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INVESTIGATING EFFECTS OF METAKAOLIN CONTENT ON THE PHYSICAL PROPERTIES OF CONCRETE, AND ITS SUSCEPTABILITY TO COLONIZATION AND BIODEGRADATION BY SULPHUR OXIDIZING BACTERIA

By

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Ryerson University, Toronto, Ontario, 2010

A thesis

Presented to Ryerson University

In partial fulfillment of the

requirements for the degree of

Master of Applied Science

in the program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2013

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Investigating Effects of Metakaolin Content on the Physical Properties of Concrete, and its Susceptibility to Colonization and Biodegradation by Sulphur Oxidizing Bacteria.
Master of Applied Science, 2013

Christopher Bentley
Environmental Applied Science and Management
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Abstract

Biogenic sulphuric acid attack on concrete is a concern worldwide, as it can lead to collapse of sewer infrastructure. Despite knowledge of the cause and the degradation pathways, not much is known about colonization patterns by sulphur oxidizing bacteria. These bacteria were grown on concrete in attempts to catalog degradation and colonization patterns. This was achieved using a battery of concrete property tests and experimenting with imaging techniques. Increased metakaolin content of concrete decreased sorptivity and chloride permeability of concrete while increasing strength and porosity. Concrete with higher metakaolin appeared more resistant to biogenic acid attack, despite increased porosity. Advances were made in protocols for imaging bacteria on a concrete surface, a challenge given the presence of autofluorescing materials in concrete. Information gained has shown that imaging bacteria on an autofluorescent surface can be achieved, and recommendations are made to further advance these efforts.

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1. Introduction

1.1 *History of Sewage Systems*

Since the inception of the modern day sewer system, an increase in public health and general street cleanliness has been achieved (Foil *et al*, 1993). The World Health Organization (WHO) attributes that sanitation systems play a significant role in keeping both homes and communities free of disease (World Health Organization, n.d.). This idea that sanitation could help a population thrive has been understood for centuries, although in the early days sanitation may have been more about the removal of smell than for the population's safety. Sanitary systems can be dated as far back as 3400 B.C. where both the Aegean and Indus people (circa 3400 B.C.E. and 2550 B.C.E., respectively) were found to be building systems into homes which would carry waste to a small hole in the ground which would be a few meters in size and these "toilets" acted much like modern day septic tanks (Foil *et al*, 1993). The later Minoan civilization (3000-1000 B.C.E.) had extensive knowledge of hydraulics and capacity to build fountains as well as sewer systems which transported wastewater, roof waste, and general drainage water long distances from the main palaces (Foil *et al*, 1993).

These systems, although very sophisticated for the times, were short lived and did not significantly influence how other civilizations handled waste. During the Middle Ages many European civilizations used simple disposal methods such as tossing it out the nearest window or collecting waste, of all kinds, in refuse piles which existed within the borders of the city (Foil *et al*, 1993). England, as an example, experienced both the Great Plague of 1665, responsible for 60,000 deaths, and many cholera epidemics in the 1800s which have been attributed to the cesspool like conditions of the Thames River, which was used as early London's refuse area. It was during these times the familiar nursery rhyme

"Ring around the rosie" became popular as it described the symptoms of the 1665 epidemic (Schladweiler, 2002)[1].

Despite early evidence showing that disease outbreak was triggered by poor sanitation; it took until two separate discoveries for proper sanitation to take flight. These two discoveries were the clear correlation between a clean water supply and disease (discovered by John Snow) and the discovery that germs caused disease (discovered by Louis Pasteur) (Alphin & Verstraete, 2003; Foil *et al*, 1993; Sterling *et al*, 2009).

The first modern day sewer systems were then built in Hamburg, Germany in 1843 and in London, England in 1847. These systems were built large enough to allow for regular maintenance and laws were created for the connection of the sewer ways to the population's homes directly. The standards for health and safety that these systems created were said to have finally matched those that the Indus people had created more than 4000 years earlier (Foil *et al*, 1993).

1.2 *Environmental Impact of Sewage Systems*

As mentioned above, sewers have helped to substantially reduce human disease as populations are no longer forced to interact on a daily basis with their own filth. The effects that sewage can have, however, are not solely linked to humans; our own waste can have a wide variety of impacts on the environment and ecosystems as well.

1.2.1 Environmental Impact of Development and Construction

Infrastructure construction can have a major impact on the natural environment, and as the world increases in population the demand for further development will continue to increase. As an

[1] Credible source based on use by US EPA in document "Rehabilitation of Wastewater Collection and Water Distribution Systems", 2009

example of the ever-growing market for new housing www.newinhome.com, a website owned by the Toronto Star, shows that as of June 4th, 2013, over 150 developers are working on 1477 new developments in the Greater Toronto Area alone. These projects range from new high-rise developments to new subdivisions, each of which has a different environmental footprint. Although the scope of this paper is focused around degradation of sewer systems, full scale building operations always include the production of new sewer lines to connect the newer areas to the older infrastructure and therefore should be considered as connected in terms of total life-time impact. Notable environmental impacts of development include: displacement of species as habitats are destroyed, possible detriments to water bodies, and increases to CO_2 levels as well as other greenhouse gases (Lee *et al*, 2004).

Untouched lands like forests, thickets, and fields provide a number of species with suitable habitats. However, due to ever rising human population numbers, these lands are being consumed at rapid rates leading to the displacement of thousands of species and placing those considered "at-risk" in dire straits. Residential land development can result in a reduction of habitat quality (Kurki *et al*, 2000). This can lead to "at-risk" species disappearing, declining in numbers, or becoming more vulnerable (Robles *et al*, 2008), and can create barriers to movement (Beier & Loe, 1992), which may increase predation (Kurki *et al*, 2000). In a study looking at 34 ponds across New Brunswick it was found that amphibian abundance was negatively influenced by the presence of roadways and pre-commercial clearing projects (Jacobs & Houlahan, 2011). Although it is common for people to focus only on how development affects animals, land development projects can also have negative effects on plant diversity. It was found that development activities on lands adjacent to aquatic habitats (250 – 300m) had a negative effect on many aquatic plant species. This was attributed to destruction of plant species during development but also due to development altering dispersal routes (Houlahan *et al*, 2006).

1.2.2 Environmental Impact of Sewage on Human Health

As mentioned above (section 1.1), lack of proper sanitation has and can lead to the outbreak of disease. While most developed nations do not suffer from these outbreaks any longer due to the installation of sanitation systems, many places around the world still suffer the effects of untreated sewage. It is estimated that 3.4 million deaths per year can be linked to unsafe water, poor sanitation and hygiene (Ustün, 2008). In Varanasi, India a large amount of untreated human waste enters the Ganges River. Over an 11 year period (1993 - 2004) fecal coliform counts were found as high as 10^8 MPN/mL (most probable number). This has led to an average of 66% of the polled population suffering from water-borne/enteric disease in which cases of cholera/cholera death, dysentery, and hepatitis A were reported (Hamner *et al*, 2006). Subsequent research had also found detectable levels of *E. coli* O157:H7 located within the same body of water. *E. coli* O157:H7 is a very pathogenic bacterium which can cause hemorrhagic diarrhea and kidney failure, it has high mortality rates, and can cause infection with as little as 10 to 100 cells through ingestion (Hamner *et al*, 2007) and likely persists within the body of water through the defecation of the infected into the river, a common latrine for the poor in India. In Karnaphuli estuary, Bangladesh it was found that point sources for sewage had an enhancing effect on cultivable *Vibrio* counts (Lara *et al*, 2009), significant as *Vibrio cholerae* is the causative agent of cholera.

After hurricanes Katrina and Rita (August 2005 and September 2005 respecitively), water and sediment samples were collected from Lake Pontchartrian, Louisianna (a heavily affected body of water after these hurricanes) and from canals within the residential areas of New Orleans. Elevated levels of fecal contamination were found in many samples and in some cases were found to exceed recreational water quality requirements (Sinigalliano *et al*, 2007). Similarly a paper presenting data from the same region, following the same two storms, found that hurricanes/floods have the potential to drastically modify microbial communities in watersheds (Amaral-Zettler *et al*, 2008). Water collected from canals

showed a microbial community similar to that of raw sewage, in which some potentially pathogenic microbes were found due to discharge of sewage to flood waters (Amaral-Zettler *et al*, 2008). In both papers mentioned in this paragraph *Vibrio spp.* (Including *Vibrio cholerae)* were detected and are can be considered pathogenic, some being highly pathogenic.

The above issues highlight the extremes: poor or no sanitation system and unpredictable weather patterns causing flooding and infiltration of sewage to flood waters. However, germane to this research, the collapse/failure of sewer systems can lead to the release of similar bacteria to waterways that are used for recreation and drinking water (see section 1.10 for examples of sewer system collapse).

1.2.3 Environmental Impact

Raw sewage is a cornucopia of nutrients (nitrogen, phosphorus, sulphur, etc.), heavy metals (mercury, cadmium, copper, etc.), pharmaceuticals, personal care products and hormones. For all intents and purposes most of these products mentioned above are reduced to relative low levels at wastewater treatment plants. However, failures in the system transporting waste to the treatment plants can lead to untreated wastewater entering rivers, streams and other larger bodies of water that are used as drinking water sources, as places for water sports and recreation and as habitat for animals and plants. Failures can include ruptures or complete collapses of the sewer lines and mains or overflows due to high rainfall events. As there are many contaminants found in raw sewage a detailed description of what each may do to the environment is not possible. However, in the paragraphs below an example of how each class may affect the environment will be discussed.

1.2.3.1 Effect of Nutrients

Raw sewage can be a high source of both nitrogen and phosphorus, and biosolids (a by-product of waste water treatment) has been used as a cheap fertilizer for this reason. Although benefits do exist for sewage and biosolids containing high amounts of nutrients, overloading of aquatic systems such as streams, rivers and lakes can lead to or speed up the process of eutrophication.

Eutrophication is the process of over stimulation of primary production through the introduction of limiting nutrients and organic matter (Nixon, 1995). This can lead to anoxic/hypoxic conditions and an increase in the turbidity of water (Cloern, 2001). A study was conducted on the Cachoeira River estuary, Brazil, where a portion of this river receives sewage treatment effluent. The study's author believes that the sewage treatment effluent was linked to the increase of chlorophyll a concentrations (direct indicator for primary production) and the presence of anoxic zones especially during the drier seasons in Brazil (Silva *et al*, 2013). Studies of the Romanian Black Sea have found that human input, including sewage, has caused the body of water to become eutrophic and over a period of 17 years (1973 - 1990) this has led to the estimated loss of 5 million tons of fish (Borysova *et al*, 2005). In contrast to this, it was found in 2006, in the same body of water after an upgrade was performed on the Constanta treatment plant, the levels of nutrients diminished and the eutrophication levels were seen as strongly diminished. The averages from 2000 to 2010 were still higher than those recorded as the pristine conditions between 1955 and 1965 (Vuță & Dumitran, 2013). Although in both situations we find sewage treatment occurring, we still see the results of elevated eutrophication. Therefore untreated wastewater would lead, likely, to greater total effect.

1.2.3.2 Effects of Heavy Metal

Heavy metal toxicity has been known for a long time and certain heavy metals like lead and mercury can have severe effects at high doses. As an example mercury can cause loss of feeling in

extremities, ataxia, tunnel vision or blindness, and in serious cases can cause convulsion, seizures, paralysis and death (Honda *et al*, 2006). As an example of just how toxic mercury can be, in the 1950s it was found that Chisso Corporation dumped an estimated 27 tons of methylmercury into Minamata Bay, Japan over the course of 37 years. The long exposure time led to over 900 people losing their lives and over 2 million people were left with health effects with the most severe being permanently paralyzed (McCurry, 2006).

Lim *et al* (2013), found, in Masan Bay, Korea, that sediment collected near a sewage treatment plant's effluent was the most heavily polluted with heavy metals, where mercury made up 32% of the mean contribution. When spatial variations were compared between an Ecological Risk Index and a Biological Pollution Index, both showed serious concern for the areas surrounding the sewage treatment plant. Ripoll River, Spain receives input of sewage discharge. It was found that within this river fish species contained levels of mercury within their tissues that exceed European and Spanish legislation on content per gram of fish (Maceda-Veiga *et al*, 2013). The levels of heavy metals in sewage treatment effluent will vary depending on population and industrial activities within a catchment. The relevance to this work is that a failure of sewage treatment infrastructure due to biological degradation of concrete sewer pipes could contribute to release of metals to the environment.

1.2.3.3 Effects of Pharmaceuticals and Hormones

Pharmaceuticals encompass a large range of drugs to treat just as many disorders. There is mounting concern in recent years of these pharmaceuticals and their effects on natural ecosystems. The interest comes alongside the plethora of information showing the presence of these drugs in drinking and recreational water supplies as well as within tissues of fish and other aquatic organisms (Benotti *et al*, 2009; Gelsleichter & Szabo, 2013; Gonzalez Alonso *et al*, 2010; Metcalfe *et al*, 2010). Verlicchi *et al* (2012) compiled data for 118 pharmaceuticals from 17 different classes and from 78 peer-reviewed

pieces of literature. Their results showed that raw sewage has the potential to contain very high levels of many pharmaceutical and personal care product classes, as summarized in Table 1.

Table 1: A highlight of pharmaceutical concentrations found within wastewater treatment plants influent (all data was compiled by Verlicchi *et al* (2012))

Drug Class/Type	**Example of Drug Class/Type**	**Concentration Minimum - Maximum**
Analgesics/Anti-Inflammatories	Ibuprofen, diclofenac, and naproxen	0.0016 - 373 µg/L
Antibiotics	Ofloxacin, roxithromycin, and ciprofloxacin	0.001 – 32 µg/L
Psychiatrics	Carbamazepine and fluoxetine	0.0025 – 25 µg/L
Hormones	Estrogen, estradiols, and estriols	0.002 – 3 µg/L

Most research being conducted on the subject has concentrated on determining the quantity of specific pharmaceuticals within influent, effluent, and receiving bodies of water and not much has been done in terms of effects to ecosystems. However, this is slowly changing. Brodin *et al* (2013), found that while using two different concentrations of oxazepam (anti-anxiety medication) 1.8 and 910 µg/L that European perch experienced altered behaviors. With the environmentally-relevant concentration of 1.8 µg/L, fish were more active, less social, and ate more than those not exposed. In Wascana Creek, Saskatchewan a study was conducted to determine the reproductive effects to fish in streams that are impacted by municipal wastewater effluent. Tetreault *et al* (2012) found that all species of fish studied experienced altered gonadal development and delayed spawning. Male fathead minnows showed fewer secondary sexual characteristics, with high levels of hormones within the water body considered a contributing factor.

1.3 *Ontario's Sewage System History (1800s – 1930s)*

Like the majority of the world at this time, sewage treatment was in its most basic form. During these times most homes used outhouses or privies and these systems usually led down drainage ditches

into waterways such as streams and lakes. In the case of Toronto (one of the largest municipalities at the time) outlet pipes from public and private privies led straight into the harbour where the waterfront was described as "…a source of unpleasantness and a danger to public health…" (Ontario Provincial Board of Health, 1885). Due to the increasing interest in public health the Ontario government formed the Provincial Board of Health (PBH) in 1882, which was asked to help address the problems of sewage and contaminated water supplies (Canadian Public Health Association (CPHA), n.d.).

With the introduction of the PBH many municipalities began improving their water quality as well as sewage treatment. The cost for these systems was high and many municipalities disagreed on what cost they should shoulder to build them (CPHA, n.d.). This caused issues with the PBH because they could only pursue litigation against municipalities and the businesses within the cities as nuisances rather than pursing more desired full lawsuits. At this time the PBH struggled to accomplish anything as it was hard to convince a jury that the actions of an individual or business were truly affecting public health rather than being merely offensive to the senses in terms of look and smell (Benidickson, 1999).

By the end of the First World War, only one out of every three of Ontario's 284 cities, towns, and villages had something resembling sewage treatment. It was at this time that the PBH increased efforts to convince remaining municipalities of the benefits of sewage systems. Coupled to this push for better sanitation was a push for cities to introduce facilities to clean the drinking water supply. From this era arose many new technologies for cleaning water such as filtration, sedimentation, and the addition of certain chemicals (Benidickson, 1999).

1.4 *Modern Sewer Systems*

In developed areas, such as Toronto, one can find up to three different sewer systems: the sanitary sewer, storm sewer, and the combined sewer. The sanitary sewer is responsible for the transport of all wastewater released by toilets, sinks, washing machines and dishwashers, from both

residential and commercial buildings, to a municipal treatment plant. Storm sewers are those which catch rainwater or snowmelt and transport this water to the nearest watercourse including streams, rivers, and creeks which ultimately lead to larger bodies of water such as lakes. Due to the concern of pollutants being transported within storm water, as it washes over oil, gas and other contaminants on its way to a storm sewer, most cities employ the use of storm water retention areas which are designed to capture a certain quantity of water from each storm and slowly release it into the storm water system over a prolonged interval. This is done mainly to reduce the volume of water going down the pipes, but also many more dense or hydrophobic pollutants and particulate matter will settle in these retention ponds, allowing for a reduced volume of pollutants reaching the waterway. Combined sewer systems are those which allow for the transport of both sanitary and storm water within the same pipe system. Combined sewers during dry periods bring all water to a treatment plant for processing, however during stormy periods the amount of water may exceed the capacity of the treatment plant and so the overflow may be diverted directly into the body of water untreated (City of Toronto, n.d.).

All sewer types operate by the flow of gravity in which smaller diameter pipes, such as those found in homes, lead to huge trunk mains that lead to a final destination. If at any point a sewer system encounters an area in which the ground slopes up, pumping stations are used to pump the water up the hill where it can again flow by gravity. In the specific case of Toronto this wastewater can flow through these systems at a rate of two meters per second, with the flow rate determined by how full the pipe is. In both summer and winter the ambient temperature of a Toronto sewer system is roughly 13 degrees Celsius (City of Toronto, n.d.). A rich supply of organic matter and nutrients, renewed with high flow, and relatively constant, benign temperatures provide good conditions for growth of bacterial biofilms on the inner surface of sewer pipes.

Most sewer systems are designed using reinforced concrete or steel, but a multitude of materials are available for use such as plastic and cast iron. The material used depends on a variety of factors such as type of effluent, flow characteristics (uphill or downhill), strength of material, life expectancy (resistance to degradation), ease of handling the material, and cost. In most cases concrete or corrugated steel is used because they both provide great durability; concrete however, tends to be used most often because the smooth interior provides for good velocity though the system while the cost typically remains lower.

