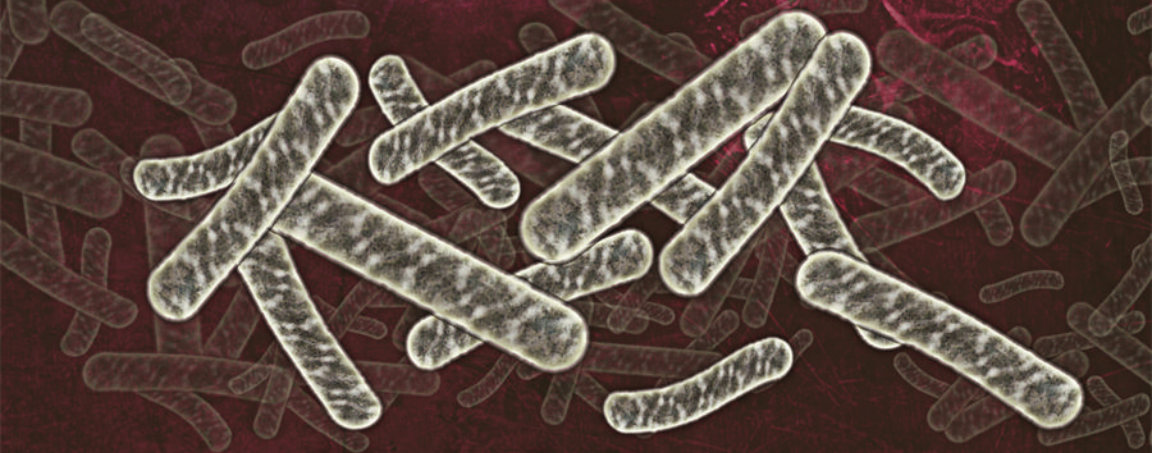
Microbiology Research Advances

PROTEOBACTERIA

Phylogeny, Metabolic Diversity and Ecological Effects



Maria L. Sezenna
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PROTEOBACTERIA: PHYLOGENY, METABOLIC DIVERSITY AND ECOLOGICAL EFFECTS

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CONTENTS

PREFACE

Proteobacteria are a major group (phylum) of bacteria. They include a wide variety of pathogens, such as Escherichia, Salmonella, Vibrio, Helicobacter, and many other notable genera. Others are free-living, and include many of the bacteria responsible for nitrogen fixation. This book presents current research from across the globe in the study of proteobacteria, including phylogeny and activity of proteobacteria in sediments from Lake Furnas; proteobacteria forming nitrogen-fixing symbiosis with higher plants; proteobacteria and the endosymbiotic origin of mitochondrion; and interpreting diversity of proteobacteria based on 16S rRNA gene copy number.

Chapter 1 - Sediments are sites of intense bacterial activity fostered by the presence of several organic and inorganic electron donors and acceptors that can be metabolised under either aerobic or anaerobic conditions. Among the bacterial groups populating sediments, the Proteobacteria phylum is one of the most abundant. Thus, the aim of the present work was to evaluate the contribution of Proteobacteria to nutrient (N and P) and iron cycling in sediments from the Azorean Lake Furnas (Portugal). The combination of denaturing gradient gel electrophoresis and cloning of the bacterial 16S rRNA gene fragment identified the Proteobacteria phylum as a dominant member of the sediment bacterial community from Lake Furnas. Using quantitative PCR to determine the relative amount of sediment bacteria affiliated to specific groups of Proteobacteria, it was inferred that 4.6% to 7.3% belonged to ammonium-oxidizing bacteria of the Beta-Proteobacteria phylum, Nitrobacter-like nitrite-oxidizing bacteria of the Alpha-Proteobacteriaphylum amounted to 0.3% to 6.0%, Geobacteraceae-like iron reducing bacteria of the Delta-Proteobacteria phylum amounted to 1.0% to 2.4%, and Rhodocyclus-like phosphorus accumulating organisms of the Beta-Proteobacteriaphylum

accounted for 0.2% to 0.5%. Experiments with homogenized sediments in batch conditions indicated that bacteria performing autotrophic nitrification, heterotrophic denitrification, iron-reduction, and biological phosphorus storage/release were active in sediments from Lake Furnas. Lake Furnas is an advanced stage of eutrophication despite the considerable efforts that in the last decades have been made by local authorities towards the reduction of phosphorus inputs to the lake. Conventional remediation measures focused on the amount of P adsorbed into iron oxides that are released to the water column under anoxic conditions. The present work suggested that biological P storage/release by denitrifying bacteria in sediments might as well contribute to the release of phosphorus from sediments. Future measures towards lake restoration should include in addition to the classical procedure an evaluation of the contribution of biological processes in sediments to the eutrophication problem.

