Bacterial Modification of Calcium Carbonate

a proposal

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**Introduction**

This study addresses the role of microbial activity in geological environments and the impact on geological processes. Specifically, we will investigate the process of biological weathering of limestone. As weathering is most literally defined as the breakdown of rock *in situ*\(^1\) [1], it would follow that biologically-mediated weathering would be the decomposition of rock as a result of biological action, generally due to bacterial attachment and the creation of a microenvironment resulting from their activity and/or the release of metabolites. Our main focus is on how bacteria modify surfaces, metabolise minerals and alter lattices in freshwater settings. An additional point of focus is on the modification of the most stable calcium carbonate polymorph, calcite, given its ubiquity in sedimentary environments and high reactivity.

**Background**

Connections between microbiology and geology have several ramifications. Bacteria can influence rock weathering while simultaneously producing and consuming various gasses [2]. Bachofen (1991), Kasting, (1993) and Kasting and Siefert (2002), are among researchers who brought the importance of the role of bacteria on earth surface evolution, including the uppermost lithosphere and the hydrosphere, to light [2-6]. As a result of such findings, the role of bacteria in the formation of our current atmosphere is now common knowledge. Microbes can also help provide insights into the mobility of elements and enable the application of such knowledge to environmentally relevant areas including bioremediation [7]. Wu *et al.* (2007) affirm that research has demonstrated that microorganisms are able to accelerate the release of elements from geologic materials. This reportedly may occur directly, in order to acquire nutrients necessary for biomass production (e.g. Welch *et al.* (2002)), or indirectly by excretion of metabolites that decrease pH, complex cations and/or change mineral saturation states (e.g. Barker *et al.* (1998)) [8].

Friis *et al.* (2003) and Lüttege and Conrad (2004), are among few researchers [9-13] that have dealt with the effects of biological processes on calcite dissolution, despite extensive reports on inorganic and biologically-mediated precipitation of calcite. These have established that bacterial metabolism can influence pH, alkalinity and carbonate equilibria by releasing CO\(_2\) and altering charge balance relationships during utilisation of carbon, nitrogen and additional nutrients [11, 12, 14]\(^2\). Those studies are mainly concerned with dissolution in marine environments and with model bacteria [12]. Even fewer studies have addressed the extent to which metabolising bacteria influence limestone weathering on the continent [8, 12]. A recently submitted report by Jacobson and Wu (2009), discusses the microbial

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\(^1\) Under earth surface conditions, when exposed to air, moisture and organic matter

\(^2\) And references therein
interaction with calcite in the context of continental weathering, though as with previous studies, model bacteria are employed. They report that their chosen bacterium is able to lower solution pH metabolically in the presence of glucose and ammonium, thereby accelerating calcite dissolution. Our study will focus on natural microbial consortia, which to the best of our knowledge has not been done. We will be looking at microbial colonisation in form of biofilms under conditions that would closely reflect a natural environment. A microbial biofilm can be described as an ecosystem of interacting high-density bacterial communities that associate with a surface; biofilms are structurally complex, dynamic systems [15, 16]. They can be identified by the presence of an extracellular polymer slime matrix (EPS), which is their main component [15]. It is the EPS that determines the physical properties of a biofilm while the constituent bacteria determine the physiological properties of the ecosystem [15, 16].

**Aim**

We will be looking at the interaction between the organisms, the mineral and the freshwater solution under earth surface conditions. The main question that the proposed research will answer is whether bacterial attachment and their metabolism modify calcium carbonates; or more specifically, if bacteria alter the surface of a calcite crystal in solution. Whether the microbes will attach to the mineral is a given, because microorganisms, given time, will colonise any mineral surface as supported by Lüttge et al. (2005), microbial-mineral interactions begin at the surface of the mineral. In turn, this can only occur if there is attachment [17]. Attachment would normally occur if these bacteria have a natural affinity for the mineral and if the surface is conducive for attachment. Bacteria could be interested in attaching for reasons of stability, so as to better resist shear forces applied in any aqueous system or for nutrition, where these bacteria could use the mineral as a source of nutrients. The research question may be addressed through a number of equally important queries.

Critical questions include:

1. Is there preferential attachment of some bacteria in nature?
2. What is their taxonomy?
3. Do they affect the solid surface?

The first and third questions are currently being reviewed in a separate independent study. The second question addresses what sort(s) of bacteria thrive(s) on calcium carbonate, which have already been partly investigated through genetic assessment and characterisation of the microbial communities that may attach to the mineral surface. This will be discussed in the methodology.
Methodology

This study was initiated by an extensive literature search on bacterial attachment and interactions with calcium carbonate surfaces. A synthesis of the readings is found in the introduction.