The City of Toronto operates 4 wastewater treatment plants, 82 pumping stations, 358 km of trunk sewers, and 4,397 km of sanitary sewers. Within the water treatment and supply sector Toronto has 4 water treatment plants (separate from the wastewater plants), 18 pumping stations, 548 km of trunk water mains, and 5,480 km of distribution water mains. Toronto operates 1,301 km of combined sewage lines, 4,305 km of storm sewers, 463,300 km of sewer service connections, 546 km of roadside ditches, and 371 km of watercourses. This totals 479,689 km of total sewer-pipe length that must be maintained annually (City of Toronto, 2012). One of the main challenges in maintaining this network is that sewer pipes degrade, causing risk to public health, ground water quality, and ecological integrity of receiving waters. Concrete is used for the majority of the network's length because of cost, but is subject to degradation by microbial biofilms colonizing the surface and producing sulphuric acid, described more fully below.

1.5 *Concrete and Concrete Properties*

Cement and concrete are often used as synonyms but, in fact, describe two different things. Cement is used as a binder in creating concrete, while concrete is a fully finished product. Concrete is designed using three main ingredients: cement, course aggregate and fine aggregate. Other materials can be added to give the concrete different properties once cured (M. Lachemi and E. Ozbay, personal

communication, January 18, 2011). As an example silica dust can be added to a mix to produce a concrete with a compressive strength exceeding 100N/mm^2, this is well above standard concrete which can range 32.5 – 52.5N/mm^2 (Barkauskas, 2002).

Concrete is one the most widely used building materials in the world and cement, its binder, is produced and sold worldwide. In 2007 the United States alone produced roughly 95 million tons of cement. However, due to recent residential market slumps these high outputs have reduced significantly to around 64 million tons produced in 2009 (van Oss, 2012). Cement contributes to roughly 10 - 15% of the total volume (and mass) of concrete (Portland Cement Association, n.d.), therefore, the US provided the materials to create roughly 653 - 980 million tons of concrete in 2007, and 426 – 640 million tons in 2009.

Cement comes in various strength classes. Minimum compressive strengths of 32.5, 42.5 and 52.5N/mm^2 after 28 days of curing are used to designate the strength classes. These strength classes are then further subdivided by the initial strength (2 days of curing) by the use of the letters N and R, N being lower initial strength and R being higher (Barkauskas, 2002). Cement used in sewer systems are designed by the contractor to meet the needs of the job, but must maintain specific features. All concrete that is not silica fume overlays and high performance concrete (HPC) will use Portland cement as their binder. These concrete mixes must then achieve a strength rating of 40 MPa (= 40N/mm^2) after 28 days of curing. For high performance concrete, blended hydraulic cement containing silica fume is to be used. Portions of it (silica fume) can be replaced by Portland cement type N (GU in new naming scheme). HPC must achieve 50 MPa in terms of strength and be below 1000 coulombs when a rapid chloride permeability test is applied after 28 - 32 days of curing (Ontario Provincial Standard Specifications (OPSS), 2007).

Aggregates come in a variety of different shapes and sizes and are distinguished by their densities or size depending on the country in which it is being used (Barkauskas, 2002; Ontario Provincial Standard Specifications (OPSS), 2011). Aggregates can be very fine, like sand, or they can be larger, like crushed stone and take up 25 - 35% and 30 - 40% of the finished concrete's weight, respectively (Barkauskas, 2002). In Ontario the creation of aggregates is managed by the Ontario Aggregate Act of 1990 and classification of the aggregate for use in making concrete is governed by the Ontario Provincial Standard Specification (OPSS) 1002 of 2011. In Ontario, aggregate is distinguished by size and is lumped into two main categories, fine and course aggregate. OPSS 1002 determines whether it is fine or course by passing it through sieves in which specific percentages of the load must pass through each consecutive sieve (OPSS, 2011) (Tables 2-4).

Table 2: Gradation Requirements for Fine Aggregate (from OPSS, 2011)

MTO Sieve Designation	**Percentage Passing**
9.5 mm	100
4.75 mm	95 – 100
2.36 mm	80 – 100
1.18 mm	50 – 85
600 µm	25 – 60
300 µm	10 – 30
150 µm	0 – 10
75 µm	0 - 3 natural sand, 0 - 6 manufactured sand
Note: Fine aggregate shall have no more than 45% passing any sieve and retained on the next consecutive sieve	

Table 3: Gradation Requirements (LS-602) - Coarse Aggregate for Structural Concrete, Sidewalks, Curb and Gutter (OPSS, 2011)

Nominal Maximum Size	**19.0 mm**	**16.0 mm**	**13.2 mm**	**9.5 mm**	**6.7 mm**
MTO Sieve Designation	**Percentage Passing**				
26.5 mm	100	-	-	-	-
19.0 mm	85 - 100	100	100	-	-
16.0 mm	65 - 90	96 - 100	-	-	-
13.2 mm	-	67 - 86	90 - 100	100	100
9.5 mm	20 - 55	29 - 52	40 - 70	85 - 100	-
6.7 mm	-	-	-	-	75 - 100
4.75 mm	0 - 10	0 - 10	0 - 15	10 - 30	40 - 80
2.36 mm	-	-	-	0 - 10	0 – 20

Table 4: Gradation Requirements (LS-602) - Coarse Aggregate for Concrete Pavement or Concrete Base (OPSS, 2011)

Nominal Maximum Size	**37.5 mm**	**19.0 mm**	**Combined**
MTO Sieve Designation	**Percentage Passing**		
53.0 mm	100	-	100
37.5 mm	90 - 100	-	95 – 100
26.5 mm	20 - 55	100	-
19.0 mm	0 - 15	85 - 100	35 – 70
9.5 mm	0 - 5	20 - 55	10 – 30
4.75 mm	-	0 - 10	0 – 5

Once a concrete mixture has been created it is deemed fresh concrete and is deemed so as long as it is still workable. The workability of concrete is determined by its consistency. For example concrete lacking fluidity will require compaction to ensure it fills the entire space required of it while concrete that is very fluid will require nothing more than the power of gravity to fill the space. This property is directly defined by the water/cement ratio and each use of concrete will specify a different water to cement ratio. As an example, in reinforced concrete this ratio should not exceed 0.75 (Barkauskas, 2002). The water to cement ratio has a large effect on the concrete once cured as well. This ratio affects strength, permeability, resistance to weathering, bonding strength between concrete and reinforcement, shrinking and cracking (Camp, 2012). A common property of concrete that is affected by

the water to cement ratio, and is commonly manipulated for different desired effects, is its porosity. This is because the final strength of concrete is directly related to its volume of all void space contained within, which includes pores. This influences strength because the total density of the concrete itself is reduced by the presence of these pores (Bentz & Aitcin, 2008). Where less strength is required, increased porosity may be desirable for more economical use of concrete. Sewer systems themselves do not normally require high compressive strength, and increasing porosity may be desired to reduce cost. However, increased pore size and abundance may allow for greater microbial colonization which can eventually lead to further degradation.

Following the fresh concrete stage comes the curing stage. At this stage the hardening concrete is provided with a moist environment to prevent rapid moisture loss. This stage is critical because it is at this stage that concrete develops its strength. It has been shown that a concrete sample left instantly to dry will only achieve 40% of its possible strength, while the same sample left for only three days of curing in a moist environment can achieve 60% of its possible strength (Zemajtis, n.d.). Common practice is to cure concrete for 28 days. At this point it will have gained 95% of its possible strength, and beyond this point increase in strength of the concrete is marginal. Due to the wide uses of concrete not all curing processes are the same and many are designed specifically for use in specific places. For example in one application someone may cover the concrete in a material that prevents moisture loss, while somewhere else one may consistently keep the concrete surface wet. It is dependent on whether the concrete was pre-cast or whether it is being poured on site, and whether that site has direct access to a large water supply (Zemajtis, n.d.).

Concrete, once cured, can fall victim to many forms of degradation. These include fire, aggregate expansion, salt water degradation, leaching of calcium, physical damage, chemical damage, and biodegradation (Howsam, 1990). These degradation pathways do not always act independently but

instead the degradation can be a conjunctive effect of some, if not all, the other pathways (Howsam, 1990). As described in greater detail below, a rich supply of organic matter creates conditions for high rates of microbial metabolism. This may facilitate growth of sulphur oxidizing bacteria on the concrete surface. This pathway has been shown to be capable of removing up to 4.3 – 4.7mm per year of thickness at the air to sewage interface and 1.4mm per year of thickness at the crown (top) of a sewer pipe (Mori *et al*, 1992). Commonly, the sewage-air interface is the most damaged area, as this zone has the best conditions (highest concentrations of H_2S, nutrients and water (splashing onto the concrete from the sewage), and adequate O_2) to support growth of sulphur oxidizing bacteria (Mori *et al*, 1992).

1.6 *Metakaolin*

As mentioned above, many different materials can be added to concrete to alter its final properties. One commonly used additive is metakaolin. Metakaolin is a pozzolanic material that is created by calcinating kaolinitic clay at around 700-800°C (Joorabchian, 2010). Pozzolanic materials are silica or aluminum based and have no cementitious properties on their own. With the introduction of water, however, pozzolanic compounds will react with calcium hydroxides ($Ca(OH)_2$) and form calcium aluminate hydrates and or aluminium silicate hydrates of a gel like consistency (Kim *et al*, 2007). These products then act much like cement and can bind material together when dried. Metakaolin on its own is much smaller in size than standard cement (particle size of 1.3µm vs. 10µm in Portland cement) (Zhang & Malhotra, 1995). This size difference accounts for two different property enhancements for concrete which are directly connected. Due to its small size, metakaolin is able to fill the pore spaces that normally exist when using cement alone. This provides an enhancement in concrete's durability and strength. Further, the concrete will be more durable as it will be less likely to absorb outside liquids, reducing physical degradation associated with salt intrusion or cracking due to expansion of water when it freezes. These enhancements may not be as relevant for production of sewer pipes as they are for

surface construction, as sewer temperatures are above freezing and salt concentrations are relatively low. However, as this is a common additive for making high performance concrete, other properties will be affected that may or may not contribute to increased bacterial colonization.

1.7 *The Cost of Bacterial Concrete Degradation*

The biodegradation pathway mentioned above goes by many names: biogenic sulphuric acid corrosion, hydrogen sulfide corrosion and microbial induced corrosion. This pathway occurs as a result of bacteria converting H_2S gas into sulphuric acid which then attacks the concrete surface. This pathway is so destructive that in 2003 it was estimated that the United States spends 4.5 billion dollars per year rehabilitating its wastewater infrastructure, a number which has been growing by 8 to 10 percent annually over the last 10 years (Sterling *et al*, 2009). While Sterling *et al* (2009) did not specify how much of the rehabilitation need directly attributed to sulphuric acid degradation, the study did suggest this was the primary cause of infrastructure degradation. Germany as well has documented this problem, finding that 40% of all the degradation occurring to their waste water systems was due to sulphur-oxidizing bacteria and has cost them a total of $100 billion (USD) (Kaempfer & Berndt, 1999). Canada, as well, is no stranger to this issue. Estimates show that it could cost upwards of $123 billion (Cdn) to bring all systems within the country to a “fit for use” level. This is due to the fact that 79% of the estimated life of Canadian required infrastructure (which includes wastewater removal) has been spent (Mirza, 2007).

1.7.1 *Revenue for Municipalities and Infrastructure Ownership*

Due to the high maintenance costs associated with these systems, it seems fitting to discuss how a municipality gains funds in Ontario, and who owns infrastructure (including sewer systems). This discussion is intended to provide context for understanding challenges associated with funding infrastructure repairs. The largest revenue stream for municipalities is property taxes, accounting for roughly 53.3% of all revenue for Toronto in 2004 (Kitchen & Slack, 2006). Ontario municipalities can also

receive transfers from the provincial government in the form of conditional and non-conditional grants, through licensing, permits, fines and penalties, investment income, payments that do not involve taxation, and development charges.

During the Mike Harris era (1995 - 2000), there were significant changes in funding of municipalities through transfers and grants, and in mandates for the different levels of government. The changes relevant to this thesis stemmed from a seemingly unrelated issue. The Conservative Party believed that funding for education needed reworking (Garcea & LeSage, 2005). The goal was to provide more equal funding to schools across Ontario by severely reducing schools' dependency on municipal property tax and increasing the amount of Provincial transfers municipalities received, earmarked for schools. This plan was to be 'revenue-neutral' because the Province would find the extra money to fund the schools through a reduction of other transfers to municipalities. Municipalities would then re-direct revenue from property taxes, rather than funding schools. This revenue, along with structural amalgamations of municipalities to reduce redundant spending, was to meet the short-falls associated with loss of provincial transfers earmarked for other purposes (Garcea & LeSage, 2005).

An outcome of this reform that began in funding of schools was a restructuring of who (i.e. what tier of government) paid for what services. Relevant here, municipalities took on greater ownership of infrastructure over time. In 1961 the Federal and Provincial Government owned 69% of infrastructure stock while municipalities owned 31%. By 2002, however, Federal and Provincial ownership dropped to 48% while the municipal ownership rose to 52% (Federation of Canadian Municipalities, 2006). This transfer of ownership was independent of the Harris government reforms of the mid-1990s. However, as a result of these reforms, municipalities took over the burden of maintaining the infrastructure which they now owned. Further, municipalities took on greater responsibility for public housing and public health. Given an aging and growing population in Toronto, these new mandates have created serious

funding challenges for other municipal mandates, including infrastructure repair. It was estimated in 2006 that the national municipal infrastructure deficit was between 60 to 100 billion dollars and growing at the rate of 2 billion dollars a year (Federation of Canadian Municipalities, 2006).

1.8 *Sulphur-Oxidizing Bacteria*

As mentioned before, the leading cause of degradation within a sewer system has been linked to sulphur-oxidizing bacteria. These bacteria normally are found in soil or sediments at the transition from aerobic to anaerobic conditions. These zones can often be located by colour which is relevant to the by-products being created. Sulphur-oxidizing bacteria can often be found in bands of soil or sediment that are either purple/reddish or green dependant on the abundance of oxygen and light penetration (Anderson & Hairston, 1999). While there is a diversity of sulphur oxidizing bacteria, three species in particular make up a large portion of the population in sewer pipes, and are consequently well represented in the literature; they include: *Thiobacillus thiooxidans, Halothiobacillus neapolitanus* and *Thiomonas intermedia* (Mori *et al*, 1992; Okabe *et al*, 2007). *Thiobacillus thiooxidans* is an obligate chemolithotroph, which gains energy through the oxidation of reduced sulphur compounds while gaining its carbon source from fixing CO_2. It is also considered to be acidophillic, meaning that it can survive in environments with low pH (shows no growth when pH is greater than 4.3), and has been found in coal mine drainage and mineral ores. This species has been shown to be very useful for industrial applications and in combination with *Thiobacillus ferrooxidans* can be used to remove sulphur compounds from coal (Jin *et al*, 1992). This is an important function because sulphurous coal, when burned, creates hydrogen sulfate a compound which can become sulphuric acid on contact with water in the atmosphere. This was the leading cause of many deaths in early England when poor quality coal was burned in abundance (Bell *et al*, 2004).

Halothiobacillus neapolitanus, like *T. thiooxidans*, is an obligate chemolithotroph but is only a moderate acidophile. It can live in acidic conditions up to a certain point (highest yield of growth around 6.5 with a steady decrease as pH lowers to 4.5). These bacteria, much like *T. thiooxidans*, can be found where sulphur is abundant (mineral and coal mines). This species was formerly known as *Thiobacillus neapolitanus* until 2000, when it was reclassified as a bacterium that is tolerant to high salt concentrations, signified by the "halo" prefix (Wood *et al*, 2005). This species currently has no industrial application that can be found.

Thiomonas intermedia is a facultative chemoorganotroph, which can create energy through the oxidation of sulphur compounds while fixing CO_2 but can also create energy using other organic compounds for both its energy and carbon needs (London, 1963). These bacteria are moderately acidophilic and, like *T. thiooxidans*, have been found in mine drainage and soil in both the terrestrial and aquatic environment (optimal pH of 6 while increases and decreases from this value resulted in slower growth) (Wentzien & Sand, 2004). Currently, this species is being tested for application in bioremediation in cases where arsenite levels are high (Duquense *et al*, 2007). This is because the *Thiomonas* genus seems to be capable of oxidizing arsenite to arsenate which will then precipitate out of the aqueous phase.

1.9 *Biodegradation of Concrete*

Biodegradation of concrete occurs when conditions of sufficient nutrients (particularly reduced forms of sulphur) and high moisture levels allow for the growth of sulphate-oxidizing bacteria. These reduced forms of sulphur (normally H_2S gas) are produced by sulphur-reducing bacteria that reside within the sludge on the bottom of the pipe or in a biofilm like slime existing just above the sludge layer. These bacteria, under anaerobic conditions, convert sulfates to sulfides and release them into the surrounding environment (Bertolini *et al*, 2004). This H_2S gas then releases from the sludge layer either

through turbulence of the sludge itself or dissolution of the gas due to the alkaline conditions, which consequently reduces the pH of the environment (Vincke *et al*, 1999). This gas release allows for abiotic neutralization on the surface of the concrete reducing the concrete's pH. This reduction of pH on the concrete surface allows for the first microbial colonizers, which are normally heterotrophic, neutrophillic, and halo-tolerant bacteria species, to form colonies (Okabe *et al*, 2007). These bacterial species, which include neutrophillic sulphur-oxidizing bacteria, then allow for biogenic oxidation of the gases produced which continues to lowers the pH of the concrete surface, allowing for acidophilic bacteria to colonize. These bacteria then assist in the further reduction of pH leading to the release of polythionic and sulphuric acid (Wei *et al*, 2010). These acids provide the necessary conditions for the breakdown of the cement portion of concrete (Mori *et al*, 1992; Shiping *et al*, 2010).

The pathways for degradation, which will follow, have been well cataloged due to the wealth of research on the quantification and identification of the bacteria colonizing the sewers. These studies have looked into which species are present, at what abundance, leading to suggestions of which bacteria seems to contribute most to degradation. These studies, however, are all done *ex situ* in which swabs of the surface of a sewer pipe have been examined or concrete samples directly from a site are ground down to a paste and then examined (Amann *et al*, 1995; Diercks *et al*, 1991; Islander *et al*, 1991). Patterns of colonization of the concrete surface have not been examined. The course of development for acidogenic biofilms is important to understanding the patterns of concrete degradation. Moreover, the properties of concrete (e.g. pore structure) that influence the pattern of biofilm development are likely to affect susceptibility to degradation. Studies on the patterns of colonization of concrete have been hampered by challenges of imaging sulphur oxidizing bacteria on concrete surfaces and pores. In this thesis, some progress has been made to allow direct visualization of these bacteria on concrete surfaces and pores to improve our understanding of colonization patterns.