Chapter 2 - The ability to fix nitrogen is inherent only to prokaryotes and within them several Proteobacteria are able to establish symbiosis with legumes and the non-legume Parasponia in which structures specialized in nitrogen fixation named nodules are formed. Within Proteobacteria, the species able to induce nitrogen-fixing nodules are classified in two different classes, alpha and beta. All “classical” rhizobia are alpha Proteobacteria whereas several species recently described as legume endosymbionts have been classified within the beta Proteobacteria. Although several gamma Proteobacteria have been isolated from nodules, they cannot induce nodule formation in legumes. The ecological relevance of Proteobacteria forming nitrogen fixing endosymbiosis with higher plants is related to that of the legumes which colonise very different habitats being the legume symbiosis the main source of terrestrial fixed nitrogen. In this chapter we revised the current known Proteobacteria forming nitrogen-fixing endosymbiosis with legumes.

Chapter 3 - The acquisition of mitochondrion is one of the decisive steps in the evolution of the eukaryotic cell. Today, people almost have believed that mitochondrion had a bacterial endosymbiotic origin. But what kind of bacteria is the endosymbiont? With the accumulation of evidence from the studies of morphology, cell biology, biochemistry, and molecular biology, especially from the recent studies of molecular phylogeny and genomics, proteobacteria, in particular Rickettsiales or their close relatives, are proposed to be the endosymbiont. In this chapter, the authors review the progress of the studies in this field.

Chapter 4 - The application of the 16S rRNA gene diversity analysis has revealed the immense microbial diversity of our planet. At the same time, and

after of more than two decades of using this methodology along with several important improvements and new techniques, there is still no universal golden rule on how to estimate prokaryotic diversity in a natural sample, as there is in macroecology. A general assumption during studies of prokaryotic diversity is that each found 16S rRNA gene found corresponds to one cell. However, in this paper it is shown that recent genomic data reveal that this is not the case for several bacterial phyla. Since the Proteobacteria, along with the Firmicutes, are the most abundant and diverse bacterial phyla, in this paper the average 16S rRNA gene copy number is presented at the sub-phylum (α-, β-, γ-, δ- and ε-Proteobacteria), order and family level of the Proteobacteria phylum. At the sub-phylum level the average 16S rRNA gene copy number varied between 2.1±1.3 and 5.8±2.8. Since the 16S rRNA gene copy number affects the relative abundance of each proteobacterial species/phylotype found in a clone library, and subsequently the estimation of diversity, the corrected relative abundances of the found proteobacterial phylotypes were estimated in 37 clone libraries from six different natural habitats. It is suggested, that at least in the cases where Proteobacteria consist 50-75% of the clone library, the corrected abundances should be used for diversity estimations.

Chapter 5 - High-altitude Andean lake (HAAL) ecosystems of the South American Andes are almost unexplored systems of shallow lakes formed during the Tertiary geological period, distributed in the geographical area called the Puna at altitudes from 3,000 to 6,000 m above sea level, and isolated from direct human activity. They present a broad range of extreme conditions which makes the indigenous microbial communities exceptionally interesting to study physiological mechanisms of adaptation to chemical and physical stresses such as hypersalinity and high levels of UV radiation. Previous work have revealed the outstanding diversity of these environments, being Proteobacteria the most extended and best represented microbial taxa within the extremophilic communities. The aim of this work is to review the microbial diversity of Proteobacteria present at the HAAL and to describe their multiple resistance properties towards the extreme factors that these microbial communities thrived in their natural environments. A special reference to the representatives of the genus Acinetobacter found at the HAAL is also presented.

