundances of Crenarchaeota

Fig. 2: Molecular phylogenetic analysis of bacterial 16S rDNA by Maximum likelihood method. Clones for the white (W, also labelled in bold) and grey (G) mats are included, and redox conditions for the mats are noted if more than one clone formed a clade, ANO for anoxic mats and OXI for oxic mats. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 100 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained by applying the neighbour joining method to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 154 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 113 positions in the final dataset.

were greater in the white mat (36.1% relative abundance in the community) compared to the aerobic part of the channel (15% relative abundance), but the abundances of sequences affiliated with Woesearchaeota were greater in the grey mat (36.8% of the community) compared to the white mat (22.2% of the community).

Although the observed species richness for both bacterial and archaeal libraries was underestimated ac-

Fig. 3: Molecular phylogenetic analysis of archaeal 16S rDNA by Maximum likelihood method. Clones for the white (W, also labelled in bold) and grey (G) mats are included, and redox conditions for the mats are noted if more than one clone formed a clade, ANO for anoxic mats and OXI for oxic mats. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 100 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained by applying the neighbour joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 208 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

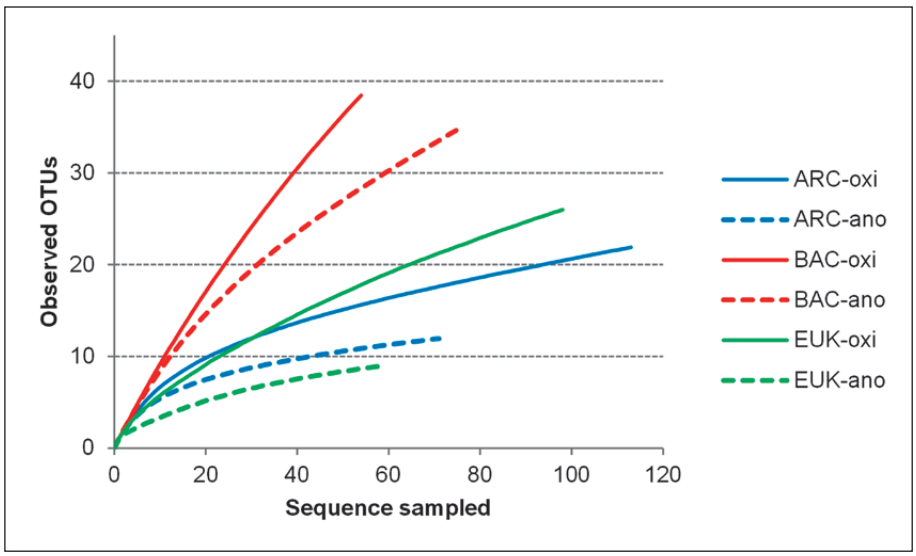

Fig. 4: Sampling saturation profile for archaea (ARC), bacteria (BAC) and eukarya (EUK) from white mat under anoxic condition (ano) and grey mat exposed to oxygenated conditions (oxi). Phylotypes were defined to encompass clones that exhibited at least 97% sequence similarity.

cording to rarefaction curves (Fig. 4), and more clones would need to be screened to retrieve the full diversity, approximately one third of bacterial and archaeal phylotypes overlapped between the two mat types (Fig. 1). Specifically, 12 bacterial OTUs were shared between the white mats upstream (anoxic) and grey mats downstream (oxic), and eight archaeal OTUs were shared between the anoxic and oxic subhabitats.

18S rRNA GENE SEQUENCE DIVERSITY AT THE SULPHIDIC TO OXIC ECOCLINE

A double set of primers was essential to reveal wider eukaryotic diversity, although much greater diversity than was retrieved likely exists because the total number of clones screened in this study was insufficient to uncover total richness based on rarefaction curves (Fig. 4). Few of the retrieved 18S rRNA gene sequences were related to previously known taxa at >97% sequence similarity according to BLAST and $SILVA_{NGS}$ analysis pipeline (Fig. 5), specifically for the downstream mats that had 61.2% unclassifiable sequences (Fig. 1). However, among the groups with robust taxonomic assignments, eukaryotic groups from the upstream white mats were affiliated with the Ciliophora (79.7% of the sequences in the community), which includes the subgroups of ciliates, dinoflagellates, and apicomplexans. The order Hymenostomatida represented most of the ciliophores, at 72.9% of the total eukaryotic sequences from the white mats. The closest relative was the ciliate *Glaucomides* spp. (96-99% sequence similarity for all matching clones). Nematoda and Ascomycota comprised the remaining 15.3% of sequences from the white mats. The closest nematode relative was *Rhabdolaimus* sp. (97-99% sequence similarity), which have been previously retrieved from microbial mats on lava tube cave walls from Hawai'i (GenBank Accession no. EF032796), and are commonly found in soil and springs, including those with low oxygen concentrations (Tahseen 2012).