Apparatus

Sampling of biofilms in the Montreal region was followed by preliminary experiments to observe the behaviour of the contained bacteria and their attachment (or lack thereof) to materials of interest. This is discussed below.

Preliminary tests using bioinformatics techniques have been used in an attempt to identify what strains exist in the samples. This will enable identification of bacteria that thrive in freshwater\(^3\) carbonate systems. Characterising the bacteria would enable the identification of the nutrients required to prepare a suitable growth medium.

Subsequent analyses later in the year were meant to include the use of a 24-well plate micro reactor, which allows the control of parameters including pH, temperature and dissolved oxygen. The bacteria would be combined with cleaved Iceland Spar calcite rhombs to which they should attach. Solution and growth media compositions would be varied in order to investigate the effect of pH, temperature, dissolved oxygen and shear flow forces on bacterial attachment to the mineral surface. However, the micro reactor proved more complex than necessary and a more rudimentary set-up will be employed, as is discussed in the protocol.

Visualisation of the mineral samples, following batch experiments, will be performed in order to compare the mineral samples to mineral controls, or pristine mineral slides. Atomic Force Microscopy (AFM) (or scanning force microscopy (SFM)) can be used to observe any surface modification that has occurred as a result of attachment.

Procedure

Sampling was carried out in the Montreal area in September, 2008. Limestone is a predominant rock type in Montreal’s landscape. It was important that the chosen environment be exposed to precipitation or moisture so as to support bacteria that survive in aqueous conditions and therefore the biofilms that we are interested in. The south face of Mount Royal, particularly the Trenton carbonate formations is

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\(^3\) Meteoric water in this case
where samples were collected. Three samples were collected from carbonate formations along the Ormsted Trail as follows:

**Site One:** Weathered material (wet) collected from a rock wedge in the metamorphosed Trenton carbonates along the access trail leading to the Ormsted trail up from Pine and Peel streets.

**Site Two:** Slime scraped from a rock surface in the stream on the north side of the Ormsted Trail above the Pine/Peel access trail, below the cliff.

**Site Three:** Weathered material (damp) collected from rock wedge in the metamorphosed Trenton carbonates one or two meters up the cliff on the north side of the Ormsted Trail above the Pine/Peel access trail.

Preliminary tests, including characterisation of the collected samples, were conducted over a span of 12 weeks. Note that only samples collected from sites one and three were included in the preliminary tests and are identified as FA and FB, respectively. There was no chemical analysis (i.e. pH measurements, etc.) of the collected samples prior to microbial analysis.

### Preliminary Tests

Preliminary tests using bioinformatics techniques have been conducted to identify what bacterial strains exist in the collected weathered samples; i.e. identify bacteria that thrive in freshwater carbonate systems. Characterisation is an important which enables identification of nutrients required for preparation of suitable growth media.

There are several techniques involved in genetic diversity assessment as depicted in Figure 1 in the appendix [18]. Only a few were employed in our tests due to constraints regarding time and apparatus. Applied techniques include diversity fingerprinting techniques such as Polymerase Chain Reaction (PCR) and Terminal Restriction Fragment Length Polymorphism (T-RFLP), analysis of a population of interest including Cloning. Both techniques made use of the 16s rRNA that is a conserved ribosomal RNA sequence common to all prokaryotes [19]. Most community characterising techniques make use of 16S rRNA sequence analysis, as otherwise profiling would prove difficult, given the diversity and complexity of bacterial communities [20]. A brief description of each process is provided in the appendix including the advantages and the limits of each one.

### Current Findings

The aim of the preliminary tests was to become familiarised with sequencing and bacterial characterisation techniques and identify their limitations. It was established that both weathered
material samples are robust with typical soil dwelling microbes belonging to the taxonomic group Actinobacteria, consisting of gram positive heterotrophs. A description of this phylum is found in the appendix.

A more reliable and detailed characterisation of the bacterial communities could be advised. A revision of the employed techniques as well as their execution may prove useful as there were a few mishaps with implementation in the lab. Majority of the bacteria during the database analyses were returned as ‘unclassified’, indicating one of three factors: perhaps the bacterium is identified as belonging to an undetermined phylum; it is the reflection of a chimera; it is simply an incorrect sequence. Thus the results are solely preliminary and there is still room for refinement.

Proposed Protocol

The proposed protocol is based on the literature search carried out, in combination with the preliminary results of the regolith sample characterisation. This set-up is also limited by challenges which are discussed in the appendix.