Concrete structures outside of sewers typically do not undergo bacterial degradation. Generally, concrete does not make a good substrate for bacteria because pH is usually quite high, around 12 (Sand *et al*, 1987). Sewer systems, however, provide conditions in which pH can be reduced because moisture levels are high and nutrients are abundant (Lahav *et al*, 2004; Matos & Aires, 1995; Nielsen *et al*, 2005; Zhang *et al*, 2008). These conditions permit high levels of metabolic activity in the sludge layer above the concrete surface, as well as wetted surfaces above the sewage-air interface. Bacterial activity initially generates carbonic and small organic acids that lower the pH of the environment, including the concrete surface. Degradation does not begin until the colonization of sulphur-oxidizing species which normally occurs at pH below 7. At pH levels between 7 and 5, *H. neapolitanus, Thiobacillus intermedia*, and other similar species are able to colonize the concrete surface above the sewage-air interface (or below this interface if oxygen concentrations permit) (Mori *et al*, 1992; Okabe *et al*, 2007). The production of acids by these bacteria facilitate the establishment of *Thiobacillus thiooxidans* which begins to grow at pH of 5 or below. *T. thiooxidans* produces a great deal of sulphuric acid, dropping the pH to as low as 1.5 (Sand & Bock, 1984). There has been observed a positive correlation between the abundance of *T. thiooxidans* and the amount of corrosion in sewer pipes (Milde *et al*, 1983). The corrosion is caused when sulphuric acid is formed which then reacts with free lime in the concrete to form gypsum (Aviam *et al*, 2004).

$$CaO + H_2SO_4 \leftrightarrow CaSO_4 + H_2O \qquad \text{(eq. 1)}$$

This gypsum layer is then referred to as the corroding layer. The corroding layer then reacts with the calcium aluminate contained within concrete to form ettringite:

$$3CaO + Al_2O_3 + 3CaSO_4 + 32H_2O \rightarrow (CaO)_3(Al_2O_3)(CaSO_4)_3 \bullet \quad 32H_2O \qquad \text{(eq. 2)}$$

Ettringite is responsible for the increase in internal pressure of the concrete causing cracks. These cracks are considered a problem because they then lead to increased surface area on the concrete allowing for further bacterial-driven degradation (Aviam *et al*, 2004).

This same process can actually occur to the outside surface of a pipe (meaning the surface touching the surrounding soil) as well, but is highly dependent on the surrounding environmental properties such as depth of ground water, sulphur content of ground water or pore water, redox status, and how permeable the soil is to the flow of sulphuric acid (Skalny *et al*, 2003). However, the necessary conditions for significant rates of sulphur oxidation are much more likely to occur within the pipe than outside the pipe. Non-biogenic acid degradation is also possible both within and outside the pipe. This process is dependent on the oxidation of sulphur containing molecules by free oxygen. For example pyrite (FeS_2) can interact with free oxygen and water to create sulphuric acid (Skalny *et al*, 2003):

$$2FeS_2 + 7O_2 + 2H_2O \leftrightarrow 2FeSO_4 + 2H_2SO_4 \qquad \text{(eq. 3)}$$

The sulphuric acid would then interact with the concrete in a similar manner to the process described earlier. This non-biogenic process has been implicated in degradation of sewer pipes as well as the foundations of some buildings, depending on their relative proximity to groundwater.

1.10 *Examples of Major Failure*

In some places around the world collapsing sewer systems have been a reality due to an inability to maintain an aging system (due to cost and man power available, discussed further in section 1.7 and 1.7.1). Many of these incidents are small and cause temporary disturbances through road closures or pot holes. However, in some cases these systems can cause catastrophic sinkholes. Such cases include Sterling Heights, Michigan where in 2004 a sinkhole opened up (60ft wide x 160ft long x 30ft deep) after an 11 foot diameter sewer interceptor broke. In Portland, Oregon during December, 2006, a sinkhole

swallowed a city work truck and caused a natural gas line to leak after a sewer backup caused the sewer to collapse. In Guatemala City, Guatemala, three people lost their lives and 1,000 people were told to evacuate when a 330 foot deep sinkhole ripped the neighborhood apart. The incident was blamed on heavy rain fall and a ruptured sewer main (Figure 1; Schladweiler, n.d.). As these systems age this will become more and more common; although the number of deaths directly associated with these incidents has been small, incidents such as gas line leaks that may result have the potential to end in greater tragedy. Further, a failure of sewage lines has potential to release untreated human waste into surface water and groundwater, which could increase localized incidents of communicable diseases and environmental degradation.

Figure 1 - Examples of sinkholes caused by sewer system damage or collapse. A) Sinkhole in Sterling Heights, Michigan (2004) B) Sinkhole in Portland, Oregon swallowing up a service truck (2006) C) Sinkhole in Guatemala City, Guatemala with a depth of roughly 330 feet (2007) (All images sourced from: http://www.sewerhistory.org/grfx/misc/disaster.htm)

1.11 *Attempts to Alleviate Biogenic Sulphuric Acid Attack*

As mentioned before in regards to cost of repair, it is obvious that a solution is required so that expenditures on such an issue can be significantly reduced. This problem, however, is twofold. The first problem is that money is lacking for the rehabilitation of the current infrastructure, and so if concrete was produced to withstand sulphuric acid, it is likely that a complete overhaul of the system would not be within the budget of most cities. Secondly, the entire problem is not fully understood and although all the chemical processes leading to the degradation are well chronicled, many of the biological aspects

are not (e.g. What patterns of colonization facilitate establishment of large acidogenic populations? What properties of the concrete affect these colonization patterns?).

As mentioned in section 1.9, it is the anaerobic conditions that lead to the creation of H_2S gas which, under aerobic conditions, is then converted to sulphuric acid and starts the degradation of the concrete. These anaerobic conditions exist because of long retention times of the wastewater itself and the settling of material in areas that are uneven in grade. It has then been suggested that methods be explored for the prevention of anaerobic conditions but all methods that have been tried are very expensive (Ramsburg, 2004). One such method was to reduce the retention times of the sludge, but this would require an entire system overhaul and is well outside most cities' budgets. Another method was the addition of oxygen, hydrogen peroxide or nitrates to attempt to oxygenate the system. This method did provide relief to the system but was never considered for permanent introduction due to its expensive nature (Ramsburg, 2004).

So the prevention of anaerobic conditions is unlikely, but other researchers have attempted to address the problem from the other end by preventing the colonization of sulphur-oxidizing bacteria. This prevention method was attempted using liners that are sprayed onto the inner surface of concrete pipes to prevent growth. This, much like the addition of oxygen, saw great success (Ramsburg, 2004), but was again an impractical solution because the material itself was expensive, as was the labour. Further, these liners are susceptible to delamination and thinning due to friction with products flowing past, and therefore careful monitoring of these pipes is required to ensure that the liners remain intact (Ramsburg, 2004).

Finally a method to produce conventional concrete that is more resistant to sulphuric acid degradation has been attempted. As an example Joorabchian (2010), attempted using metakaolin and lime stone filler to increase concrete's ability to resist acids. This was successful in that it created a

concrete that was more resistant to the acid, demonstrated by conventional testing (i.e. prolonged submersion of concrete in dilute sulphuric acid). The addition of metakaolin also modified many other characteristics of the concrete such as increasing porosity but decreasing its sorptivity (Joorabchian, 2010). Addition of metakaolin could offer a relatively inexpensive improvement on resistance of concrete to acid degradation, provided the results of conventional testing translate to a reduction in biological degradation.

An important component of this thesis research was development of techniques to better simulate environmental conditions within a sewer pipe, so that bacterial colonization (related to concrete properties) and corresponding biological degradation could be studied. The techniques include construction of a test chamber, as well as methods for direct visualization of sulphur oxidizing bacteria on concrete surfaces, and selective probing of these bacteria.

1.12 *Imaging Techniques*

A wide variety of imaging tools exist with potential for the investigation of the *in situ* status of sulphur-oxidizing bacteria. These imaging tools provide a high level of detail and information on both the microscopic and macroscopic environment. These tools include confocal laser microscopes, scanning electron microscopes, ultrasound, and x-ray.

Confocal laser microscopy is a technique used for obtaining images of the microscopic world in both three-dimensions and in high-resolution (resolution being defined as the ability to distinguish two separate entities from one another). This is accomplished by a computer which pieces together images from numerous individual planes (focal depths) in a point by point fashion until a three-dimensional image is created. The advantage to this technology is twofold. One advantage is that, due to its use of lasers to scan at very specific depths, highly detailed images can be created of a single layer at a time. This is advantageous over normal light microscopy in which, if focusing on the middle of a cell, both the

bottom and top layers are visible and blur the image. The second advantage is that one can use filters to select very specific wave-lengths of light. This is important because it allows for the use of fluorescent tagging of an entire bacterium or even very specific sequences of DNA (Pawley, 2006). As concrete is an auto-fluorescent material this provides an interesting avenue of research. A specific wave length known to excite concrete might be used to gain topographical information while a fluorescent probe, excited by a second wave length that does not excite concrete, might be used to image bacteria on its surface. These two lasers, used in conjunction, might allow construction of an image showing the physical relationship of the bacteria to the substrate. This may then provide a better understanding of where on the concrete surface bacteria are colonizing.

In this case laser confocal microscopy presents an interesting avenue in investigating the colonization of bacteria on a concrete surface. A technique commonly used in microbiology for the identification of specific taxa within a mixed community is fluorescent *in situ* hybridization (FISH). FISH uses nucleotide probes that bind directly to specific regions of DNA in target taxa (usually genus or species level specificity). This is important because one can use these probes to identify the locations of specific groups of bacteria on the surface or pore spaces of concrete. By using different probes for different taxa, it is possible to produce snapshots of community composition and, over time, to gain information on patterns of colonization by different taxa (e.g. microbial succession). This technique can also be used to explore how the concrete surface is colonized. The bacteria may colonize a surface indiscriminately, or may preferentially colonize pore spaces. The reason this is important is if the bacteria that produce sulphuric acid preferentially colonize the pore spaces, then creating a concrete with less pore space could be a simple and cost effective solution to a problem that is current plaguing all developed cities worldwide.

Scanning electron microscopy is a technique used to get highly detailed images at the 1 nanometer scale, or upwards of 500,000 times magnification. This is done by shooting a high-energy beam of electrons at the sample and collecting the information that reflects back. The electron beam itself interacts with the atoms that surround the sample and creates a backsplash of energy which is read by a computer as a signal and is constructed into an image. This normally occurs within a vacuum because any atoms other than those of the sample in question would cause interference. However, environmental scanning electron microscopes do exist which allow for a low moisture level to be present. This technology is advantageous in that it provides amazing detail on the surface make up in high definition of any object you place under it (Goldstein, 2003). So, for example, environmental SEM can allow the visualization of concrete's pore space and could provide accurate data on pore structure (number, size, etc).

This technology can not only allow for one to catalog the sizes of the pores present on newly produced concrete, but also to compare pore structure after defined intervals in a degradation study. Currently the only data on concrete loss is of how much is removed annually in total from a surface; an increase of pore structure size has never been investigated and could prove very useful in terms of designing concretes that will be stronger against sulphuric acid. Further, in combination with Laser Confocal Microscopy, it can be used to investigate whether colonization of pores by sulphur oxidizing bacteria modifies pore structure in a manner than might accelerate concrete degradation over time (e.g. by increasing total surface area as pores expand).

Ultrasound is a technique which employs the use of cyclic sound waves that are above the human detection limit for hearing. This technology is used in a large variety of areas but is most frequently thought of as a technology used for medical imaging of soft tissues such as muscles, internal organs, fetuses, etc. This technology is capable of creating images that are detailed enough to allow one

to diagnose lesions to tissue and give accurate sizes to the lesions themselves (Fish, 1990). Due to its relatively inexpensive nature and its portability, this technology is now being explored for its use as a non-destructive imaging technology in many other fields, and strides are being made to allow this technology to detect images at a microscopic level. This is done by using sophisticated algorithms to allow the computer to put together magnified images (Cartz, 1996; Akune *et al*, 2011).

X-rays, much like ultrasounds, are most known for their use in the medical fields for imaging internal structures, normally of the skeletal system. X-rays are beams created from electromagnetic radiation that exist in the wavelengths of 0.01 to 10 nanometers. In the medical field, x-rays pass through an object and are normally "caught" on a detector that can be either digital, producing an image on a computer screen, or non-digital producing an image on a film. This technology, much like ultrasound, is not used solely in the medical field and modified versions are being used for crystallography (chemical analysis of the crystal structure of a compound), industrial radiography (used for inspecting industrial parts and most commonly welds), and in an array of other fields (Goldstein, 2003).

1.13 *Imaging of Bacteria on Concrete Surface*

As mentioned in the section above, confocal microscopes can be used to image fluorescent labels within bacteria, either synthetic labels added to probes, or endogenous labels such as fluorescent proteins. Fluorescent proteins may be produced by genetically modified bacteria, carrying a gene(s) causing it to express these proteins constitutively, or under specific conditions.

1.13.1 *Gene/Plasmid Insertion*

Plasmids are vectors for the insertion of a gene that will replicate separately from the chromosomal DNA already present within a cell, normally a bacterial cell. These plasmids are double

stranded DNA that form circular shapes and house a gene of interest, in this case a gene coding for a fluorescent protein, and a gene(s) for antibiotic resistance (Prescott *et al*, 2005). The antibiotic resistance is of importance because it allows for artificial selection of bacteria carrying the gene of interest (fluorescent protein) through the use of liquid media or a solid agar medium that contains the specific antibiotic that this bacterium has now gained the resistance to (Chavshin *et al*, 2013; Chew *et al*, 2012). Plasmids can transfer between bacteria through two methods which are conjugation and transformation, which will be explained below (Barlow, 2009; Krol *et al*, 2013; Molin & Tolker-Nielsen, 2003). Once inserted these plasmids do not provide their own machinery for replication but instead piggy back on the machinery that already exists within the bacterium to continue to make copies of the gene of interest (Prescott *et al*, 2005).

Conjugation is a method of plasmid transfer through direct contact between bacteria. This occurs when a bacterium creates pili (often referred to as sex pili) which attach to and create pathways between neighbouring bacteria. Once the bacteria are firmly connected the plasmid in the donor bacterium changes from its circular shape to a linear piece of double stranded DNA and travels through the pilus into the host or recipient bacterium. Once the full sequence of DNA has entered the bacterium it will regain its circular shape and begin replication. This is not always a perfect process and many times the other cell only receives a portion of the plasmid. This is crucial because the entire plasmid is required to function correctly (Prescott *et al*, 2005).

Transformation is the process of a cell incorporating DNA from its outside surroundings rather than direct exchange with a donor cell. Certain bacteria are capable of this naturally, however most are not and require conditions that will increase the permeability of their cell walls and membranes. For these cells, a process known as electroporation is often used to make the cells competent to receive DNA. In this process an electrical current is passed across the cell membrane to increase the

permeability of the cell wall. In more basic terms the electrical current “punches” holes into the cell wall allowing plasmids to pass into the cell directly (Prescott *et al*, 2005).

In certain situations it is not of interest to have the plasmid remain within the cell once inserted but instead have the gene of interest inserted directly into the host’s chromosome. For example when observing a community of bacteria where various target species each express a different fluorescent protein, one would not want the fluorescent gene from one species to transfer into one of the other bacteria. In these cases usually a second helper plasmid is used that has the ability to move the gene of interest directly into the bacterium’s chromosome (Prescott *et al*, 2005).

This technology is being utilized heavily in the agriculture sector where many experiments are being run to understand the colonization patterns of specific bacteria and fungi on a variety of common food crops such as corn, potatoes and lettuce (Czajkowski, 2010; Du *et al*, 1999; Zavaleta-Mancera *et al*, 2007). Most of these experiments are delving into ways to prevent the bacteria or fungi of interest from colonizing the crops and destroying them, while others are investigating how the colonization of one species prevents the colonization of a second. As many of these surfaces are hard, such as the surface of a seed, the hope is that this technique could similarly be adapted for use on other hard substrates, in this case concrete.

1.13.2 *Fluorescence In-Situ Hybridization (FISH)*

In situ hybridization describes a technique in which one can hybridize a specific nucleic acid probe to a target DNA or RNA sequence within a cell, prokaryotes tend to target RNA. The premise is that a target cell is fixed in its place (e.g. its place within a biofilm, on a substrate, relative to other cells, etc), and a fluorescent tag attached to the probe will allow visualization of that cell when the tag is excited at a specific wavelength by a laser. The probes used for hybridization can be designed to be very specific, only binding to sequences with high correlation found within members of a single species, or be

designed to be more general and bind to DNA sequences conserved at higher taxonomic levels (commonly genus).

The two methods listed above, gene insert and FISH, provide two unique avenues for the visualization of bacteria on a concrete surface. Gene insertion would produce designer cells in which could be grown directly on the surface of the concrete for visualization. The bacteria could then be monitored to study the evolution of sulphur oxidizing bacteria on concrete surfaces. This approach has the advantage that fluorescent proteins, and therefore signals, will be discretely located within cells, reducing imaging problems associated with background fluorescence. FISH is simpler in concept, as it does not require successful integration of a gene into the host chromosome. Rather, cells are fixed in place at distinct times, and then probed. This technique is quite often used in medicine because it is a quick method for localizing specific suspect pathogens within a patient's tissue or fluids (Liehr, 2009; Prescott *et al*, 2005). This method is often less complicated to use when using a mixed culture because the probes being used can be designed to be specific enough to target specific species.

This technology is commonly used in the medical field and in soil and biofilm microbiology. In this field scientists are using these probes as genetic markers for disease, to examine colonization of the intestinal wall by bacteria in newborns and even to examine the colonization patterns of oral biofilm creating bacteria on enamel (Al-Ahmad *et al*, 2009; Smith *et al*, 2011). Much like the talk on gene insertion, FISH is being used on a multitude of different surfaces opening the possibility for use on a concrete surface.

1.14 *Hypotheses and Study Design*

Previous research by Joorabchian (2010) found that increasing metakaolin content affected some properties of concrete, such as porosity and sorptivity. An increase in metakaolin improved resistance to sulphuric acid attack. Despite an increase in porosity of concrete, higher metakaolin

content reduced sorptivity, and sulphuric acid may not have penetrated concrete as effectively in concrete with high metakaolin content.

Based on this research, it was expected that:

1. An increase in metakaolin would decrease sorptivity but increase porosity (confirming Joorabchian's previous work).
2. An increase in porosity would increase the potential colonization of the concrete surface by bacteria, including sulphur oxidizing bacteria.
3. An increase in colonization by these bacteria would result in greater biological degradation of concrete with high metakaolin content, reflected as a decrease in strength over time.

This last hypothesis contrasts with the work of Joorabchian, where increased metakaolin led to less loss in strength by decreasing acid attack. The principle difference in this study is that the acid is produced on the concrete surface and within pores by bacterial activity, and an increase in colonization might result in an increase in biological degradation.