Due to the isolation program held at LIMLA (www.limla.com.ar) during the past four years a one-of-a-kind collection of extremophilic strains from the HAAL was assembled. HAAL microbial diversity was investigated by sampling bacterioplankton, benthonic microorganisms, microbial-mat associated microbes as well as gastrointestinal symbiotic organisms from

flamingoes living at the lakes. Representatives of Proteobacteria has been profusely isolated from these samples, more exactly from Lakes: Azul, Verde, Negra, Vilama, Aparejos, Chaxas, Salina Grande, Socompa, Dead Man Salar, Tolar Grande, Brava, Diamante, Huaca-Huasi, all of them located above 4000 m, at the Northwest of Argentina. In addition, a more extended coverage of Proteobacteria was detected by non-culture dependent techniques (mainly DGGE), suggesting that much more efforts will be needed to isolate most novel Proteobacteria present at the HAAL.

Within Proteobacteria, all the four main groups were represented in our culture collection being the Gammaproteobacteria the class with better coverage. The gammaproteobacteria strains were classified as belonging mainly to Pseudomonas Acinetobacter, Halomonas, Stenotrophomonas, Moraxella, Enterobacter, Serratia, Salinivibrio, Pseudoalteromonas, Aeromonas and Marinobacter. 16S rDNA gene sequence comparison of some isolates with the ones presented at the database indicated an identity lower that 94%, which should point out that these extremophilic communities harbour yet unraveled species.

The extreme conditions suffered by these microorganisms at the HAAL made them resistant to factors present as well as not present in their natural environments. Exposure to UV-B radiation during 24 h revealed that most isolates were highly resistant: 33.3% of betaproteobacteria, 44.4% of gammaproteobacteria, 40% of alphaproteobacteria were able to survive through the whole exposition time. In addition, resistance to hipersalinity in most isolates was also observed.

Interestingly, antibiotic resistance was also observed in spite of the pristinely and isolation of these lakes. In light of the great adaptability strength of the strains to changing conditions in their original environment, antibiotic resistance may be considered as a consequence of a high frequency of mutational events, which also, may be enhanced by the intense solar irradiation present at the HAAL (UV index in summer: 16- 18).

A special reference can be made to the representatives of the genus Acinetobacter isolated from the HAAL. Most of these strains appeared to have multiple resistance profiles to hipersalinity, UV-B irradiation, antibiotics and even arsenic. These “superbugs” can be subjected to further studies as they can be clues to discover new ways of surviving at extreme conditions, a matter that has applications in astrobiology. On the other hand, it will be very interesting to further research on these strains biotechnological potential because as extremophiles they can be source of novel bioactive compounds.

Chapter 6 - The antibiotic resistant strain Tik3 of Pseudomonas sp. originally isolated from permafrost contains a chromosome-located composite transposon Tn5045. Molecular analysis of Tn5045 structure revealed a chromate resistance transposon as one of its constituent elements. Simultaneously it was shown that the strainTik3 is able to grow in the presence of Cr(VI). The chromate resistance transposon of P. sp. Tik3 has a similar genetic arrangement and is closely related to the transposable element TnOtChr conferring the chromate resistance of Ochrobactrum tritici 5bvl1. Both elements belong to the Tn3 family and contain a group of chrB, chrA, chrC, and chrF genes located between divergently transcribed resolvase (tnpR) and transposase (tnpA) genes. The transposon Tn5045 was translocated onto the broad-host-range plasmid pRP1.2 and transferred to Escherichia coli. In addition the high-copy number plasmids pGEM-7Zf(-) and pAK1 containing Tn5045 were created. It was found that introduction of the complete operon chrBACF on a high- or low-copy number plasmid into genome of E.coli JF238 didn′t result in a significant increase in chromate resistance. At the same time the increased chromate resistance was detected in Acinetobacter calcoaceticus BD413 cells carrying pAK1::Tn5045. The findings indicate that expression of chromate resistance is regulated differently in different species of Proteobacteria.

