A step towards the molecular detection of life on Mars

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English summary

The discovery of organisms in extreme terrestrial environments has changed our views on life, broadened our perception of habitability, and raised our expectations to find life outside our planet. Today, we know not only that life is present in unthinkable extreme environments on Earth, but also that it can be highly diverse. Our neighbor planet Mars has been selected as a main target for the search for extraterrestrial life. Robotic exploratory missions with sample return are a major goal, which pave the way for human exploration.

This thesis belongs to the fields of planetary research and astrobiology. It consists of the development, evaluation and optimization of extraction and amplification methods for biomarkers of unknown life. The research presented in this thesis employed molecular techniques of microbial ecology since these are felt to be important for evaluating the possibility of extinct or extant life on Mars. The attention was directed to complex molecules that store hereditary information, such as DNA (deoxyribonucleic acid), since all known life forms contain these molecules. The advantage of using these molecules as biomarkers is that they can be amplified more readily, allowing for detection at single molecule level. The research reported in this thesis consisted of both fundamental research and method development.

Chapter 1 comprises an introduction to this thesis, addressing relevant subjects such as the chemistry of life, the search for life beyond Earth with emphasis on Mars, and a portrayal of the planet Mars along with a description of the availability of energy and carbon sources and of water in liquid state. Molecules indicative of life (biomarkers), the selection of nucleic acids as biomarkers and issues in their detection, non-nucleic acid based life detection

methods, and the relevance of studying Mars analogue sites on Earth are also addressed in this chapter.

Chapter 2 presents a study of an extreme terrestrial environment and Mars analogue (Mars Desert Research Station in Utah), with mineral compositions and erosion processes comparable to those on the surface of Mars. Cultivation-independent rRNA gene-based molecular analyses of soil samples revealed that life in this extreme environment on Earth is diverse. Bacteria were most frequently detected and showed high alpha- and beta-diversity with *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Gemmatimonadetes* in the majority of samples. Archaeal alpha- and beta-diversity was very low. For Eukarya, a variety of organisms was identified, including fungi, green algae and several phyla of Protozoa. A wide variety of putative extremophiles of all three domains of life (Archaea, Bacteria and Eukarya) were detected (but these were not detected in all samples). A large variation in occurrence and diversity over short distances indicated the need for a high spatial density of sampling. 'Spiking' experiments, i.e. the addition of external DNA, revealed that this may relate to low DNA recovery from some samples due to adsorption or degradation. This revealed that DNA extraction methods still required optimization vis-à-vis extraction efficiency (addressed in detail in **Chapter 3**). With respect to future missions for life detection on Mars, this Chapter makes us recommend high-density sampling at any location of interest and spiking experiments to assess extractability of DNA from Martian soil.

The adsorption of nucleic acids to mineral matrices can have detrimental effects on molecular microbial ecology studies, in particular in low-biomass environments on Earth or Mars. **Chapter 3** addresses the determination of the recovery of nucleic acids from a range of minerals relevant to both Earth and Mars. Clay minerals, but also other silicates and non-silicates, allowed only very low recovery (<1%) of DNA added as spike. Consequently, optimization of DNA extraction was directed towards clays. The most efficient of the tested

methods included a high phosphate solution (1 M phosphate, 15% ethanol buffer at pH 8) at the cell lysis step of the DNA extraction protocol, in order to compete the DNA away from the adsorption sites. This method enhanced DNA recovery from clay samples up to 100-fold. This is of great relevance since clays are considered to be important targets for future Mars missions. This new protocol also enhanced the DNA recovery from several mineral samples, whilst maintaining reproducible DGGE profiles. This method also proved to be appropriate for the recovery of low molecular weight DNA (<1.5 kb). Consequently, it may also be useful for studies on ancient DNA since such DNA tends to be fragmented and degraded, and it may also be relevant for Mars because if life that once existed on Mars may have gone extinct.

Since minerals may influence both microbial community composition and the detection thereof, further investigation was performed in order to understand microbe-mineral interactions, using the previous optimized extraction method. Consequently, in **Chapter 4** an *in situ* incubation experiment was performed on a Mars subsurface analogue on Earth. This experiment comprised the placement of Mars analogue minerals in an anaerobic iron-reducing aquifer at Banisveld (Boxtel, The Netherlands, EU) serving as an analogue for Mars subsurface that is anaerobic and high in iron. The main aim was to access the impact of minerals on microbial communities. This experiment revealed that mineral composition influences microbial community structure. In particular the availability of Fe (III) in minerals had a large impact on community structure, with *Geobacter* species being dominant in Fe(III)-rich minerals. Differences in soil mineral content will contribute to differences in microbial community structure of soil and sediment samples. Phosphate-rich minerals elicited the highest diversity observed, while clay-rich minerals led to the least diversity. Therefore, mineral composition is a relevant factor to take into consideration while selecting sites for life

detection missions. Minerals containing essential nutrients for life should be considered possible targets.

In **Chapters 2-4** PCR (Polymerase Chain Reaction) based methods were used, but alternatives are desirable for life detection since PCR requires sequence information and sequence information is absent when new life is to be detected, even if that life contains DNA. **Chapter 5** presents a systematic evaluation of bias (related to differences in GC content, DNA integrity and fragment size) introduced by two different Whole Genome Amplification methods in the assessment of microbial community profiles. Both methods do not require defined primers. One of the methods is widely used (MDA - Multiple Displacement Amplification) and the other is a more recent, primer-free method (pWGA - primase-based Whole Genome Amplification). GC content, DNA integrity and size influenced microbial community profiles. MDA-based community fingerprinting was more strongly impacted by GC content than was the pWGA-based method. DNA fragmentation interfered severely with MDA and pWGA. pWGA did not work for amplification of low molecular weight DNA (<1.5 kb), whereas MDA was inefficient. pWGA results were more similar to results obtained by PCR and were less biased towards low GC content species, while neither requiring the addition of synthetic primers nor a denaturing step, and MDA is more suitable for fragmented DNA. **Chapter 5** also includes a discussion of the relevance of biases for life detection and of possible implications for other environmental studies.

Chapter 6 is a general discussion about the main findings of this thesis and how these contribute to the search for life on Mars and unknown life on Earth. It concludes that DNA detection methods are powerful but suffer from their specificity for life that contains Earthly DNA and that methods that are less dependent on standard DNA yet incorporate amplification, should be developed.