This study was designed to determine whether factors such as pore size and abundance play a role in the colonization patterns of sulphur-oxidizing bacteria, thereby contributing to biological degradation of concrete. This was done using a variety of imaging techniques relying on the pros and cons of each to create a bigger picture. The use of a gene insert was of greatest interest as it could provide a traceable fluorescent marker that would not necessitate staining and associated challenges mentioned above. As a fall back, protocols were developed to perform FISH on the concrete surface for each of the three bacteria being used in the experiment. Protocols were developed for the use of laser confocal microscopy using a surface (concrete) that is opaque while imaging both the surface and the bacteria at the same time. Scanning electron microscopy was used to see if there were any visual differences in the surfaces of the different concrete mixes and to observe the formation of crystal

structures which would be indicative of bacterial presence and biological degradation. Lastly standardized concrete tests, such as strength and porosity, were performed to actually determine if differences in the concrete types (metakaolin content) altered the physical properties of concrete exposed to conditions favouring biological degradation.

2. Materials and Methods

2.1 *Concrete Production and Curing*

This experiment was conducted using 4 different concrete designs. For each design 21 cylinders of 100 mm x 200 mm and 1 prism of 100 mm x 75 mm x 405 mm were created. 21 cylinders were created to provide 7 cylinders for each time period (0 days, 7 months and 8 months). At each time period the 7 cylinders were conditioned and prepared for a battery of standard concrete tests (described below). The prism was created to allow 10 mm +/- 0.5 mm thick "chips" to be cut from it which were used for studies of colonization patterns by sulphur oxidizing bacteria.

These concrete mixtures were created using ASTM C192 "standard practice for making and curing concrete test specimens in the laboratory" (ASTM Standard C192, 2012). A revolving drum mixer was used in the mixing of the concrete. A vibration table and a stamping rod were used during casting to ensure each mold was filled properly. Once molded the concrete was left for 24 hours to harden before cylinders were removed and placed in a curing room (22°C and 95% humidity) for 28 days. Following the 28 days, 7 cylinders of each concrete type were tested for initial mechanical properties using the battery of standard concrete tests (described below), while the remaining 14 cylinders were placed into testing chambers (acrylic boxes, described below) for incubation. Seven cylinders were removed after 7 months, and the remaining cylinders were removed after 8 months for standard tests.

All mixtures were created using St. Mary's Portland Cement Type N and the water to binder ratio was held at a constant of 0.4 for all mixture types. Total binder (cement and metakaolin) content was held constant for all mixture types (400 kg/m^3), with the proportion of metakaolin varying among concrete mixtures (0%, 10%, 15%, and 20% of binder as metakaolin). Super plasticizer content was varied to help achieve a relatively equal low slump to provide a workable concrete mix (Table 5).

Portland cement was acquired from St. Mary's Cement, which adheres to the CSA-A3001. Metakaolin was acquired from Whitemud Resources Inc. which adheres to the CSA-A3001 and ASTM C618 which approves it as a type N substitute to the cement portion of a mixture.

Table 5 – Mix proportions for the 4 concrete types created

Ingredients	**Mixture ID**			
	0% metakaolin	**10% Metakaolin**	**15% Metakaolin**	**20% Metakaolin**
Portland Cement (kg/m^3)	400	360	340	320
Metakaolin (kg/m^3)	0	40	60	80
Total Binder (kg/m^3)	400	400	400	400
Fine Aggregate (kg/m^3)	816.8	812.7	810.4	808.1
Course Aggregate (kg/m^3)	987.2	982.2	979.4	976.6
Water/Binder Ratio	0.4	0.4	0.4	0.4
Super Plasticizer (kg/m^3)	0.75	1.25	1.65	2.1

2.2 *Experimental Setup*

Five acrylic boxes (250 cm x 20 cm x 20 cm, 0.64 cm wall thickness) were constructed for use in incubating concrete under simulated sewer-like conditions (see figure 2). The boxes had a lip around the top to allow a lid to seat onto the box, ideally to create a sealed environment. The box had two holes tapped in either end of the box 5cm from the bottom of the box. Fittings with one threaded end and one 0.64 cm barbed end were screwed into these holes to provide connections for air (on one end) and vacuum (opposite end) lines.

Figure 2 – Acrylic boxes used for experimental setup

The five acrylic boxes were all hooked up to the air and vacuum line and heavy perforated rubber hose was connected on the inside of the box to the air-line to provide aeration throughout the box. Five litres of activated sludge (Peel Region, G.E. Booth (Lakeview) Wastewater Treatment Plant, reactor 2) was placed in each box and dechlorinated tap water was used to raise the liquid level above the height of the heavy perforated rubber hose (~ 3 cm total depth). To suspend the concrete above the water line and above the perforated hose, blocks of wood were used that kept the concrete roughly 5cm ± 0.5 cm above the water line. The activated sludge was used to provide “sewer-like” conditions (nutrients and organic matter) and a bacterial inoculum for concrete surfaces. The air-lines were used to provide aeration and also to create an aerosol that would deposit nutrients and bacteria upon the concrete surfaces.

As mentioned above 21 cylinders were created for each mixture design for the 3 time points under investigation (initial, 7 months, and 8 months). The cylinders were distributed among the five boxes. Alongside the cylinders 21 chips of each concrete mixture were placed into the box, 3 chips for each time interval (1 and 2 week(s) and 1, 2, 4, 6 and 8 months). Once all the samples were in the boxes, the boxes were sealed with a lid. The lid was only removed when topping up the water line with dechlorinated water (usually once a week), when re-applying new activated sludge (occurring once a month) or when samples were removed.

2.3 *Preparation of Samples when Removed from Boxes*

When chip samples were removed from the boxes at the 1 and 2 week and 1, 2, 4, 6 and 8 month marks, each sample was preserved by soaking in ethanol (95%) for 10 minutes. These samples were then placed in a sterile Whirl-pak bag and placed in the freezer (-20°C) until further processing was conducted. Cylinders, however, did not undergo any specific post-experimental preparation as all tests being conducted on the cylinders did not require the preservation of any bacterial cultures.

2.4 *Mechanical Concrete Tests*

The purpose of these tests was to determine how duration of exposure to sewer-like conditions, and associated degradation of concrete by sulphur oxidizing bacteria, would affect the physical properties of the different concrete mixtures.

2.4.1 *Water Sorptivity*

This test determines water sorptivity through a unidirectional path of the water sorption, through capillary suction. To prepare the samples for the test, 3 cylinder samples were cut from the larger 100 x 200 mm cylinders to create 50 mm (height) x 100 mm (diameter) cylinders. These smaller cylinders were then placed in an oven for 5 days at 50°C to remove moisture before testing. Samples

were weighed and then covered in aluminum tape leaving only the bottom surface available for water sorption. These samples were then placed into a container suspended above using rods and water was poured in allowing the water to cover the bottom 1 – 3 mm of the cylinder, reducing the chance of sorption of water from any other surface but the bottom (Figure 3). Cylinders were weighed after 1, 5, 10, 20, 30, 60, 120, 180, 240 and 300 minutes. These tests were run for initial samples, as well as for samples (cylinders) after 7 and 8 months of exposure. These tests adhered to ASTM C1585 "Standard test method for measurement of rate of absorption of water by hydraulic-cement concretes" (ASTM Standard C1585, 2013).

Figure 3 – Concrete specimen suspended in water while conducting a sorptivity test

2.4.2 *Water Absorption by Total Immersion*

There exist many methods for testing absorption by total immersion. To be consistent with Joorabchian (2010), ASTM C642 "Standard test method of density, absorption and voids in hardened concrete" (ASTM Standard C642, 2013) was used. Joorabchian (2010) chose this method because of the information gained about pore space volume. Similar to the water sorption test, 50 x 100 mm cylinders cut from the original larger cylinders were placed in an oven for 5 days at 100 ^{O}C. After drying in the oven, the samples were weighed to achieve an oven dried weight value. These samples were then totally immersed in water for 5 days until the concrete reached a constant weight. After constant weight was achieved the samples were weighed to gain a saturated mass value. These values were then used in the following equation to determine the water absorption percentage:

$$\text{Absorption after Immersion Percentage} = [(W_a - W_b) / W_b] \times 100$$

Where W_a represents the saturated weight while W_b represents the oven dried weight.

2.4.3 *Water Porosity Test*

In addition to the "Water Absorption by Total Immersion" test, the "Water Porosity Test" was used to determine the volume of pores within concrete. Again, for consistency with Joorabchian (2010), the AFPC-AFREM French standard was used (AFPC-AFREM, 1997). As above, short cylinders (50 x 100 mm) were cut from the original samples. These cylinders were placed in a desiccator under vacuum for 4 hours (Figure 4). After this, the desiccator was filled with water, making sure to fully immerse all samples, and the vacuum was applied. Following the immersion, the samples were weighed to achieve an immersion mass. These samples were then towel dried to achieve a saturated surface-dry weight. Finally the samples were placed in an oven at 105°C until constant weight was achieved. At constant

weight the samples were weighed to gain an oven dry weight. These values were then plugged into the following equation to gain a porosity percentage:

$$\text{Porosity Percentage} = (W_a - W_b) / (W_a - W_c) \times 100$$

Where W_a is the saturated surface-dry weight, W_b is the oven dry weight, and W_c is the immersion mass.

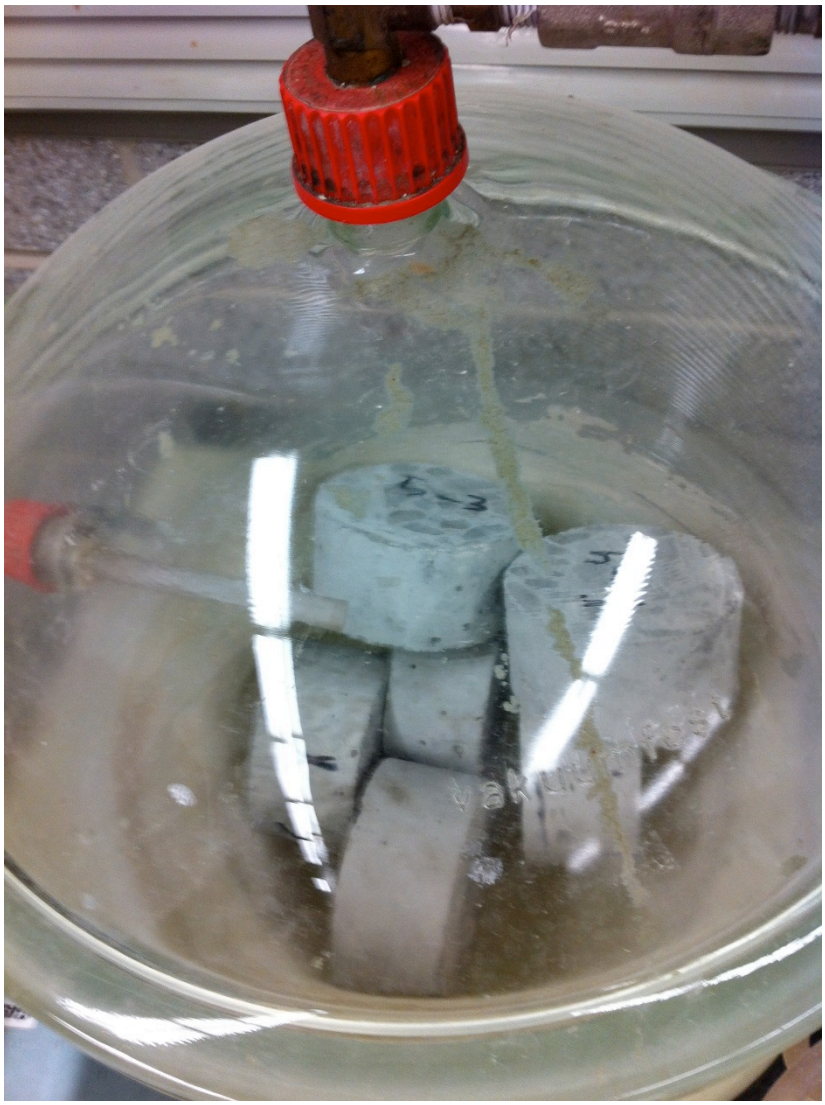

Figure 4 – Concrete within a desiccator prior to the application of water during a porosity test

2.4.4 *Rapid Chloride Permeability Test (RCPT)*

Chloride ions, as well as many other substances, can enter concrete through the porosity of the concrete itself. Therefore an RCPT test was performed to determine the concrete's susceptibility to these aggressive substances. This was performed using ASTM C1202 "Standard test method for electrical indication of concrete's ability to resist chloride ion penetration" (ASTM Standard C1202, 2012). Small cylinders (50 x 100 mm) were cut from the original samples, as before. Samples were placed in a desiccator for 3 hours. Following the 3 hours, distilled water was added to the desiccator to submerge the samples and the vacuum was applied for another hour. Vacuum was released and samples remained immersed in water for 18 ± 2 hours. After conditioning was complete, the samples were sealed in cells where one side of the cell was filled with 3% NaCl and the other side was filled with 0.3 N sodium hydroxide. A negative terminal was then connected to the 3% NaCl side while a positive terminal was connected to the 0.3 N sodium hydroxide. These terminals were then connected to a 60 V constant power supply and were turned on (figure 5). Readings are taken every 30 minutes up until the end of the 6 hours period at which point concrete is released. Coulombs that passed through the specimen were outputted by the software associated with the device. Coulombs represent the amount of charge transported as a steady state of one ampere in one second.

This information was then compared to a standardized chart (Table 6) to determine the chloride permeability.

Table 6 - Chloride ion penetrability based on charge passed (ASTM Standard C1202, 2012)

Charge Passed (Coulombs)	Chloride Ion Penetrability
≥ 4000	High
2000 - 4000	Moderate
1000 - 2000	Low
100 - 1000	Very Low
≤ 100	Negligible

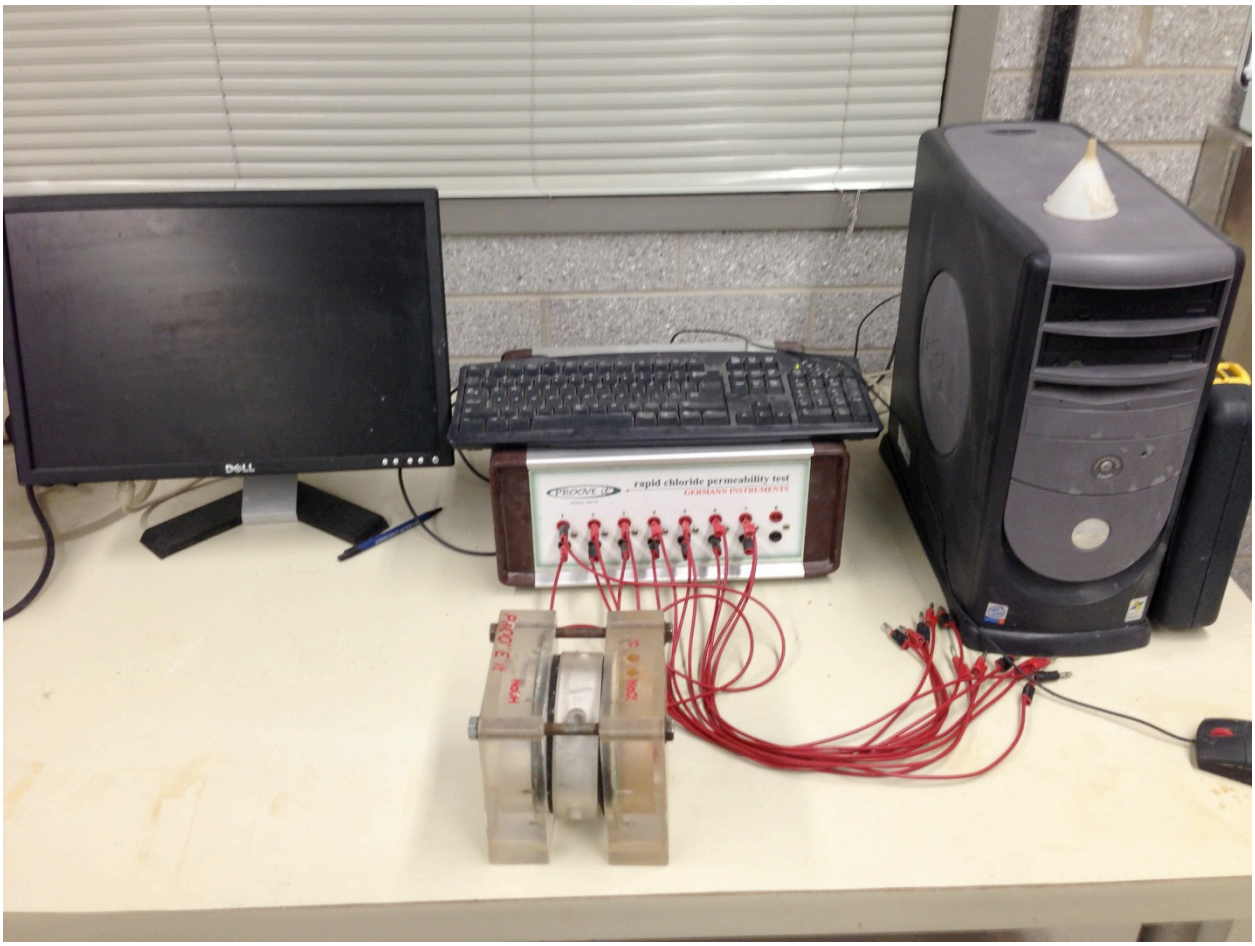

Figure 5 - Rapid chloride permeability machine with one sample housed in the testing cell

2.4.5 *Concrete Compression Test*

To test the strength of the concrete before and after incubation in the boxes, compression tests were done. This was accomplished by placing an intact concrete cylinder sample (100 x 200 mm) onto a compression machine and allowing the machine to apply a slowly increasing pressure (figure 6). Once the concrete gave way, the amount of pressure the concrete was able to sustain was recorded in megapascals (MPa). This test was performed at three different times, 0 days, 7 months and 8 months, and three samples per mixture type were tested at each of the three different times.

Figure 6 - Co

2.5 *Culturing of Bacteria*

Contents of all media found below can be found in appendix section 6.3 – 6.5.

2.5.1 *Halothiobacillus neapolitanus* (ATCC – 23638, recommended media: ATCC Medium 290 S-6)

Halothiobacillus neapolitanus were cultured on a variety of media to ensure optimal growth. Media included: ATCC Medium: 290 S-6 Medium for *Thiobacilli*, ATCC Medium: 290 S-6 media for *Thiobacilli* modified with LB broth, ATCC Medium: 290 S-6 media for *Thiobacilli* modified by increasing concentrations to 1.5x, DSMZ medium 68: *Thiobacillus neapolitanus* Medium, and thioglycollate medium. All media were done in both agar and liquid form except for the thioglycollate media which is meant to be used in liquid form. The bacteria were then incubated at 30 ^{o}C as recommended by ATCC.