Chapter 7 - Several filamentous β–bacteria form sheath, a tube-like extracellular structure, which enfolds a line of cells. The genera Leptothrix and Sphaerotilus are phylogenetically related filamentous -Proteobacteria whose members are known as typical sheath-forming bacteria in various aquatic environments. Despite the extensive studies on taxonomic properties and ecologicalimportance of these bacteria, not much is known about the structural composition of their sheaths. The sheath of both genera is readily degraded with hydrazine, releasing amphoteric heteropolysaccharides, which is made up of pentasaccharide repeating units. In the case of Leptothrix sheath, the pentasaccharide repeating unit is composed of 2-amino-2-deoxy-galacturonic acid, galacturonic acid, galactosamine, glucosamine, whereas the pentasaccharide of Sphaerotilus sheath contains glucuronic acid, galactosamine and glucose. In addition to the sugar moieties, cysteine and glycine are found as the major amino acids in acid hydrolysates of both Sphaerotilus and Leptothrix sheaths. Structural analysis of partial hydrolysates of the sheaths indicated that the glycans are attached to oligopeptides of cysteine and glycine residues through an amide bond involving the C-terminal carboxyl group of the oligopeptides and particular amino groups in the

pentasaccharides repeating units. The peptideside chains can be spontaneously connected by disulfide bond.

The sheaths of the genera Leptothrix and Sphaerotilus are somewhat similar supermolecule constructed by association of sulfhydryl glycoconjugates, which may represent a novel glycoconjugate category.

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Chapter 1

PHYLOGENY AND ACTIVITY OF *PROTEOBACTERIA* IN SEDIMENTS FROM LAKE FURNAS

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ABSTRACT

Sediments are sites of intense bacterial activity fostered by the presence of several organic and inorganic electron donors and acceptors that can be metabolised under either aerobic or anaerobic conditions. Among the bacterial groups populating sediments, the *Proteobacteria* phylum is one of the most abundant. Thus, the aim of the present work was to evaluate the contribution of *Proteobacteria* to nutrient (N and P)

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and iron cycling in sediments from the Azorean Lake Furnas (Portugal). The combination of denaturing gradient gel electrophoresis and cloning of the bacterial 16S rRNA gene fragment identified the *Proteobacteria* phylum as a dominant member of the sediment bacterial community from Lake Furnas. Using quantitative PCR to determine the relative amount of sediment bacteria affiliated to specific groups of *Proteobacteria*, it was inferred that 4.6% to 7.3% belonged to ammonium-oxidizing bacteria of the *Beta-Proteobacteria* phylum, *Nitrobacter*-like nitrite-oxidizing bacteria of the *Alpha-Proteobacteria*phylum amounted to 0.3% to 6.0%, *Geobacteraceae*-likeiron reducing bacteria of the *Delta-Proteobacteria* phylum amounted to1.0% to 2.4%, and *Rhodocyclus*-like phosphorus accumulating organisms of the *Beta-Proteobacteria*phylum accounted for 0.2% to 0.5%. Experiments with homogenized sediments in batch conditions indicated that bacteria performing autotrophic nitrification, heterotrophic denitrification, iron-reduction, and biological phosphorusstorage/release were active in sediments from Lake Furnas. Lake Furnas is an advanced stage of eutrophication despite the considerable efforts that in the last decades have been made by local authorities towards the reduction of phosphorus inputs to the lake. Conventional remediation measures focused on the amount of P adsorbed into iron oxides that are released to the water column under anoxic conditions. The present work suggested that biological P storage/release by denitrifying bacteria in sediments might as well contribute to the release of phosphorus from sediments. Future measures towards lake restoration should include in addition to the classical procedure an evaluation of the contribution of biological processes in sediments to the eutrophication problem.

Keywords:*Proteobacteria*, sediments,bacterial diversity, activity

NOMENCLATURE

amo*A* - Ammonia monooxygenase gene;

anammox - Anaerobic ammonium oxidizing bacteria;

AOB - Ammonium oxidizing bacteria;

BMFC -BenthicMicrobial Fuel Cell;

DGGE -Denaturing gradient gel electrophoresis;

DNA- Deoxyribonucleic acid;

DNB - Denitrifying bacteria;

dsr*A*- dissimilatory (bi)sulfite reductase, subunit alpha;

Fe - Iron;

IRB - Iron reducing bacteria;

N - Nitrogen;

NH_4^+- Ammonium;

nir*K*, nir*S* - Nitrite reductase *gene;*

NO_2^- - Nitrite;

NO_3^-- Nitrate;

NOB - Nitrite oxidizing bacteria;

nos*Z*-Nitrous oxide reductase gene

OM - Organic matter;