Questions that were important to the set-up include:

*What factors limit bacterial growth?*

*How is the growth of biofilms affected by factors such as shear forces and or mixing, pCO2?*

*What is the effect of the growth medium on mineral dissolution? I.e. in relation to inhibitors such phosphate, magnesium and dissolved organic matter.*

The proposed reactors will be beakers or fleakers with vented caps. Calcite rhombs will be suspended in a beaker from the cap using two (preferably) metal rods coated in a polymeric mask\(^4\) that will prevent leaching into the medium. Plastic rods will not be used as these, in case of leaching, will act as an added carbon source for the bacteria. The minimal media chosen are those used in similar experiments by Wu *et al.* (2007, 2008) and Jacobson and Wu (2009). These are discussed below. End of the run calcium measurements can be used to measure mineral dissolution. The calcium can be measured in various ways including alkalinity titrations, ICP-MS; a third way to follow the calcium would be to apply a

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chelating agent such as EDTA which will sequester the calcium present in solution. The amount of calcium chelated would be derived from the amount of EDTA added.

Control experiments will consider three scenarios involving a calcite-equilibrated solution, atmospheric pCO₂, 25°C and pH 8.1. Our system will allow free gas exchange. The point of this set of experiments would be to investigate the effect of phosphate on the rate/ nature of colonisation.

Wu et al., 2007, developed two specifically formulated media that mainly contain carbon and nitrogen which are crucial for biomass synthesis. Cation sources are kept to a minimum as these may mask the effects of rock dissolution [8]. Both media are referred to as P-limiting and P-bearing, respectively. Ca and Fe are exempted and there is minimal use of Mg, Na and K. Recipes for both are found in the appendix.

It is important to select media free from chelating agents as we don’t want anything that we cannot control influencing the dissolution of the mineral. Chelating agents may enhance dissolution by binding free Ca²⁺ ions, lowering its activity in solution [12]. Both P-limiting and P-bearing media are free of EDTA.

Our carbon source will most likely be acetic acid in lieu of glucose which is listed as the carbon source in the media recipes. Glucose is not favourable as we would like to simulate, as accurately as possible, natural growth environments for soil-dwelling bacteria. Glucose contains an artificially high amount of carbon- an amount that wouldn’t readily be available in nature. This would greatly influence the growth rate of the microbes.

The first scenario would involve calcite rhombs and a P-bearing minimal medium free from bacteria. The concentration of phosphate in solution would be varied and the rate of dissolution measured. We would observe the relationship between the concentration of phosphate in solution and the dissolution of the mineral.

The second scenario involves removing any phosphate from the medium. This will employ inoculated P-limiting medium into which a calcite rhomb, pre-treated with phosphate, will be introduced; colonisation will then be observed. We intend to derive a growth curve from this process by transferring the rhombs to fresh media at three to five day intervals and observing, using fluorescent microscopy, the attachment of bacteria. The rhomb will serve as our slide.

The third scenario also involves an inoculated phosphate-free medium, though the intention is to pre-treat the calcite slide with phosphate and to adsorb as much phosphate as possible to the surface before exposing it to the medium.

Subsequent experiments may adopt a similar set-up; this is yet undecided. Visualisation will involve the use of AFM. It is perhaps one of the best visualisation techniques to employ for the measurement of bacterial modification of surfaces in freshwater systems as it is a powerful tool for the observation of microbes in aqueous solution with nanoscale resolution [21]. This is advised to be used in conjunction with Vertical Scanning Interferometry (VSI) that offers a wider field of view and can be used to visualise
many microorganisms on a surface simultaneously, which may deem suitable for the visualisation of biofilms [21]. VSI will however not be employed as it is not available at McGill.

**Next Step(s)**

The majority of studies on the influence of microbes on mineral dissolution of calcite report no significant results regarding direct microbial action. In contrast to what we sought from the literature search, a number of studies discuss either the inhibitory effect of bacteria on calcium carbonate dissolution or no other apparent modification of the mineral [10, 17]. Most biologically-mediated dissolution that has been observed is indirect, such as is reported by Lüttge and Conrad (2004). With metabolically active microbial cells, Jacobson and Wu (2009), have observed no dissolution of calcite mineral surfaces with lactate but have reported dissolution when glucose is used instead, as a carbon source. We will try to reproduce these findings in our experiments on biofilms with acetate as an energy source.