2.5.2 *Thiomonas intermedia* (ATCC – 15466, recommended media: ATCC Medium 152)

Thiomonas intermedia were as well cultured on a variety of media to ensure the most optimal growth. These media included: ATCC Medium 152: *Thiobacillus* Medium, ATCC Medium 152: *Thiobacillus* Medium with LB broth, ATCC Medium 152: *Thiobacillus* Medium modified by increasing concentration to 1.5x, and thioglycollate medium. These were as well all done in both agar and liquid form except for the thioglycollate media. They were then incubated at 30 degrees Celsius as recommended by ATCC.

2.5.3 *Thiobacillus thiooxidans* (ATCC – 8085, recommended media: ATCC Medium 125)

Thiobacillus thiooxidans was cultured strictly using ATCC Medium 125: *Thiobacillus* medium, in which autoclaved sulphur is floated on the surface of the media. This is use to indicate growth as bacteria will colonize the surface of the sulphur eventually causing the sulphur to sink as the weight increases.

2.5.4 *Thiomonas intermedia* (DSM – 18155, recommended media: DSM Media 35a)

Thiomonas intermedia were cultured in DSM media 35a – *Thiomonas intermedia* medium. These were then cultured at 28 degrees Celsius and not 30 degrees Celsius as only one environmental chamber

was available for use and it was already set to 28 degrees for use in growing *Halothiobacillus neapolitanus* (DSM – 15147).

2.6 *Statistical Analysis*

The effects of concrete formulation on resistance to biological degradation were tested by subjecting the results of mechanical concrete test results (above) to statistical analysis. For sorptivity and all other data from physical tests, results were analyzed using two-way analysis of variance (ANOVA) with metakaolin content and time as independent factors. As sorptivity was a linear relationship the slopes of 3 lines per concrete type was used for the ANOVA analysis. Analyses were performed using Systat version 13 (Systat Inc., Chicago, IL).

2.7 *SEM Imaging*

All images were taken using a JEOL 6380LV scanning electron microscope (SEM), equipped with Oxford energy dispersive X-ray spectroscopy (EDS), and three-dimensional (3D) fractographic analysis. Images of initial concrete were taken at a magnification of 50, 300, and 800x magnification. Images were of all concrete mixes were captured to see if there were any noticeable surface differences, such as surface smoothness, texture and amount of pore space. Images at 8 months were taken of the 0% and 20% metakaolin samples, as the largest definable differences were found between these two specimens in initial scans. These images were taken at 300 and 2000x magnification. The 300x magnification was chosen to compare with those taken earlier and the 2000x times to get more detailed images of the crystal structures.

2.8 Confocal *Laser Microscopy*

2.8.1 *Fluorescent in situ Hybridization*

Images were taken using either a Zeiss LSM 510 equipped with a 351 nm, 488 nm, 514 nm, 543 nm and 633 nm laser or a Nikon Eclipse 90i equipped with a 488 nm, 532 nm and a 633 nm laser. At any point in which epifluorescence was required the Nikon Eclipse 90i was required as it is equipped with an epifluorescent detector.

2.8.1.1 *Pure Culture*

Fluorescent *in situ* hybridization was performed on three species, *H. neapolitanus*, *T. intermedia*, and *Escherichia coli*. Hybridization slides were made using Teflon-coated slides that were pre-treated in ethanolic KOH (10% potassium hydroxide) and then dipped in a gelatin solution (0.1% gelatin, 0.01% chromium potassium sulfate) at 70°C. All cultures were fixed in a 4% paraformaldehyde solution for 24 hours at 4 ^{o}C. Cultures were then washed using PBS and then stored -20°C (Amann, 1995).

H. neapolitanus culture (6 µl) was applied to each well on the pre-prepared hybridization slide and left to dry in the hybridization oven (46 ^{o}C; Okabe *et al*, 2007). Slides were then dehydrated in sequential ethanol baths (50%, 80%, and 100%). A 35% formamide-containing hybridization buffer (mix in appendix section 6.7; Okabe *et al*, 2007) was created and applied to each well of the slide (8 µl) along with the probe (1 µl at a 50 ng/µl concentration; probe: S-S-H.neap-635-a-A-19, ALEXA 647). This was placed in a moisture chamber and hybridized at 46°C for 1.5 hours. Slides were washed with appropriate wash solution (mix in appendix section 6.7) by placing them within the solution at 48 ^{o}C for 10 minutes. Slides were then stored at -20°C until examination.

Methods for *T. intermedia* (probe: S-S-T.int-0442-a-A-18, ALEXA 647) and *E. coli* (probe: ECO1167 (ECO 45A), ALEXA 647) were similar to those for *H. neapolitanus,* except formamide concentrations varied, dependant on species (10% formamide and 40% formamide respectively; Okabe *et al*, 2007; Neef *et al*, 1995).

2.8.1.2 *Activated Sludge*

FISH was performed on sludge samples procured from G.E. Booth (Lakeview) Wastewater Treatment Plant in Mississauga, ON. The sludge was probed using S-S-T.int-0442-a-A-18 to determine whether it contained *T. intermedia*. Methods for fixation and hybridization were the same as those for this species found in section 2.8.1.1.

2.8.1.3 *Pure Culture on Concrete Surface*

Protocol for imaging bacteria on concrete was developed using *E. coli* as a substitute for the sulphur oxidizing species based on the shared gram-negative characteristics and its rapid growth rates. Untested concrete chips were washed of any residue created during cutting, and left to dry. These concrete chips were then treated like slides using the methods described in 2.8.1.1 for *E. coli* staining. The epifluorescent settings on the microscope were always used prior to using laser confocal microscopy as it was easier to find focus on the concrete surface using these settings. Both a blank chip (no treatment other than a wash after being cut) and a negative control chip (all steps in FISH process with no bacteria applied) were created and examined to compare and contrast whether any products in the FISH staining process lent themselves to increased background fluorescence.

2.8.2 *Detection of bacteria on a Concrete Surface Using SYTO 62*

SYTO 62 (Catalog number: S11344; nucleic acid probe, Invitrogen Canada, Burlington, Ontario) staining was investigated using *Pseudomonas sp.* strain CT07. SYTO 62 stain was spun down to remove DMSO and applied to *Pseudomonas sp.* for 20 minutes to allow the stain to be taken up. Stained cells were then applied to the concrete surface and imaged immediately using the 488 nm laser and the 633 nm laser. This was done prior to FISH work on concrete to establish whether or not imaging of both

bacteria and concrete together was possible. This method was chosen due to its short incubation time to stain the cells while also being in the detection range desired.

3. Results and Discussion

3.1 *Concrete Properties*

3.1.1 *Sorptivity:*

Sorptivity rates of untreated concrete can be seen in figure 3.1 in which data is an average of 3 samples per concrete species. These results show a clear decrease in total sorptivity as metakaolin content is increased (Figure 7). A comparison between the control and the 20% metakaolin specimens shows a decrease in sorptivity by 67%, 0.0573mm/√h to 0.0343mm/√h respectively. It would also appear that the decrease in sorptivity rate may be negatively correlated with the amount added, in that the more metakaolin that is added the less effective it is at decreasing sorptivity. This can be seen comparing the decrease between 10% and 15% versus 15% and 20%. Here the decrease in sorptivity between 10% and 15% was found to be 16% while the decrease in sorptivity between 15% and 20% was found to be 2% which is a decrease in overall effectiveness. Concrete exposed to bacterial colonization for 7 months and 8 month showed similar patterns between sorptivity and metakaolin content. Again, concrete with no metakaolin had the highest sorptivity and the concrete with 20% metakaolin samples had the lowest. Overall, the metakaolin content did have a significant effect on sorptivity ($F_{3, 24} = 55.39$, $p < 0.0001$). Over time, the sorptivity of each concrete species decreased with further curing of the concrete ($F_{2, 24} = 6.29$, $p = 0.0064$).

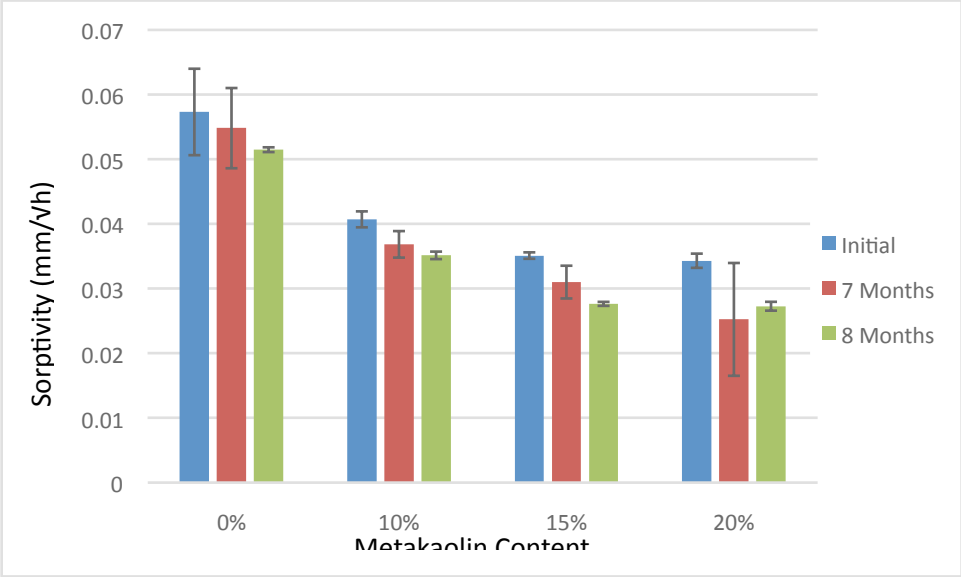

Figure 7 – Sorptivity of concrete specimens containing different quantities of metakaolin (range from 0% to 20% of cement mix). Values plotted as mean ± standard deviation (n = 3).

These results are similar to those found by Joorabchian (2010) in the parent study. Joorabchian found that concrete containing no metakaolin had the highest sorptivity while samples containing 20% had lower sorptivity rates. The decrease in sorptivity was found to be 81% between 0% metakaolin and 20% metakaolin, and a difference of 13% found between the 15% metakaolin and 20% metakaolin concretes. This outcome is consistent with various other studies as well (e.g. Bai *et al*, 2002; Ozbay *et al*, 2012; Ramezanianpour & Jovein, 2012). The decrease in sorptivity, irrespective of metakaolin content, is also consistent with other studies which examined concrete using different curing timelines. Guneyisi and Mermerdas (2007) found that when using concrete modified with metakaolin sorptivity at 28 days of curing was always greater than the sorptivity found at 90 days of curing. Ramezanianpour and Jovein (2012) as well found that the sorptivity of their concrete decreased as the specimens were allowed longer intervals of time to cure (curing times = 7, 28, 90, 180 days).

3.1.2 *Porosity:*

Overall, metakaolin content had a significant effect on porosity ($F_{3, 24} = 8.27$, $p < 0.001$). Concrete with no metakaolin had the least amount of pore space at each time period, and porosity increased with increasing metakaolin content (Figure 8). The only exception was at the 7 month mark where the 15% samples had a lower average percentage of pores compared with 10% metakaolin samples, however this difference was not statistically significant. In comparing the samples that contain metakaolin only at the initial time series, we see a difference in pore percentage of 10.3% and 2% between 10% metakaolin and 15% metakaolin and between 15% metakaolin and 20% metakaolin, respectively. This pattern appears to dampen over time as concrete further cured. Further, porosity in general decreases over time, independent of metakaolin content ($F_{2, 24} = 45.20$, $p < 0.0001$). Many authors have found that metakaolin loses its effectiveness as curing time is increased (Khatib, 2008; Dhinakaran *et al*, 2012).

The initial results agreed with those of Joorabchian (2010). In this study it was found that increasing metakaolin content increased porosity from the control (13.34%) to the 20% metakaolin mix (14.33%). Joorabchian noted that there was only a slight increase in the porosity percentage between the 15% and 20% metakaolin mixes (increase of 3.69%). This outcome is similar to other studies as well (e.g. Khatib & Clay, 2004; Kim *et al*, 2012; Paiva *et al*, 2012). This outcome, however, is in contrast to results of studies that used a Mercury Intrusion Porosimetry apparatus. Such studies have reported a general reduction in porosity with increased metakaolin content (Duan *et al*, 2012; Guneyisi *et al*, 2008; Megat Johari *et al*, 2011). This difference in reported porosity may be due to the average pore size in the concrete samples. In all studies that reported pore size, samples with metakaolin added had a smaller average pore size than those lacking metakaolin (Guneyisi *et al*, 2008; Megat Johari *et al*, 2011; Paiva *et al*, 2012). Therefore although more pore space may be present in concrete containing metakaolin, the reduction in the average pore size may prevent intrusion of mercury. This would be consistent with a

decrease in sorptivity with increasing metakaolin content observed in this study. The decrease in porosity over time is consistent with a study by Paiva *et al* (2012) that showed a reduction in total porosity by 1 - 5% in concrete cured 28 days versus 7 days. Khatib and Clay (2004) similarly found that with increased curing time came a decrease in the porosity of the sample.

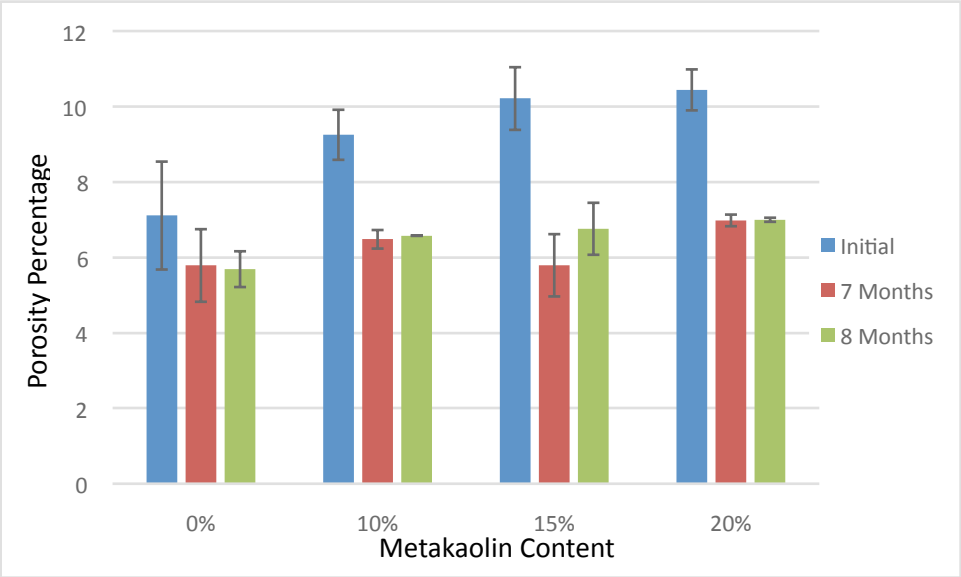

Figure 8 - Comparison of the percentage of pore space in 4 different concrete species varying in the metakaolin content. Values plotted as mean ± standard deviation (n = 3).

3.1.3 *Absorption through Total Immersion:*

Conceptually, this technique is another way to measure pore space, and the results should correspond to those of the porosity test (above). In general, this was true. Concrete samples tested for absorption of water through total immersion showed an increase in absorption with an increase in metokaolin content (Figure 9) ($F_{3, 24} = 23.81$, $p < 0.0001$), consistent with an increase in porosity. Across time a slight reduction in absorption can be seen in all mixes except for the 10% mix. This slight reduction in absorption may be attributed to longer curing times experienced by the mixes across the time series. This trend, however, was found to not be significant ($F_{2, 24} = 0.83$, $p = 0.430$). It was also

found that the absorption percentage using this method was much lower than predicted by porosity (section 3.1.2). A reduction of around 5 - 7% was seen. This is likely due to the use of a desiccator in the porosity methods in which a vacuum is created allowing water to flow more easily throughout the structure of the concrete.

Again these results coincide with those found by Joorabchian (2010). Joorabchian (2010) found that the 0% metakaolin concrete absorbed the least amount of water while the 20% metakaolin specimens absorbed more. On top of this a general reduction of about 6% was found when comparing the results of the total immersion absorption test to the results of the porosity tests.

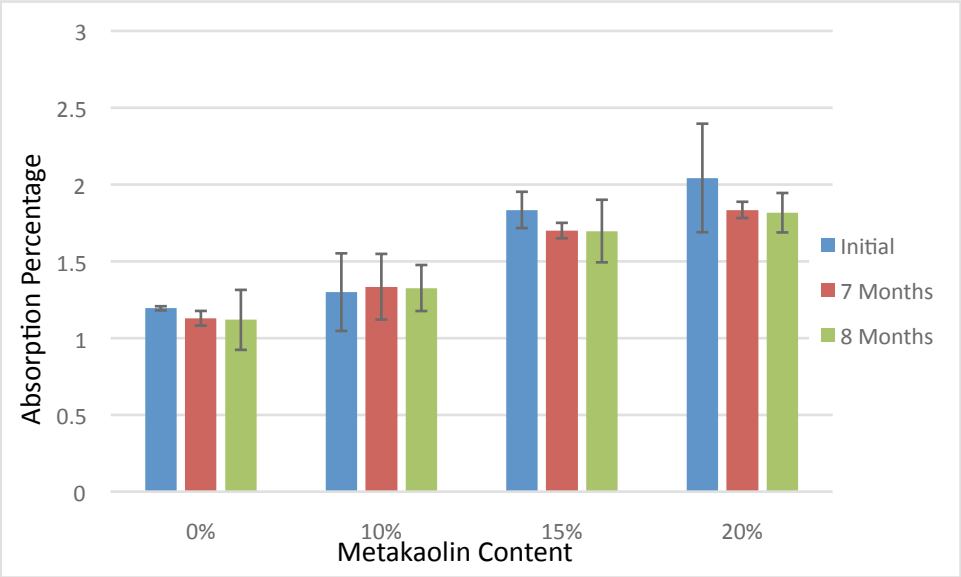

Figure 9 - Comparison of the percentage of change in mass with water absorbance in 4 different concrete species varying in metakaolin content. Values plotted as mean ± standard deviation (n = 3).

3.1.4 *Rapid Chloride Permeability:*

Metakaolin content had an effect on chloride permeability ($F_{3, 24} = 680.76$, $p < 0.0001$). Increasing the metakaolin content decreased the amount of coulombs (Figure 10). These results, in turn, agree with those of the sorptivity. As metakaolin content increases, the concrete becomes less

penetrable even though the porosity and absorption tests showed that it had increased pore space. The suspected curing time effect was seen here as well. As time progressed the total amount of coulombs decreased ($F_{2, 24} = 48.97$, $p < 0.0001$). This occurred primarily in the 0% metakaolin concrete, and to a lesser degree in the 10% metakaolin concrete. In another study a similar pattern was noticed (Dhinakaran *et al*, 2012). In this study replacement levels of 5, 10, and 15 percent metakaolin were studied for their effects on chloride permeability. Similar to this study, the more metakaolin added the greater the effect. This was consistent with time as well, as time went on the permeability decreased. The author, however, makes mention that the effects of the metakaolin seem to become less and less important as time passes (Dhinakaran *et al*, 2012). This as well aligns with the parent study by Joorabchian (2010), in which it was found that the 0% sample had a large increase in permeability vs. those that contained metakaolin. Joorabchian also noted a reduction in the permeability over time.