From the downstream grey mats in oxygenated water, which were more diverse at the level of OTUs than the white mats (Fig. 1), more than 80% of the sequences were affiliated with the Fungi/Metazoa group (Opisthokonta). Fungi represented 44% of the sequences, with most being related to the early diverging fungal lineage Cochlonemataceae, and specifically to the genus *Cochlonema* (87-90% sequence similarity). Among the metazoans (Holozoa), 54% of the sequences were affiliated with the Naididae (sludge worms), 23% with the Nematoda (round worms), 17% to the Chaetonotidae (gastrotrichs), and 3% each to the Maxillopoda (copepods) and Macrobiotidae (tardigrades). The nematode sequences retrieved from the grey mats were related to an unnamed species previously identified from Lake Huron (GenBank accession no. EU910601) (Nold *et al.* 2010b). The majority of the Naididae were annelids belonging to the Tubificinia, and were related to the genus *Slavina* (98-99% sequence identity). Among the Chaetonotidae, most of the sequences were related to the genus *Chaetonotus* (90-99% sequence similarity). Copepod sequences were related to the genus *Bryocamptus*; this group was identified previously from the spring microbial mats (Mulec *et al.* 2015). The remainder of the community was comprised of Cercozoa and Alveolata, at ~5% each, and sequences affiliated with the Stramenopiles, Amoebozoa, Apusozoa, and Euglenozoa represented 3% or less of the community. Only two OTU-based phylotypes were shared between the two mat types (Fig. 1).

DISCUSSION

According to niche theory, ecological tolerance influences wide or narrow distributions of organisms within a habitat and across habitats (Heino & Soininen 2006; Soininen & Heino 2007; Behnke *et al.* 2010; Orsi *et al.* 2011). Because some novel aquatic microeukaryotic lineages occupy distinct habitat types, the suggestion has been that microeukaryotes may have high degrees of endemism (Dawson & Pace 2002; Edgcomb *et al.* 2002; Lopez-Garcia *et al.* 2003; Stoeck & Epstein 2003; Bass *et al.* 2007; Boenigk *et al.* 2007; Orsi *et al.* 2011). Indeed, finding microeukaryotes living in specific habitat conditions continues to challenge global dispersal dogma (Behnke *et al.* 2006; Martiny *et al.* 2006) because some populations appear to have global distributions (Finlay 2002;