**Timeline**

<table>
<thead>
<tr>
<th>Date</th>
<th>Activity</th>
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<tbody>
<tr>
<td>July 2008</td>
<td>Literature search</td>
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<tr>
<td>August 2008</td>
<td>Literature search</td>
</tr>
<tr>
<td>September 2008</td>
<td>Literature search, sampling of sludge and preliminary tests</td>
</tr>
<tr>
<td>October 2008</td>
<td>Preliminary tests and drafting of proposal</td>
</tr>
<tr>
<td>November – January 2008</td>
<td>Drafting of proposal and preparation of oral presentation</td>
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<tr>
<td>February 2009</td>
<td>Drafting of proposal, preparation of oral presentation and 24-well micro reactor training</td>
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<tr>
<td>March 2009</td>
<td>Familiarisation with 24-well micro reactor</td>
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<tr>
<td>April 2009</td>
<td>Control experiments</td>
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<tr>
<td>Summer 2009</td>
<td>Control experiments/ additional experiments</td>
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<tr>
<td>Autumn semester 2009</td>
<td>Additional experiments/ data analysis</td>
</tr>
<tr>
<td>Winter semester 2009-2010</td>
<td>Thesis writing</td>
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References

Appendix

Bioinformatics Techniques

Figure 1: Diagram of molecular approaches for assessment of genetic diversity and structure of microbial communities [18]
PCR, introduced in 1985, is a technique that allows for the in vitro exponential amplification of genetic material [22]. This has become a base technique that is carried out during cloning and sequencing procedures and thus was a recurring technique used in the preliminary tests conducted. DNA sequencing enables the determination of the order of nucleotides on a given piece of DNA. The limitations common to this technique include PCR bias and artefact formation. With regards to the bias, for techniques carried out in conjunction with PCR, usually the template to be amplified contains homologous genes, most commonly the 16S rRNA gene. If there is a mismatch of the primers used in amplification with the sequence of interest, or a degeneracy of the primer that prevents it from binding efficiently to the target, there will be selective amplification, equating to reduced amplification efficiency which is interpreted as a ‘amplification bias’. In other words, the relative abundance of homologues present in the amplified product will not be representative of the gene ratio of the template [23, 24]. Artifacts are basically the result of single stranded DNA that has folded in on itself and re-annealed.

T-RFLP refers to Terminal Restriction Fragment Length Polymorphism and is a procedure used in profiling bacterial communities. It allows for comparative analysis of bacterial communities in a given sample. It is more of a fingerprinting technique, thus a means of determining what is present in the sample as a function of numerical dominance; fingerprinting serves to provide a global picture of the genetic structure of the bacterial community [18, 20]. The technique is conducted as follows:

DNA from the samples in question is extracted and purified using a provided kit\(^5\). The 16S rRNAs present in the extracts are amplified using PCR (where primers specific to a desired segment of the DNA are used); the 5’ primer is labelled with a fluorescent marker. The amplicons are then cleaved using restriction enzymes. The restriction sites for each individual differ and as such the lengths of the resultant fluorescent fragments will also differ. The polymorphic fragments are then run on electrophoresis (in this case, Capillary electrophoresis [personal communication D. Frigon]) and then and then depicted graphically in form of an electropherogram, where the generated peaks represent the abundance of a given microbial community present in the sample. In essence, the lengths of the different fragments distinguish bacterial communities.

There are several limitations associated with this technique, whether as a result of human error or of problems with the apparatus. Since it makes use of extraction techniques to extract the DNA and subsequently PCR to amplify the DNA to a quantity sufficient for further characterisation, the limitations of both procedures affect T-RFLP. The most relevant problems may be those related to sizing. It is possible that digestion with restriction enzymes may not be specific enough or that incomplete digestion may occur. In addition, it is advised that all parameters be standardised so that any differences in community profiles will relate solely to the differences in phylogeny as opposed to differences in sample preparation [20]. Artifacts, if present, may show up as ‘false peaks’ on the electropherogram; note that in our T-RFLP procedure false peaks are not considered to be a problem as the DNA was

\(^5\) Mol Bio PowerSoli kit®
digested with Mung Bean extract following PCR, so as to eliminate any artifacts that may likely have formed [25].

**Cloning** is another means of amplifying genetic material, though more time consuming and labour intensive than PCR; an added drawback of cloning coupled with sequencing would relate to the expense of the procedure [18]. Cloning coupled with sequencing, like T-RFLP allows for microbial characterisation and can be distinguished from the latter technique as it is more a means of amplifying an already identified sequence of interest rather than profiling an unknown number of communities. It enables assessment of the diversity of the community in terms of the number of different species and, to a lesser extent, the relative abundance of these species; Sequencing allows a fine identification of uncultured bacteria as well as an estimation of their relatedness to known culturable species [18]. Here upon extraction and purification of DNA from samples FA and FB, this is then amplified using PCR. In accordance with a Cloning Kit<sup>6</sup>, provided vectors<sup>7</sup> are then spliced with the amplicons and these are introduced into TOP10 *E. coli* bacterial cells which are then plated on agar with an LB nutrient medium and allowed to proliferate. Transformed cells are selected for using ampicillin resistance.