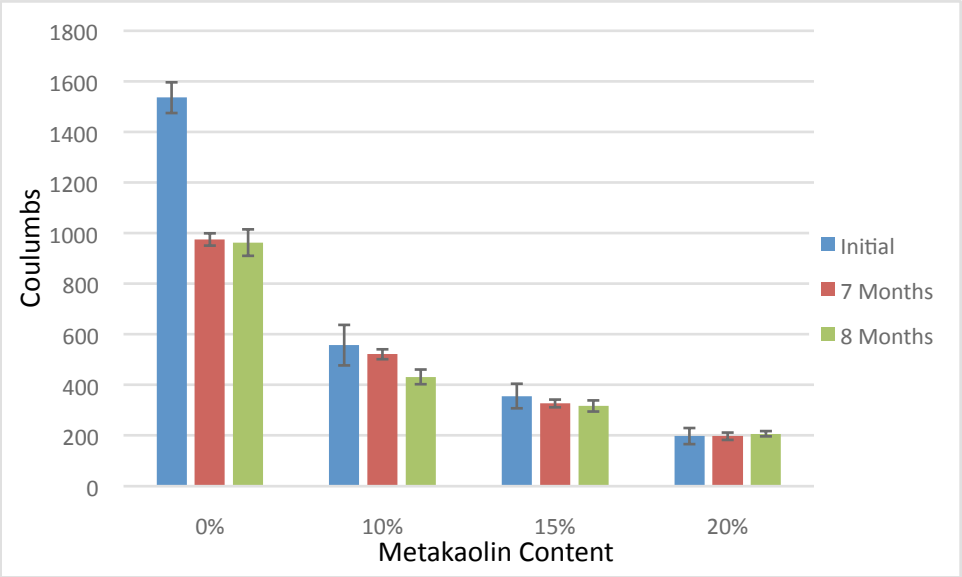

Figure 10 – Comparison of the amount of coulumbs that passed through concrete samples containing different quantities of metakaolin. Values are plotted as the mean ± standard deviation (n = 3)

3.1.5 *Concrete Compression Strength:*

Concrete strength was affected by metakaolin content ($F_{3, 24} = 95.23$, $p < 0.0001$). An increase in metakaolin content resulted in greater strength (Figure 11). This data agrees with expected outcomes, but not for expected reasons. Due to the filling nature of metakaolin, a more dense concrete, and therefore a stronger concrete, was expected. However, the porosity and water absorption data suggest that concrete with increasing metakaolin content is simultaneously less dense and stronger. As the testing progressed a general increase of strength was also observed ($F_{2, 24} = 72.17$, $p < 0.0001$). This can be attributed to the moist conditions of the study which likely allowed the concrete to continue curing while the experiment proceeded, leading to a stronger product by the end of the experiment. The initial results were as expected in which a general increase of the strength of concrete corresponds with increased metakaolin content. This trend was found in many other peer reviewed studies (e.g. Duan *et al*, 2008; Guneyisi *et al*, 2008; Megat Johari *et al*, 2011). Duan *et al* (2008) found that a substitution of 10% metakaolin resulted in an increase of total strength regardless of curing method (salt or fresh water). Guneyisi *et al* (2008) found that they could gain an increase in strength of 5 - 30% which was attributed to the amount of metakaolin used and the water to binder ratio applied. These two papers as well found that an increase in the length of time allowed for curing produced stronger concrete regardless of metakaolin content.

One thing to note is that the overall strength development of the samples seemed to occur mostly between time 0 and 7 months for those concretes containing metakaolin, while beyond 7 months the gain in strength seemed to slow down. This is in contrast to the concrete lacking metakaolin where there was apparently slower strength development between time 0 and 7 months, but a much greater increase between 7 and 8 months. This observation was captured statistically by a significant time*treatment interaction ($F_{6, 24} = 2.61$, $p = 0.043$). It may be that concrete lacking metakaolin was

merely catching up in strength, whereas concrete with high metakaolin cured more rapidly and would realize its maximum potential strength earlier. It is also possible that concrete with higher metakaolin content, with greater porosity, offered greater potential for colonization by sulphuric acid generating bacteria. In this case, the time*treatment interaction may suggest bacterial generation of acid was offsetting gains in strength associated with curing more in concrete with high metakaolin content than in concrete lacking metakaolin.

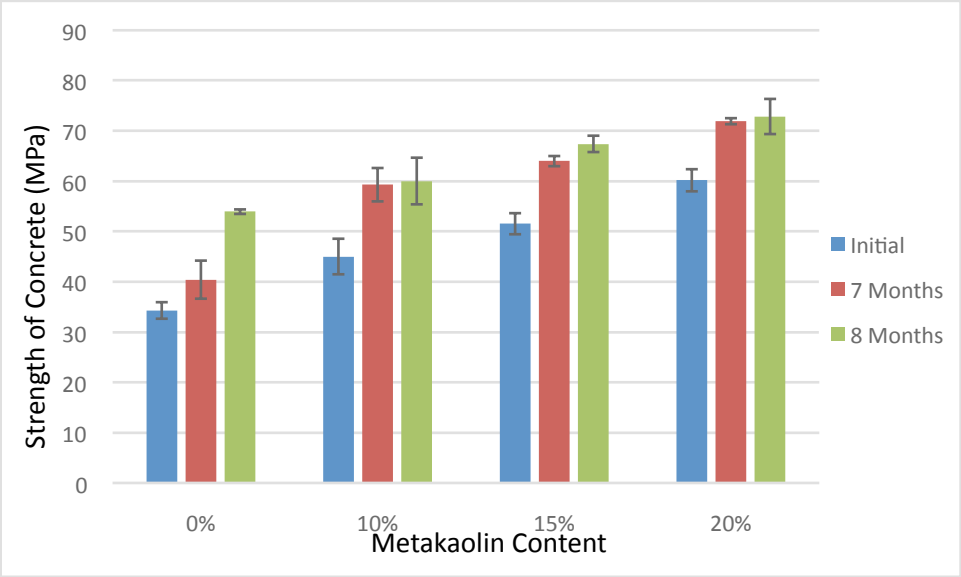

Figure 11 - Strength development of concrete over an 8 month period in which the metakaolin content of the concrete has been varied. Values plotted as mean ± standard deviation (n = 3).

3.2 *Visual Analysis of Concrete Samples*

3.2.1 *Scanning Electron Microscopy:*

Pictures of the surface of concrete were taken using a scanning electron microscope at different magnifications. Figure 12 shows concrete with no exposure to bacteria magnified by 50x using a scanning electron microscope. Visually the specimens with no metakaolin seem to contain larger pores than the specimen with 20% metakaolin. The surfaces of concrete samples containing metakaolin also

appear to be smoother than concrete that contained no metakaolin. This is expected as the size of metakaolin to Portland cement is smaller and therefore it should fill in space more effectively than the Portland cement. This corresponds with the studies on concrete physical properties which suggested greater metakaolin content was related to a general pore size reduction. Full sized images as well as images from different magnifications (300x and 800x) can be found in Appendix 6.2.1.

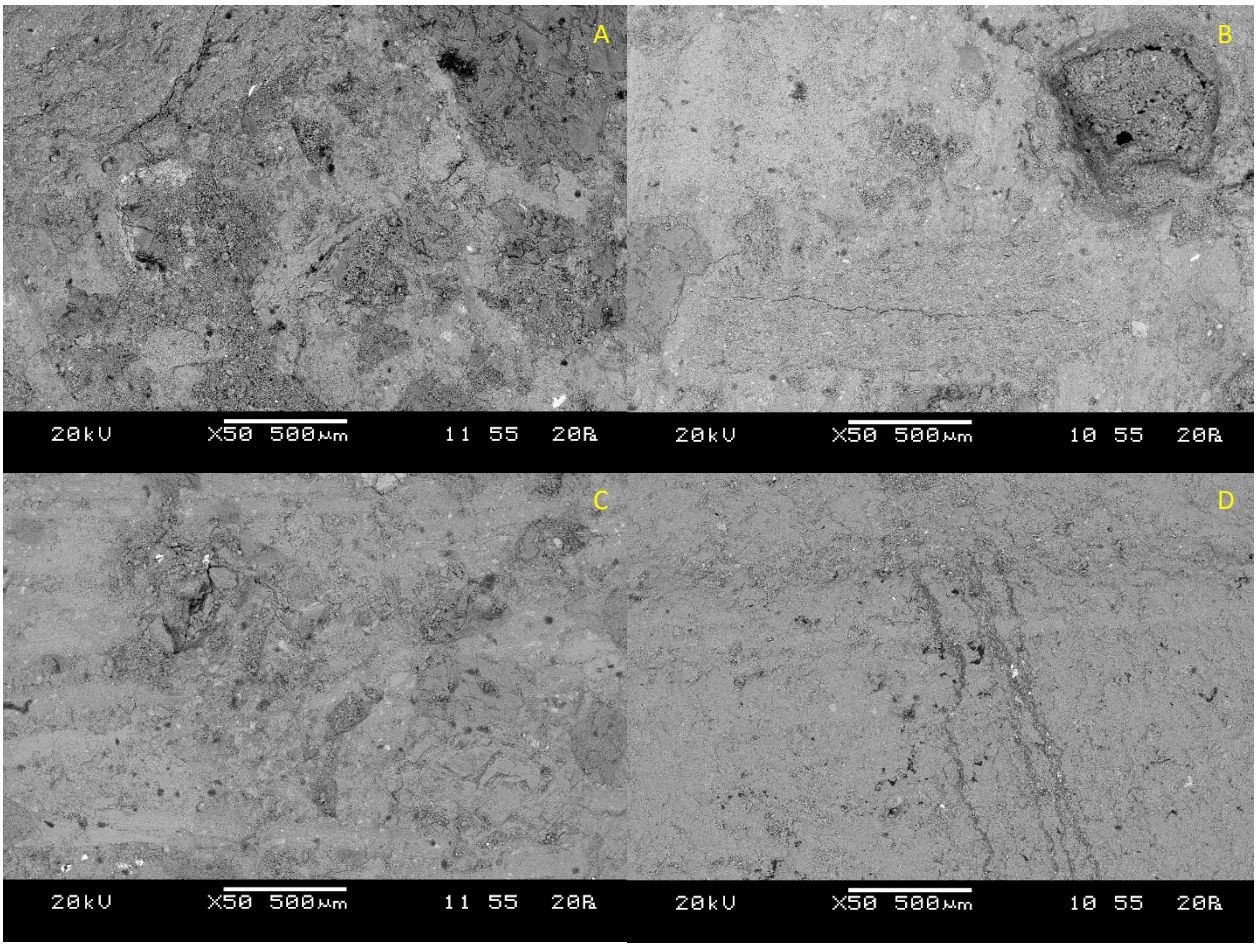

Figure 12 – Scanning Electron Microscope images at 50x magnification of 4 different concrete mixes. A) 0% metakaolin content b) 10% metakaolin content c) 15% metakaolin content d) 20% metakaolin content.

Due to the cost of use for the SEM, images of concrete exposed to microbial inocula, and potentially colonized by bacteria, were not taken at every time interval planned for concrete chip removal. Instead images were taken at the end of the 8 month cycle as a comparison of long term

exposure among the four different concrete mixtures. Also because bacterial colonies take a long time to become established many of the planned time intervals would likely not have shown great change.

Figures 13 and 14 show images of the 0% and 20% metakaolin mix before and after exposure to bacterial inoculation and colonization. The first striking difference is the change in the surface texture. The uncolonized concrete has a multitude of different pores and a range of individually identifiable particles that visually look like stones and sand grains. The colonized samples, however, appear more uniform in texture and also look to have been degraded, as the surface appears to be pock marked. The second identifiable feature is the presence of crystal structures indicative of the acid attack on concrete. As discussed in section 1.9 when concrete is exposed to acid, two different crystals can form due to the reaction between the acid and lime (gypsum) and the gypsum and calcium aluminate (ettringite). Further images of crystal structures at 300x and 2000x can be found in appendix section 6.2.

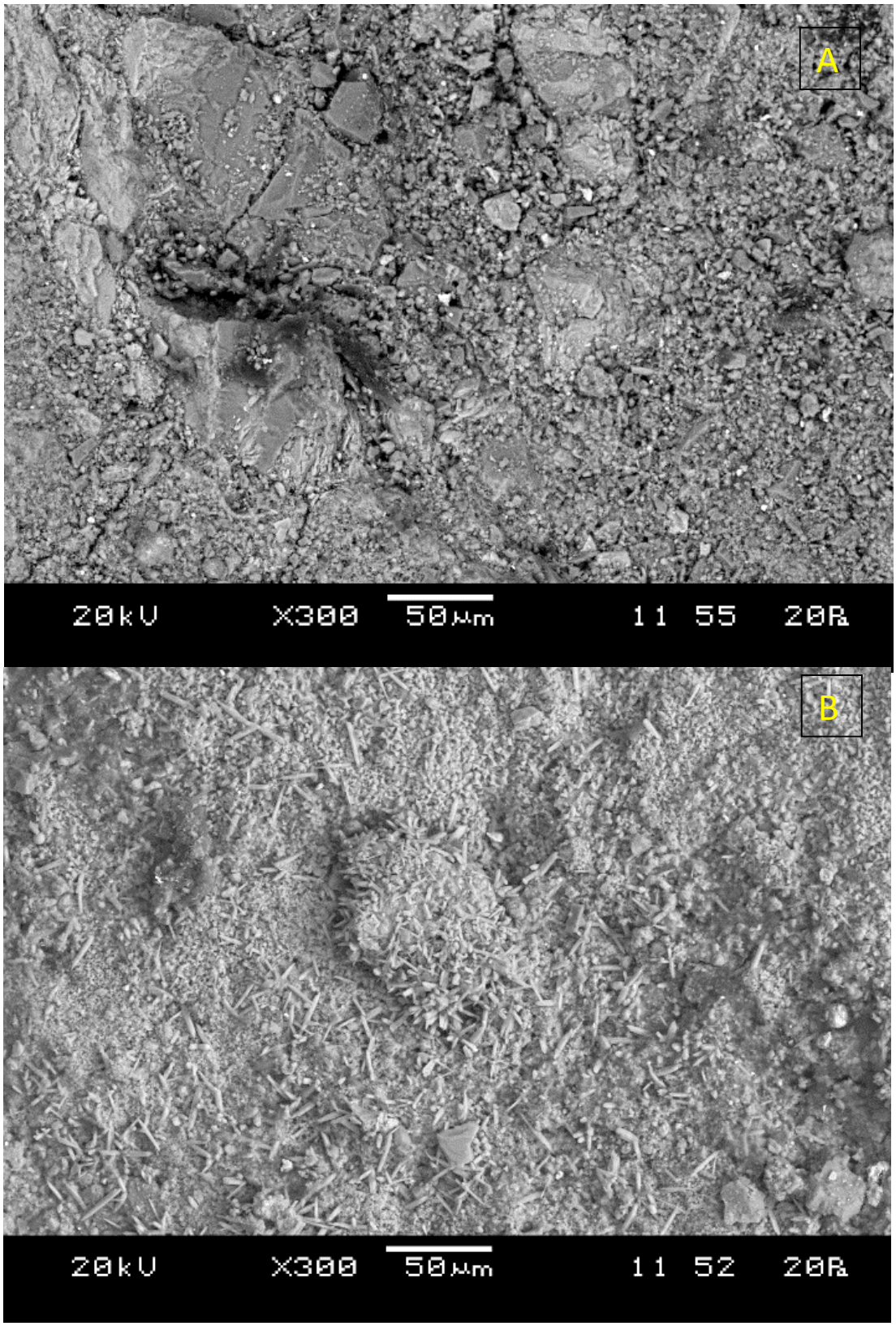

Figure 13 – Comparison of A) uncolonized and B) colonized concrete containing no metakaolin. 300x magnification.

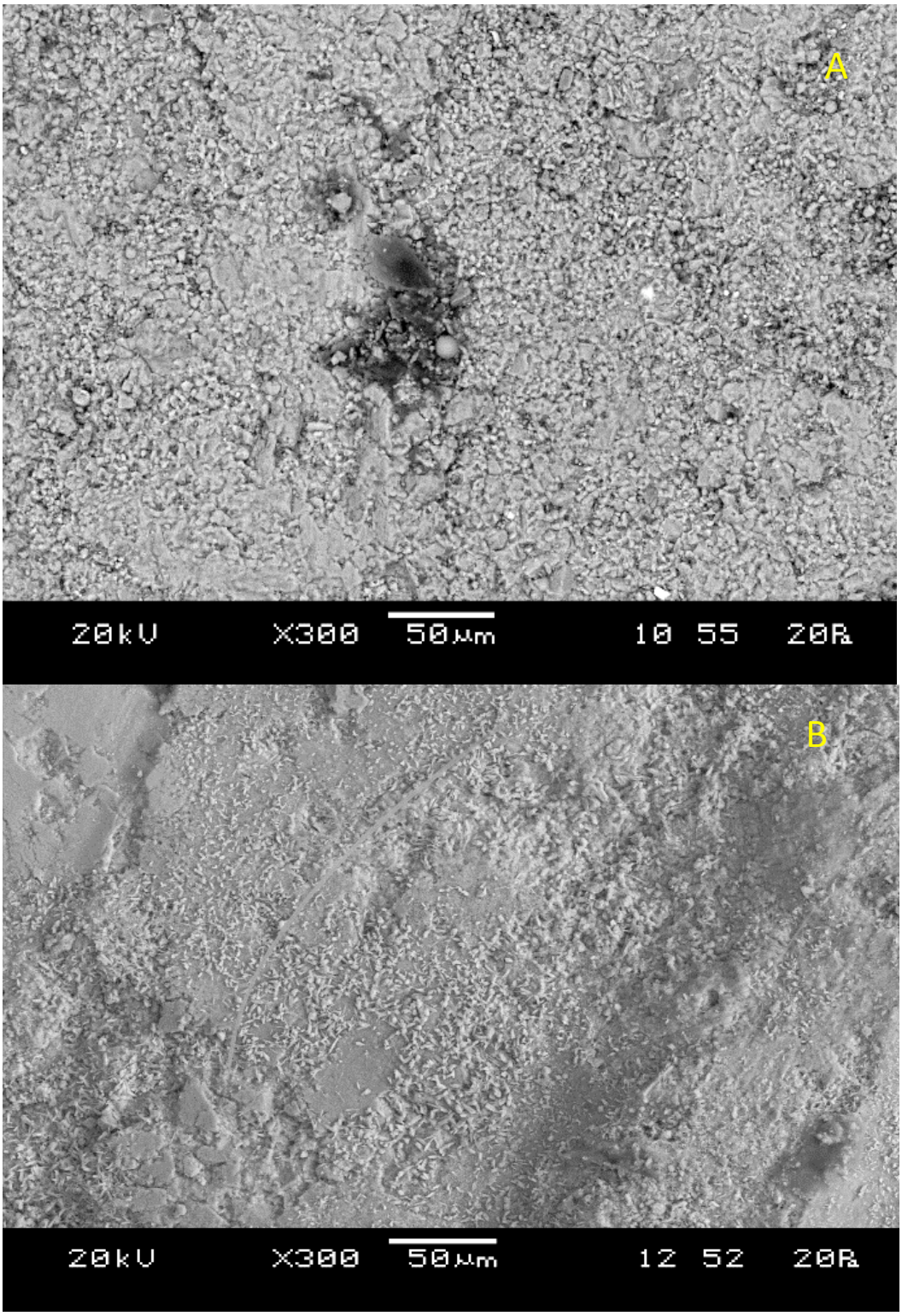

Figure 14 – Comparison of A) uncolonized and B) colonized concrete containing 20% metakaolin. 300x magnification.