P - Phosphorus;

PAO - Polyphosphate accumulating organisms;

PCR- Polymerase chain reaction;

pHzpc -pH at the point of zero charge;

PO_4^{3-}- Phosphate;

qPCR - Quantitative polymerase chain reaction;

RFLP - Restrictionfragment length polymorphism;

RNA - Ribonucleic acid;

rRNA - Ribosomal ribonucleic acid;

SRB - Sulphate reducing bacteria;

SSCP - Singlestrand conformation polymorphism;

TFe - Total iron;

TGGE - Temperature gradient gelelectrophoresis

TN - Total nitrogen;

TP - Total Phosphorus;

t-RFLP -terminal-restriction fragment length polymorphism

1. INTRODUCTION

Sediments are repositories of the overlying water body (for example, ocean, lake, river, or reservoir) composed of organic and inorganic materials (Huettel *et al.*, 2003; Chen and White, 2004). The composition of sediments is influenced byseveral factors namely the chemistry of the overlying water, the level of primary productivity, runoff or rivers, as well as biogeochemical transformations occurring inside sediments (Huettel *et al.*, 2003; Chen and White, 2004; Reitzel *et al.*, 2007). They receive substantial inputs of both allochthonous material (both organic and inorganic) and autochthonous material (mainly organic) from primary productivity (Huettel *et al.*, 2003; Chen and White, 2004).Bacteria in sediments interact with metals and minerals, which are dragged to sediments by settling of materials from runoff, changing their physical and chemical states (Gadd, 2010). Many minerals (calcium carbonates, silicates and iron oxides or sulphides) can also be products of microbial metabolism (Nealson, 1997; Gadd, 2010), as for examplemagnetite that is an end product of Fe(III)-reducing microorganisms in sediments (Lovley, 1991).

1.1. Biogeochemical Profiles in Sediments

The composition of sediments in terms of organic matter (OM) (Nelson *et al.*, 2007), total nitrogen (TN) (Haukka *et al.*, 2006; Herrmann *et al*., 2009) and total phosphorus (TP) (Li *et al.*, 2005; Zeng *et al*., 2009), as well as in situ physical-chemical parameters, dissolved oxygen and pH(Koretsky *et al.*, 2006), have been reported to drive the dynamics of microbial communities in sedimentary environments. Thus, a better characterization of biogeochemical parameters in sediments combined with sediment microbial communities assessmentmay help to clarify the environmental function of microorganisms in sediments.

In general, the determination of biogeochemical profiles in sediments requires sedimentsampling and execution of physical-chemical analyses (Bufflap and Allen, 1995; Laskov *et al.*, 2007). The most common profiles obtained in the solid phase and the respective analytical methods are the OM profile determined by weight loss at ignition (Nelson *et al.*, 2007; Zeng *et al.*, 2009), TN and TP profiles obtained by acid/alkaline or microwave digestion (Andersen, 1976; Johnes and Heathwaite, 1992; Zeng *et al.*, 2009), metals (Tessier *et al.*, 1982; Kostka and Luther, 1994; Koretsky *et al.*, 2006), and

individual P species profiles performed by sequential extraction(Psenner and Pucsko,1988; Ribeiro *et al.*, 2008). Profiles in sediment pore-water usually require sampling that can be done either ex situ by squeezing or centrifugation, or in situ using vacuum filtration or dialysis (Bufflap and Allen, 1995). The amount of water sampled by these techniques is relatively small, limiting the number of analyses that can be performed. The use of microsensors shortcuts the necessity of pore water sampling, but its availability might still be limited considering the large list of water parameters to be analysed (Stockdale *et al.*, 2009). The most common elements measured with microsensors are O_2, pH, NO_3^-, NH_4^+ and H_2S (Altmann *et al.* 2004; Nakamura *et al.* 2006; Preisler *et al.*, 2007). Microsensors also allow identifying the precise localization of the thin sediment surface layer where aerobic processes take place that might be otherwise overlooked by conventional pore water analysis or mass balance studies (Ahltman *et al.*, 2004). Voltammetric microsensors have also been used to study biogeochemical processes in sediments (Luther *et al.*, 2008; Himmelheber *et al.*, 2008). In a voltammetric work, current is measured while scanning the entire voltage range of the solid-state electrode, which allows the measurement of more than one species at a given time in the same region of space (Brendel and Luther, 1995). To date, O_2(aq), H_2O_2, Fe^{2+}, Mn^{2+}, $S_2O_3^{2-}$, ΣH_2S [$H_2S + HS^- + S^{2-} + S^0 + S_x^{2-}$], organic-FeIII(aq), and FexSy(aq) species are the commonly elements measured using this technique (Luther *et al.*, 2008; Himmelheber *et al.*, 2008; Himmelheber *et al.*, 2009).