**Actinobacteria**

Actinobacteria, one of the largest groups of bacteria, are comprised of gram positive microbes, implying that their cell walls consist of a thick peptidoglycan layer. Actinobacteria are known for their high Glycine-Cytosine (GC) content (greater than 55 mol%) [26-28]. They are soil- dwelling microorganisms, widely distributed in aquatic and terrestrial ecosystems and divided into 39 families and 130 genera [26]. These genera cover a wide range of morphologies (including coccoid, rod-coccoid and fragmenting hyphal) and physiologies [26, 27]. In nature Actinobacteria play important roles in decomposition and humus formation. They also play additional roles in medicine, industry and environment, where some species produce antibiotics and few others are pathogens [26, 27].

**Potential Challenges**

One challenge we have encountered thus far in the planning stages is the inherent lack of reference literature on the use of mixed cultures in studies on the physiological nature of microbial mineral attachment; at least, not to our knowledge.

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<sup>6</sup> Invitrogen<sup>®</sup> TOPO TA Cloning Kit<sup>®</sup>

<sup>7</sup> pCR<sup>®</sup>-4-TOPO<sup>®</sup>
In addition, the apparent methodological flaws of those few groups that have investigated the growth of bacterial strains and their subsequent influence on mineral surface dissolution - this is most apparent in the choice of growth media which contain phosphate, a known calcite dissolution inhibitor (it adsorbs to the surface of the mineral and prevents dissolution at neutral to basic pH) [29]. As described by Stumm and Morgan, 1996, dissolution of ionic solids occurs by detachment of ions at kinks in monomolecular steps on the crystalline surface. These kinks are points of excess surface energy and the preferred sites of adsorption of inhibitors. Kink detachment is hindered by the adsorption of organic solutes and phosphates, thus retarding mineral dissolution [14]. The adsorption of phosphate on calcite is seen to be strongly pH-dependent and occurs in under-saturated solutions [14, 29-31]. Although not confirmed by way of chemical analysis, phosphate is a main constituent of standard microbial growth media such as Luria Bertani (LB broth). Finding a suitable alternative, that doesn’t serve as a phosphate source, serves a challenge.

A second challenge would be the visualisation of the surface topography of the mineral as dissolution occurs during colonisation as even the most sophisticated visualisation techniques including AFM and VSI can only be applied to exposed surfaces. This implies that microbial action is as best only studied at a given time but not as a process or mechanism that elicited the change [17]. It should be noted though that it is difficult to visualise the changes in mineral surface topography of a colonised surface. The abovementioned techniques can only be applied to exposed surfaces. Thus an interesting aspect to investigate would be an efficient means of removal of microbes from the mineral surface prior to visualisation. This is discussed by Buss, et al., 2003 among others, who establish based on tests with a variety of detergent that for strictly topographic analyses such as AFM, treatment with sodium dodecyl sulphate (SDS), followed by acetone, would be the most suitable for non-residual removal of microbial matter [17, 32].

### Media Recipes

Table 1: P-limiting medium showing major constituents in g and minor constituents in μg [8]

<table>
<thead>
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<th>Constituent</th>
<th>Concentration[^8]</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.04 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.0005 g</td>
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<tr>
<td>MgSO₄</td>
<td>0.0005 g</td>
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[^8]: per litre of solution
### Tables 2a and 2b: P-bearing media showing major constituents in g and minor constituents in µg [8]

<table>
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<th>Constituent</th>
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<tbody>
<tr>
<td>MnCl₂·4H₂O</td>
<td>12 µg</td>
<td>CoCl₂·6H₂O</td>
<td>4.8 µg</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>1.2 µg</td>
<td>Na₂MoO₄·2H₂O</td>
<td>3.6 µg</td>
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<tr>
<td>ZnCl₂</td>
<td>2.4 µg</td>
<td>LiCl</td>
<td>0.6 µg</td>
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<tr>
<td>H₂BO₃</td>
<td>1.2 µg</td>
<td>KBr</td>
<td>2.4 µg</td>
</tr>
<tr>
<td>KI</td>
<td>2.4 µg</td>
<td>Cl⁻</td>
<td>2.4 µg</td>
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<tr>
<td>BaCl₂</td>
<td>0.6 µg</td>
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9 per litre of solution
10 per litre of solution
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