Further observation suggests that the crystal structures tended to be smaller on the surface of the 20% metakaolin mix than on the 0% metakaolin mix. This might be attributed to properties of metakaolin not lending itself to acid degradation, or perhaps colonization of the 20% concrete surface may occur at a slower pace and therefore the quantity of acid produced may be reduced (see figure 15 for a comparison at 2000x). These observations are in contrast to the hypothesis that the concrete with greater porosity (20% metakaolin) would be more readily colonized, and therefore, biologically degraded, than the concrete with lower porosity (0% metakaolin).

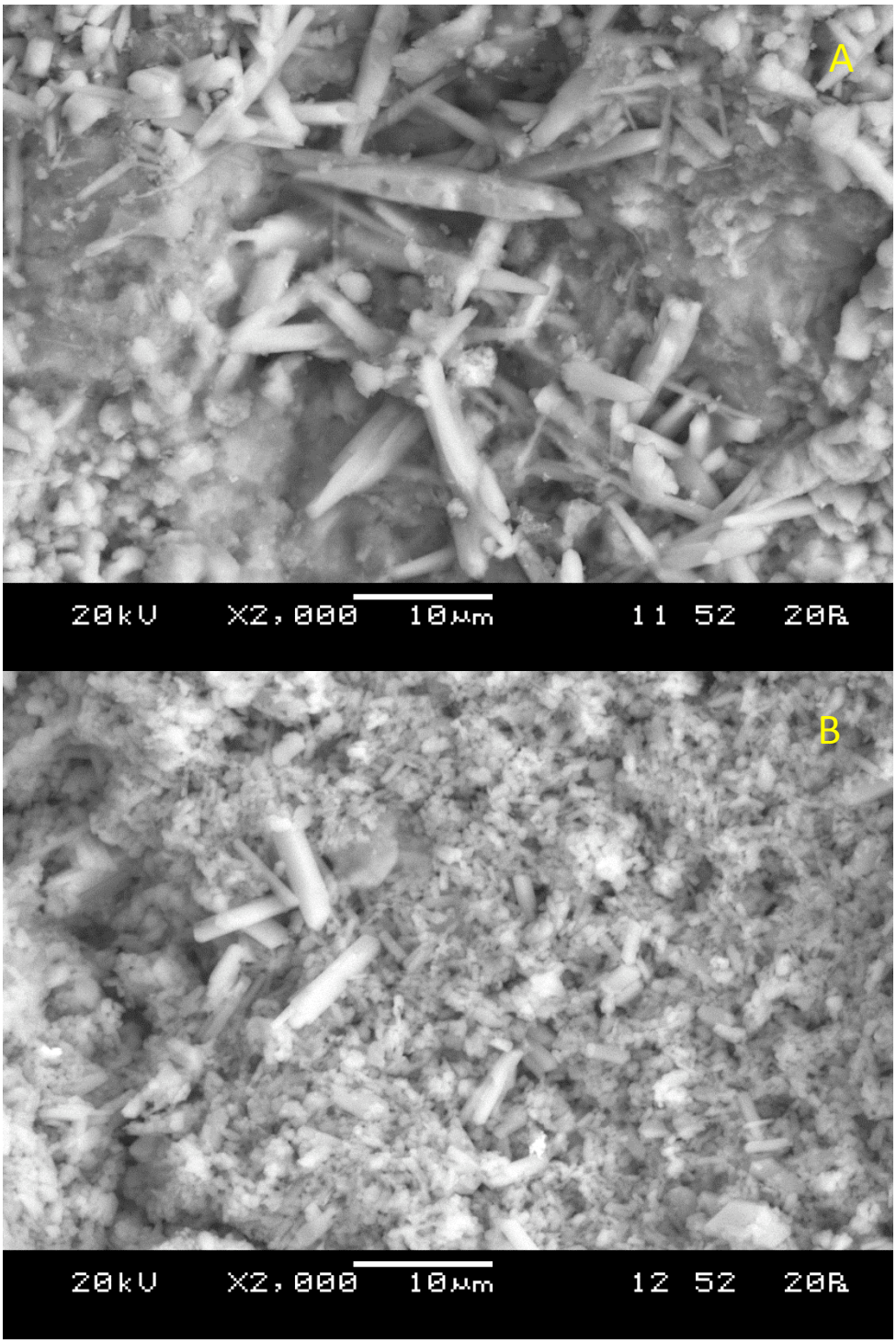

Figure 15 – Comparison of the size of crystals formed on different concrete mixes differing metakaolin content. A) Depicts a 0% metakaolin sample where the size of the crystals is visibly different from B) which depicts a 20% metakaolin sample

3.2.2 *Macroscopic Observation – Images from Digital Camera:*

A digital camera was used to analyze colony formation on the surface of concrete chips. Due to complications growing the three different species of bacteria used in this study, discussed further below, it is impossible to state whether the images to be discussed contain these bacteria and analysis will take note of this.

Seen in Figure 16 is a variety of different chips all showing some form of "staining". These stains have been attributed to the formation of bacterial colonies. This was determined when scrapped samples were gram-stained, and showed the presence of bacteria in scraped material. Although no research has been done on the patterns of bacterial colony formation on the surface of concrete, these images would suggest, as expected, that the colonies tend to form around and in cracks and pore space. This is likely because these portions of the surface provide an area in which colonization can occur and which offers protection from erosion or abrasion. This pattern of initial colonization may also be true of sewer systems where these cracks and pore structures would provide protection from flowing waters and debris.

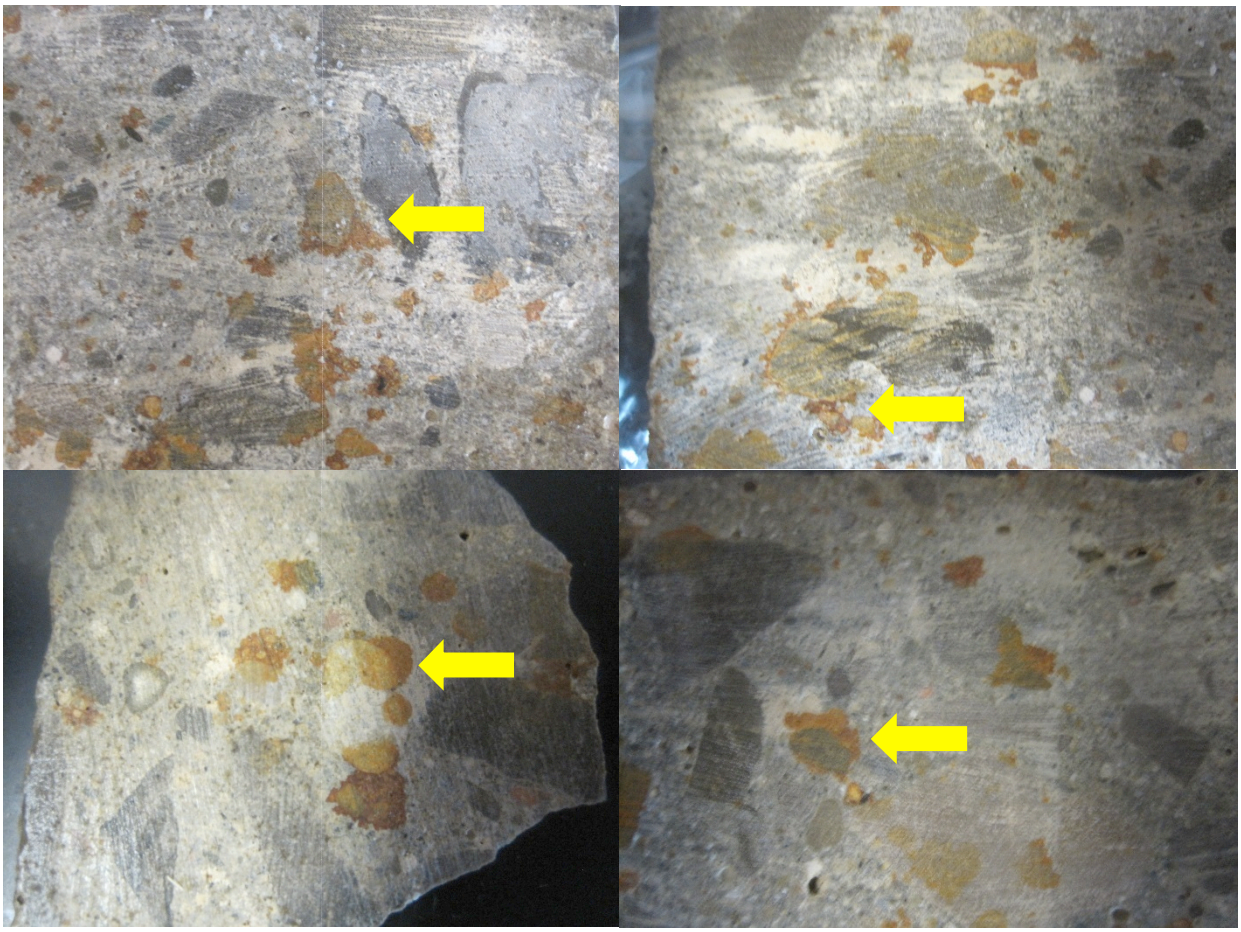

Figure 16 - Images of different concrete chips showing the presence of bacterial colonies. Yellow arrows are used to point out examples of colonies on each image.

3.2.3 *Bacterial Growth:*

As mentioned in the Materials and Methods section, three species of sulphur oxidizing bacteria were used in this experiment to investigate the colonization patterns on concrete. It was intended that these bacteria would be cultured and used as an inoculum on the concrete surface. Unfortunately successful growth of these bacteria was never achieved prior to the investigation starting. These cultures were received from ATCC by way of Cedarlane Corporation (Burlington, ON). Cultures were purchased on 2 separate occasions in which 2-5 generations were successfully grown before total collapse. These bacteria were grown on a multitude of different media (documented in section 2.5) to

try to regain growth as suggested by Cedarlanes. This was to no avail. Upon receiving the second batch of cultures, cultures were grown on the media described, as well as in a thioglycolate media which separates bacteria based on their oxygen demand, meaning those that require oxygen would grow near the surface while those that grow anaerobically would grow towards the bottom. This was done based on the fact that these bacteria occupy the soil boundary layer between oxygenated conditions and de-oxygenated conditions, and might require microaerophilic conditions. The results of the culturing activities showed that they grew best near the surface, and therefore further culturing continued in an oxygen rich environment.

Initial attempts to insert plasmids containing information for producing green fluorescent proteins were done prior to the start of the larger overlying experiment. These were conducted prior in hopes that if successful, the cultures used for inoculation could be pre-labeled, allowing for explicit identification under the laser confocal microscope. This insertion was unsuccessful and was likely not do to the procedure employed but instead was more likely caused by the culturing issues experienced. Due to the lack of success with this experiment fluorescent *in situ* hybridization (FISH) became the next route for examining bacterial colonization. This lack of bacterial growth also led to the use of activated sludge as the inoculum as it is a complex mixture of bacteria.

Due to the complications of maintaining active cultures, the experiment was run without pure culture for inoculation of concrete. Instead, activated sludge from the G.E. Booth (Lakeview) wastewater treatment plant in Mississauga, ON was used as an inoculum. It was assumed that this sludge would include sulphur oxidizing bacteria given the high content of organic and inorganic sulphur in sewage waste. To test this assumption, fluorescent *in situ* hybridization (FISH) staining was performed using a sample of the activated sludge. The target was *T. intermedia* (probe used was S-S-T.int-0442-a-A-18, ALEXA 647). Figure 17 shows 4 different images all of which show the complex community present in

activated sludge. The important aspect here, however, is the presence of the a red signal native to the ALEXA 647 which would indicate the presence of *T. intermedia* based on the FISH staining techniques. As pure culture was not achieved, this probe was not tested for specificity (ability to discriminate among sulphur oxidizing bacterial strains). However, the complex nature of the communities in the images, especially images B and C, show that it has only bound to select bacteria and therefore this was taken as evidence that the probe was sequence specific, and discriminatory to some extent. As this probe was gained from literature (Okabe *et al*, 2007) and has been used by Wei *et al (*2010) the robustness of this probe is apparent. With the tentative conformation that sulphur oxidizing bacteria, presumably *T. intermedia,* was present, activated sludge was used as the inoculum in hope that as air passed through the aeration tubes, bacteria within the activated sludge would become aerosolized and deposit upon the concrete surface suspended above the waterline.

A third culture of *H. neapolitanus* was ordered at the 4 month mark of the experiment in hopes to gain some pure culture to serve as a positive control in FISH studies. This culture was chosen based on the fact that it seemed to be the most successful of the three species before crashing. It had previously survived the most generations before collapse. However, upon making a request for another batch an email was sent from Cedarlanes that read "Unfortunately ATCC item #23638 has failed QC testing and has also been pushed back to late August." (Full email can be found Appendix section 6.8). This leads to the belief that the cultures ordered prior were likely affected by this failure of quality control, ultimately leading to the failure of culturing. Upon cancelling the order a culture that was labelled March of 1957 arrived which also failed to grow.

Due to this frustration a second company was contacted, DSMZ in Germany, and a culture of *T. intermedia* was ordered from them instead. It was interesting to note that the culturing instructions from this company were vastly different from those received from ATCC and bacteria were received as

live culture rather than freeze dried culture. These cultures were readily culturable and survived as many generations as required.

It is, as well, interesting to note that the formula that ATCC requires for growth of these species has since changed and now resembles the growth media DSMZ suggests.

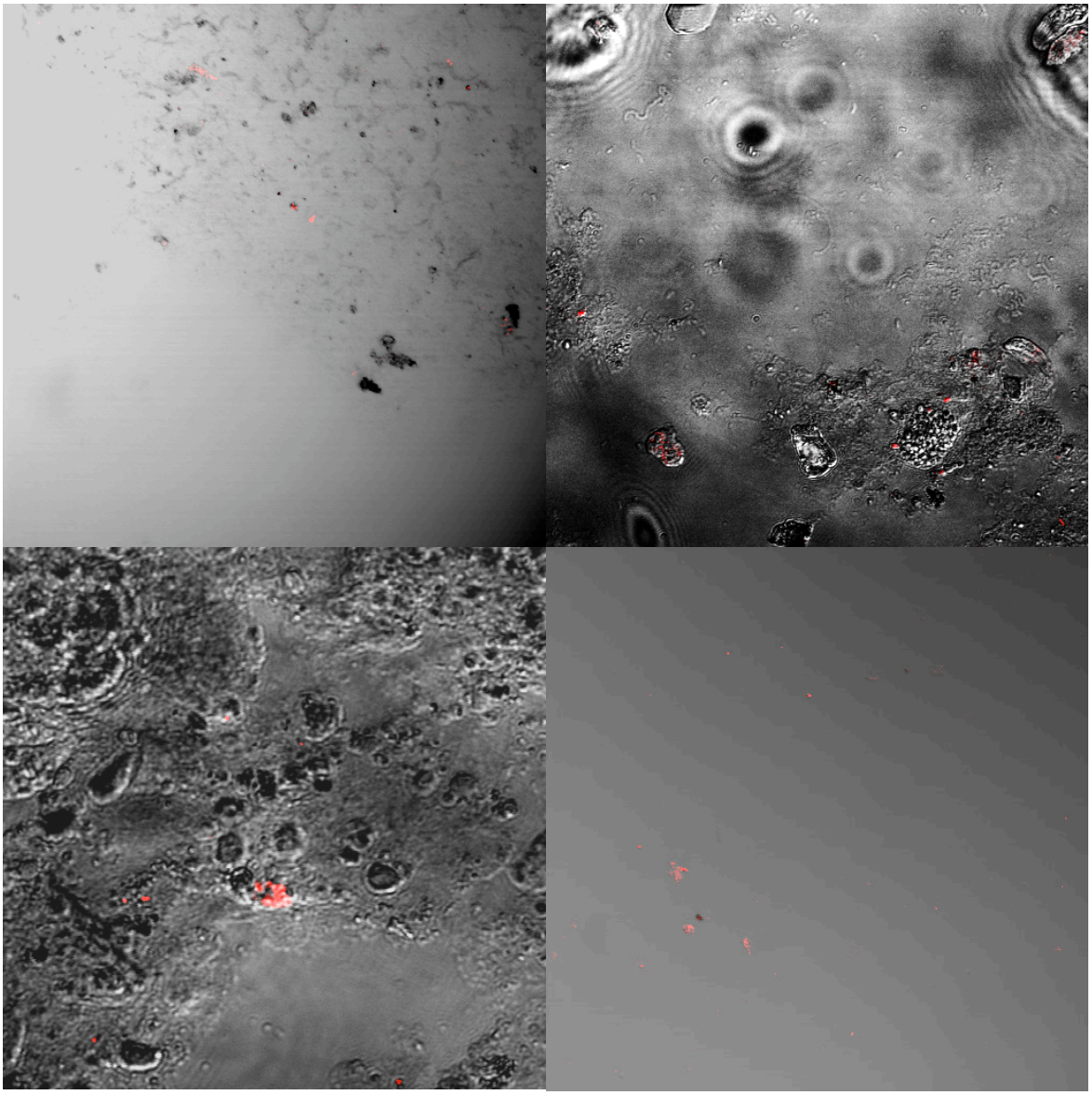

Figure 17 – Images depicting the presence of *T. intermedia* within activated sludge samples. Images A – C show complex communities of bacteria (black areas), within the complex communities red signals are seen indicative of *T. intermedia*. D) Red signals present are considered to be *T. intermedia*.