1.2. Biogeochemical Processes Driven by *Proteobacteria* in Sediments

Proteobacteria comprise one of the largest divisions within prokaryotes and account for the vast majority of the known Gram-negative bacteria (Gupta, 2000). Phylogenies based on 16S and 23S rRNA geneshave led to the division of the proteobacterial group into five subdivisions or subclasses that have been arbitrarily designated *Alpha*, *Beta*, *Gamma*, *Delta*, and *Epsilon* (Woese, 1987; Gupta, 2000). Nowadays, genetic fingerprinting techniques are routinely used to explore the diversity of microbial communities in the environment (Muyzer *et al*., 1993; Zak *et al*., 2006; Moura *et al*., 2009). These techniques provide a profile of the community diversity based upon the physical separation of unique nucleic acid species (Stahl and Capman, 1994). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE),both based on differences between taxa in denaturation(chemical for

DGGE and temperature for TGGE) of a PCR-amplified gene, have frequently been used to characterize the bacterial diversity in lakesediments(Kojima *et al*., 2006;Nelson *et al*., 2007; Qu *et al*., 2008;Martins *et al*., 2010). Restriction fragment length polymorphism(RFLP) and terminal-RFLP (t-RFLP) are other techniques that profile the microbial community composition based on differences among taxa in the length of restriction fragments of a PCR-amplified gene (Zak *et al*., 2006) and have been used for comparing the community composition and relative abundance of sequences within a targeted microbial group (Polymenakou *et al*., 2005; Schwarz *et al*., 2007). Single strand conformation polymorphism (SSCP) is different from the community profiling techniquespreviously described, in that it is based on electrophoretic separation of the original sample RNA, rather than PCR products derived from RNA or DNA. This technique is based on conformational changes in single-stranded DNA, which result from single-point mutations that can be detected as shifts in migration time (Atha *et al*., 1998) and has been used for profiling microbial communitiesfrom estuarine sediments (MacGregor and Amann, 2006). Another molecular biology tool commonly applied is a metagenomic clone library that is used to screen for functional or taxonomic genes and could result in the discovery of new enzymes, metabolic pathways and organisms with impacts on biogeochemical processes (Zak *et al*., 2006). As an example, the almost complete assembly of *Kuenenia stuttgartiensis* genome, an uncultured bacterium, has revealed unique metabolic adaptations associated with anaerobic ammonium-oxidation as well as iron and manganese respiration (Strous *et al*., 2006). However, in a complex community, it is necessary to analyze an enormous clone library to overcome random sampling of many genomes.

The application of the above mentioned techniques suggested that *Proteobacteria, Bacteroidetes, Chloroflexi* and *Actinobacteria* are the most common phyla in sediments from different environments such as shallow eutrophic lakes (Tamaki *et al*., 2005; Zeng *et al*., 2009), eutrophic reservoirs (Qu *et al*., 2008), meromictic lakes (Nelson *et al*., 2007), meso-eutrophic monomictic lakes (Schwarz *et al*., 2007), freshwater and brackishwater lakes (Kojima *et al*., 2006) and freshwater suboxic ponds (Briée *et al*., 2007). Other phyla currently retrieved from freshwater sediments include *Acidobacteria*, *Deferribacteres*, *Firmicutes* and *Nitrospirae* (Tamaki *et al*., 2005; Schwarz *et al*., 2007; Qu *et al*., 2008; Zeng *et al*., 2009).

The oxidation of organic matter in sediments is coupled to a succession of increasingly less energetically-favourable terminal electron acceptors, namely O_2, NO_3^-, Mn(IV), Fe(III), and SO_4^{-2} resulting in a vertical pattern of redox