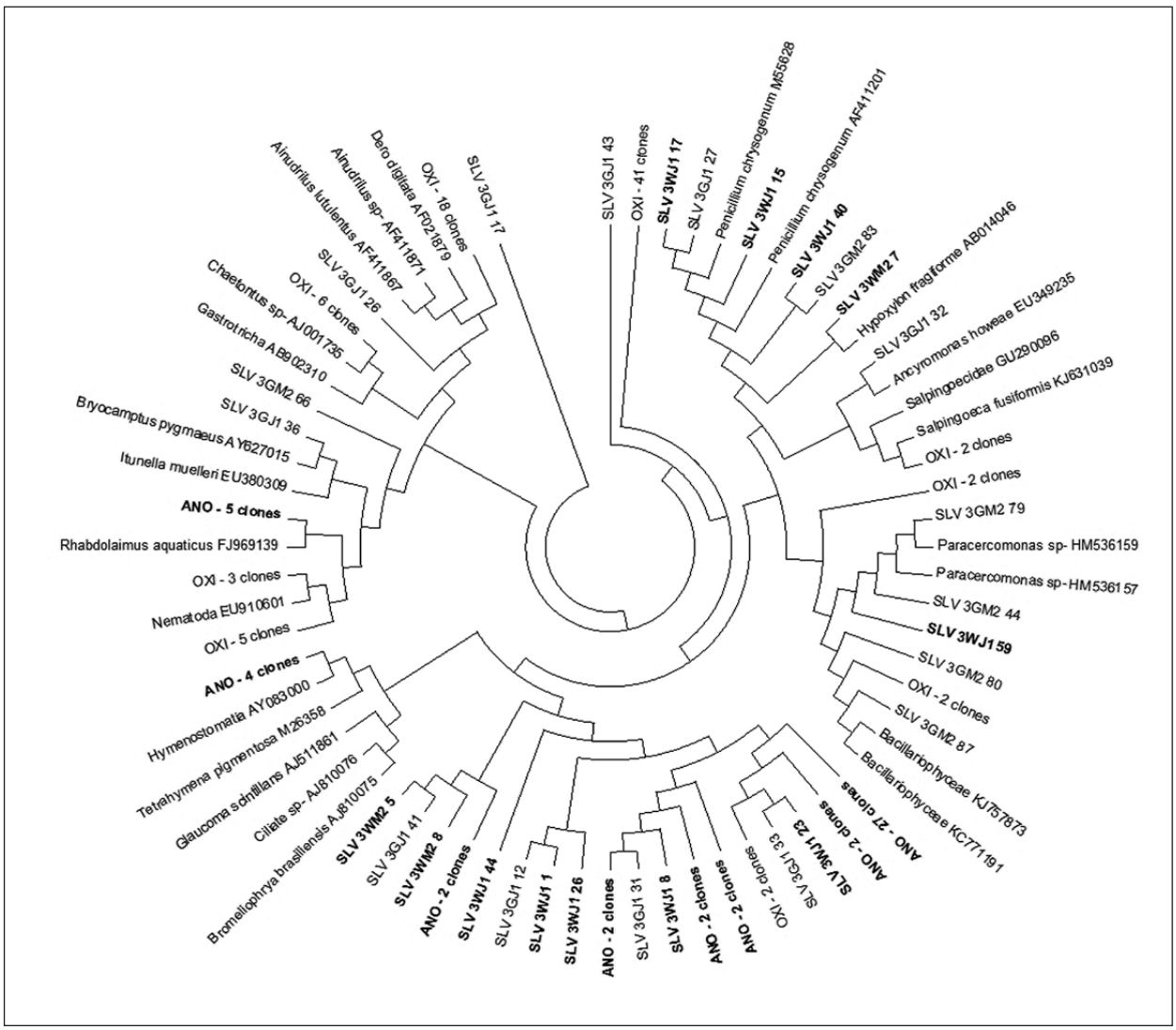

Fig. 5: Molecular phylogenetic analysis of eukaryotic 18S rDNA by Maximum likelihood method. Clones for the white (W, also labelled in bold) and grey (G) mats are included, and redox conditions for the mats are noted if more than one clone formed a clade, ANO for anoxic mats and OXI for oxic mats. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 100 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained by applying the neighbour joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 180 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1528 positions in the final dataset.

Bass *et al.* 2007; Guillou *et al.* 2008). In general, spatial distributions of organisms within a habitat are influenced by feeding and grazing options and pressures within a food web (Sherr & Sherr 1994; Sherr & Sherr 2002; Pernthaler 2005; Orsi *et al.* 2011) and dispersal mechanisms (Richards & Bass 2005), which relate strongly to organism size (Fenchel & Finlay 1995; Finlay 2002; Soininen & Heino 2007). Food web structure is also influenced by organism size, with bacteria and smaller protozoa serving as food for larger organisms, as well as the scale of the habitat and environmental extremes that could lead to specialized metabolisms, particularly in ecotonal habitats (Edgcomb *et al.* 2002; Griebler *et al.* 2014). However, interpretations regarding endemic versus cosmopolitan spatial occurrences of microeukaryotes may be premature because their diversity and ecology from many different habitats is generally understudied. Knowledge regarding microeukaryotes in karst systems is especially poor (Engel 2010).

Therefore, the purpose of our study was to evaluate microeukaryotic diversity from the undersampled environment of a karst spring. The Žveplenica karst water mean transit time of 1.0 to 1.5 years (Mulec *et al.* 2015) may be long enough to develop karst AMEC (Farnleitner *et al.* 2005; Pronk *et al.* 2009) that can be sampled at the spring. By studying the microbial mats at the spring, unique microeukaryotic diversity was uncovered that paved the way to study eukaryotic AMEC. Collectively, the implications of this study relate to community dynamics, such as grazing dynamics, in sulphidic to oxic ecotones and potential patterns in microbial phylogeography related to microeukaryote host dispersal mechanisms.

We chose the Žveplenica karst spring because results could be compared to those from previous studies in geochemically similar lakes and marine systems where diverse microeukaryotic groups occupy sulphidic to oxic ecotones (Edgcomb *et al.* 2002; Lopez-Garcia *et al.* 2003; Stoeck *et al.* 2003; Luo *et al.* 2005; Behnke *et al.* 2006; Zuendorf *et al.* 2006; Nold *et al.* 2010a; Nold *et al.* 2010b; Oikonomou *et al.* 2015). However, most of the gastrotrichs, ciliophora, cercomonads, and nematodes from the spring samples had <95% sequence identity to previously described taxonomic groups. This implies a level of novelty for the Žveplenica spring microeukaryotes diversity. Conversely, and perhaps obviously, because this type of habitat is undersampled, lineage novelty may simply be due to a lack of information about these groups. Future research should attempt to address the level of endemism for the microeukaryotes retrieved from the Žveplenica karst spring to verify AMEC membership.

Ecologically important eukaryotic groups were retrieved from the spring that are known to be anaerobic and that occupy sulphidic to oxic ecotones, such as members of alveolates and fungi (Stoeck & Epstein 2003). Limited overlapping taxonomic representation between the white and grey mats, and lower diversity in the white mats from the anoxic and sulphidic portion of the stream, suggested niche specialization. The specific microeukaryote ecology and potential niche occupation based on geochemistry may result in a less complex food web structure in the white mats, with fungi significant in cross-feeding, anaerobic ciliophora, and bacterivorous nematodes like *Rhabdolaimus* spp. Moreover, *Cochlonema* spp. are obligatory zoopagalean parasites of free-living amoeba (Koehsler *et al.* 2007), although no sequences for amoeba were retrieved from the white mats. This corresponds to previous unsuccessful attempts to isolate amoebae.

In contrast, the diverse grey mat food web structure may be more complex because the mat was comprised of a mix of anaerobic and aerobic stramenopiles, alveolates, cercomonads, and fungi (Stoeck & Epstein 2003), in addition to nematodes, sludge and round worms, gastrotrichs, copepods, and tardigrades that all participate in various trophic interactions as decomposers, predators, and parasites. In general, gastrotrichs are broadly distributed in aquatic habitats, typically are associated with sediments and benthic substrate, and consume bacteria and smaller protozoa as detritivores (Ricci & Balsamo 2000).

Previously, six faunal groups were identified from the filtered sulphidic water through a container equipped with 60 μm mesh, oligochaetes, crustaceans (Cladocera, Cyclopoida, and Harpacticoida), gastropods, and Trichoptera (Mulec *et al.* 2015). The impact of microeukaryotic grazing on microbial mats has been well established (Dalby *et al.* 2008; Takishita *et al.* 2010; Edgcomb & Pachiadaki 2014), such that the types of bacteria and archaea that grow in a habitat need not only to tolerate the ecological conditions, but also to grow faster than grazing occurs. The presence of larger organisms, including flagellates and euglenids in the grey mats, may assist the dispersal of some bacteria during grazing, as previous evidence suggests that some Epsilonproteobacteria representative can survive and/or replicate within protozoa (Winiecka-Krusnell *et al.* 2002; Axelsson-Olsson *et al.* 2005).

In conclusion, the microbial mats occurring within the sulphidic to oxic ecotone of the Žveplenica spring are an important local biodiversity hotspot. Diverse bacteria and archaea associated with chemolithoautotrophy and primary productivity occupy the mats and serve as a rich food source for microeukaryotic heterotrophs that are likely sourced from the karst aquifer. Because of the sulphide-oxygen gradient, the microeukaryotic diversity of microbial mats is distinct between the anoxic versus oxygenated portion of the stream, such that the association

of some microeukaryotes with the bacterial and archaeal diversity may be related to grazing options, in addition to ecological tolerance of the geochemical conditions. These results expand what is known of microeukaryotes in continental karst systems and ecotonal habitats (Fenchel & Finlay 1995; Dawson & Pace 2002; Edgcomb *et al.* 2002; Stoeck *et al.* 2003; Luo *et al.* 2005; Behnke *et al.* 2006; Epstein & Lopez-Garcia 2008; Behnke *et al.* 2010; Creer & Sinniger 2012; Parris *et al.* 2014; Wang *et al.* 2014; Oikonomou *et al.* 2015).

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