3.2.3.1 *Confirmation of T. intermedia Probe using E. coli as a Negative Control*

Originally each probe was going to be tested with the other two sulphur-oxidizing bacteria as negative controls. This was chosen as they are reported to exist at the same time within complex communities and would serve as good negative controls due to their cohabitation with one another. However, complications led to the use of *E. coli* as a surrogate (Figure 18). The robustness of the probe is discussed in section 3.2.3.

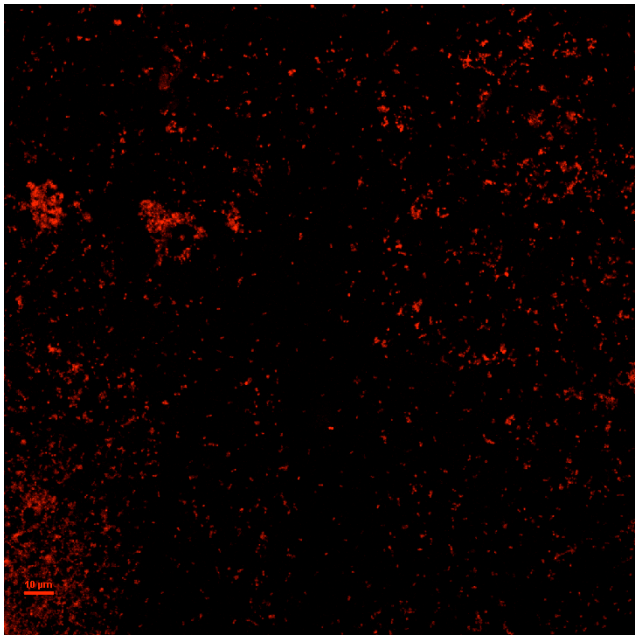

Figure 18 – Use of *T. intermedia* probe (probe: S-S-T.int-0442-a-A-18, ALEXA 647) on pure culture of *T. intermedia*.

3.2.4 *Use of Confocal Laser Microscopy to Simultaneously Image Concrete Surface Topography and Bacterial Presence*

Brief scans of the blank concrete surface were taken using all lasers to determine which produced the least amount of auto-fluorescence. As concrete is known for its autofluorescence the goal was to find a wavelength that showed the lowest intensity of autofluorescence. This was done in hopes

of finding a wavelength in which bacteria could be labelled so that images of both the surface of concrete (done with one laser) and the bacterial presence (done with laser that showed low autofluorescence) could be taken at the same time. The second objective was to make sure that this wavelength also showed nothing that could possibly be mistaken for bacteria (particles of the same shape and size showing fluorescence). At the time it was determined that the 633nm wavelength had the least amount of auto-fluorescence and was chosen as the desired wavelength to target with probes (Figure 19).

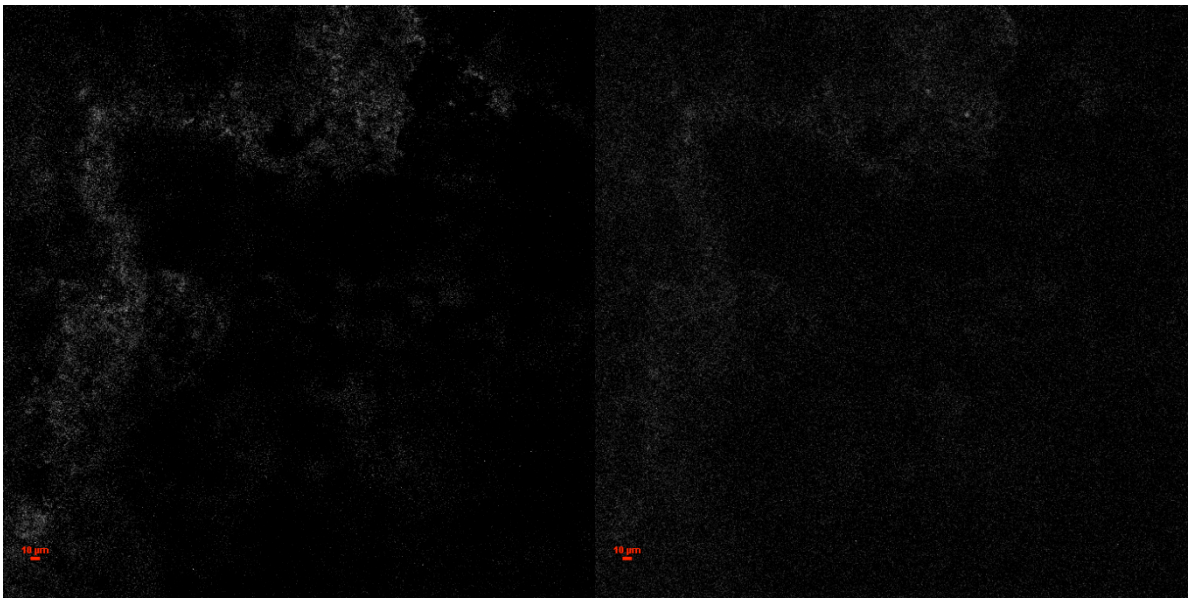

Figure 19 – Side by side comparison of the same concrete surface's autofluorescence under the 488nm wavelength (left image) and the 633nm wavelength (right image). Images were compared for brightness of autofluorescence and clarity of image (clarity being defined as the sharpness of edges and general fuzziness).

3.2.4.1 Staining on Concrete Surface using SYTO 62

A sample of *Pseudomonas* sp. stained with SYTO 62 was placed on a concrete surface was performed to test whether or not the 633nm wavelength was indeed exploitable for the detection of both parameters, concrete surface and bacteria, simultaneously. This produced an image where the

topography can be seen in 3-D space (Figure 20). This image allows one to see how the concrete surface seems to ascend and rise suggesting the presence of pore space. A single red dot can also be seen on the image; this was assumed to be bacterial based on its size (~2µm) but also based on the fact that it is not present under the other two emission wavelengths. Another indication that it is bacterial is that it seems to sit above the surface of the concrete itself, much like we would expect. From a top down angle (Figure 21) we again see the red dot standing out on a background of greens and yellows. From this angle it is easy to see that it is the only truly distinct red signal, this information was also used as an indication that it was bacterial. It is also interesting to note that this image seems to suggest that this bacteria is within the pore space. This however, is likely coincidental as the bacteria was not native to the concrete and therefore patterns are likely to be undefined.

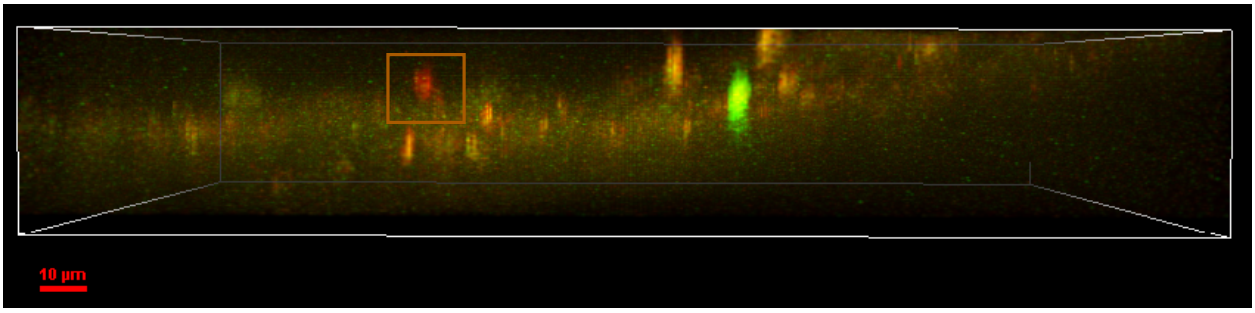

Figure 20 – Image of concrete surface in 3 dimensional space using a confocal laser microscope showing possible presence of a bacteria (shown encompassed in the yellow box)

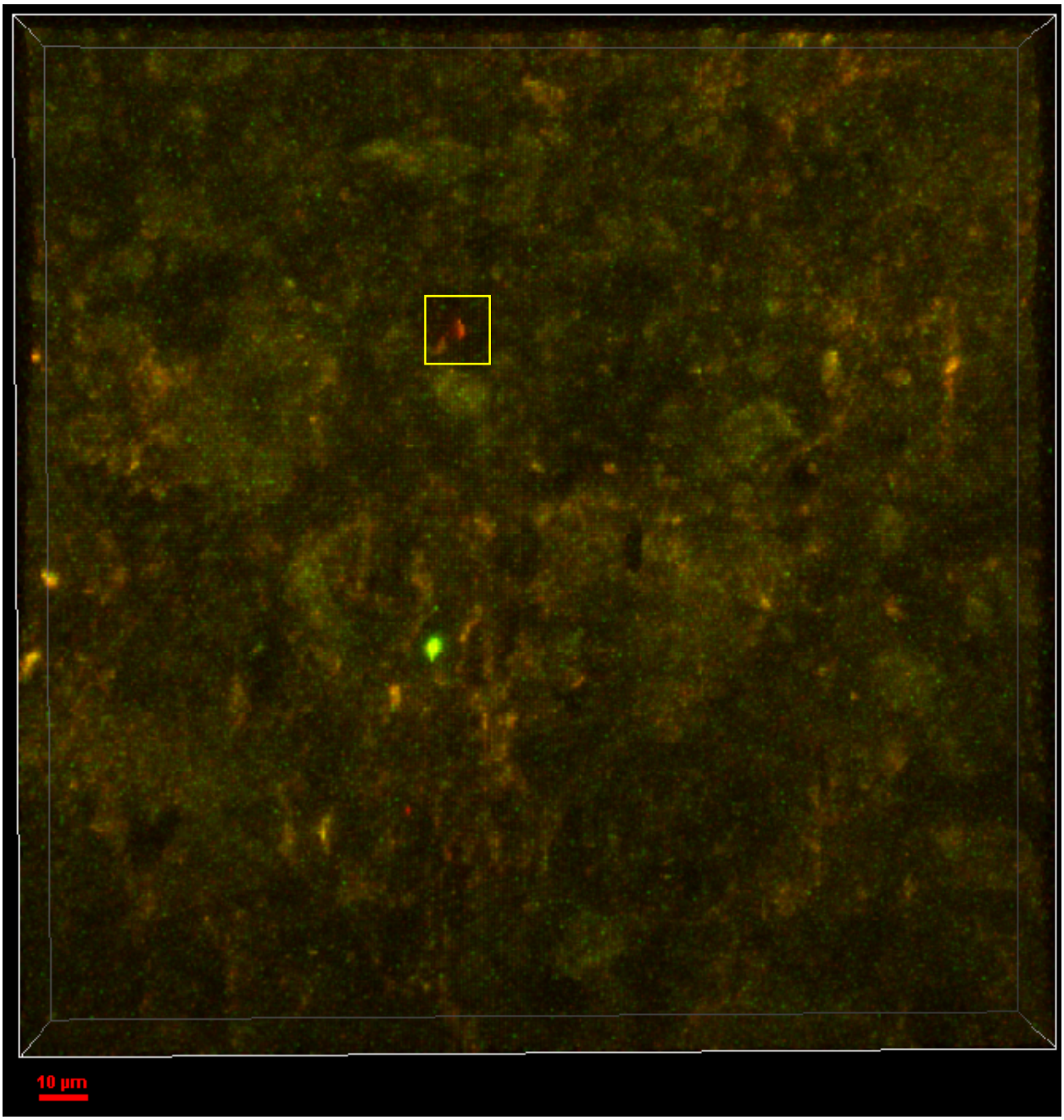

Figure 21 – Image of a concrete surface from a top down angle, a red dot is depicted on the surface and is considered bacterial

3.2.4.2 *Fluorescent in situ Hybridization of Concrete Surface*

Following confirmation that two lasers might be used simultaneously to image concrete surface and bacteria stained using SYTO 62, FISH was used to explore the possibility further. An overnight

sample of *E. coli* was used as a surrogate to SOB due to its rapid growth and their shared gram-negative cell membranes. Figure 22 shows side by side images of the same surface of concrete under the 488 laser (left image) and the 647 laser (right image). These images were both taken using the 650 LP detector to compare and contrast the background fluorescence at this detection limit but also to try and determine bacterial presence. Highlighted by yellow boxes there appears to be bright spots that are roughly of 1 - 2µm in size and do not appear in the same position on the image opposite. Their exclusion in the image on the left suggests that these are bacteria as we expect to only see bacteria under the 647 laser. Their general size is also a good indication that it is indeed bacteria that is present. However, unlike past images there seemed to be a greater presence of auto-fluorescence emitting from the 647nm wavelength laser. This was thought to be due to a chemical present in the FISH staining procedure that may have adsorbed onto the concrete producing an increased auto-fluorescence.

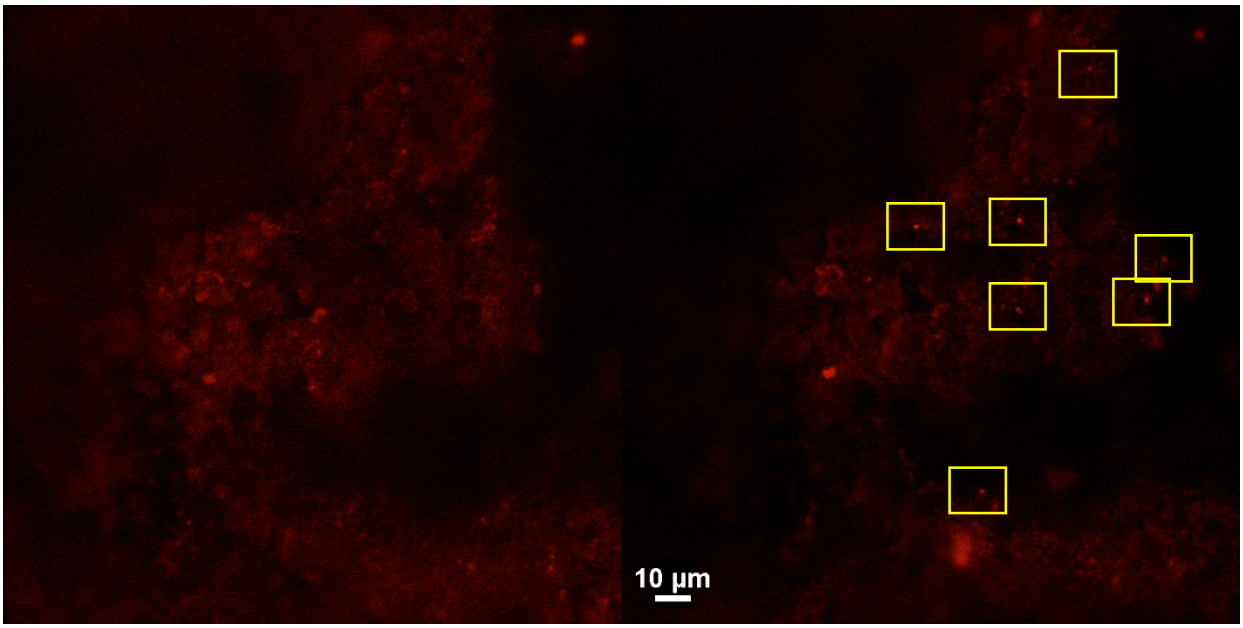

Figure 22 – Side by side comparison of the background fluorescence of a concrete sample using the 488 (left) and 647 (right) wavelength lasers both captured at the 650 LP detector.

Two further samples were then tested, one which was not exposed to the FISH probes (Figure 23) and one used as a control which had the entire FISH process completed with the exclusion of *E. coli* (Figure 24). Seen in figure 23 the images of the blank concrete seem to resemble one another with no major difference in auto-fluorescence but the auto-fluorescent level was still much higher than in previous tests. This was determined to be due to upgrades and a realignment of lasers on the microscope between the SYTO 62 staining and the FISH staining projects. It is thought that the realignment of the lasers allowed for greater strength of signal detected and therefore was causing the increase in auto-fluorescence.

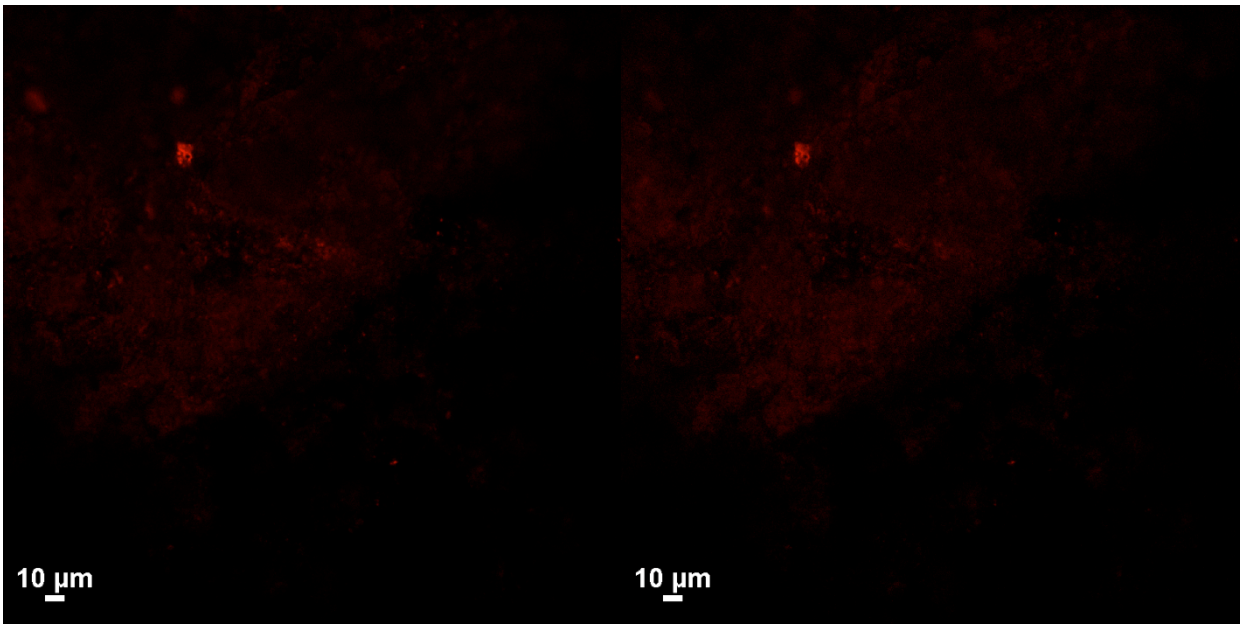

Figure 23 – Comparison of a blank piece of concrete under the 488 (left) and 647 (right) laser being detected using a 650LP detector.

The sample that had FISH performed seemed to suggest that there is a product in the FISH process that is not fully washed from the concrete and which lends to an increase in auto-fluorescence. Also these pictures seem to depict particles around the size of 1 – 2µm. Most of these spots, if not all, seem to appear in both images. However, because they do exist it is likely not safe to consider those

from the above image (Figure 22) to be bacteria and no definitive way with current tests has the ability to truly distinguish these as bacteria versus some particle inherent to the concrete matrix.

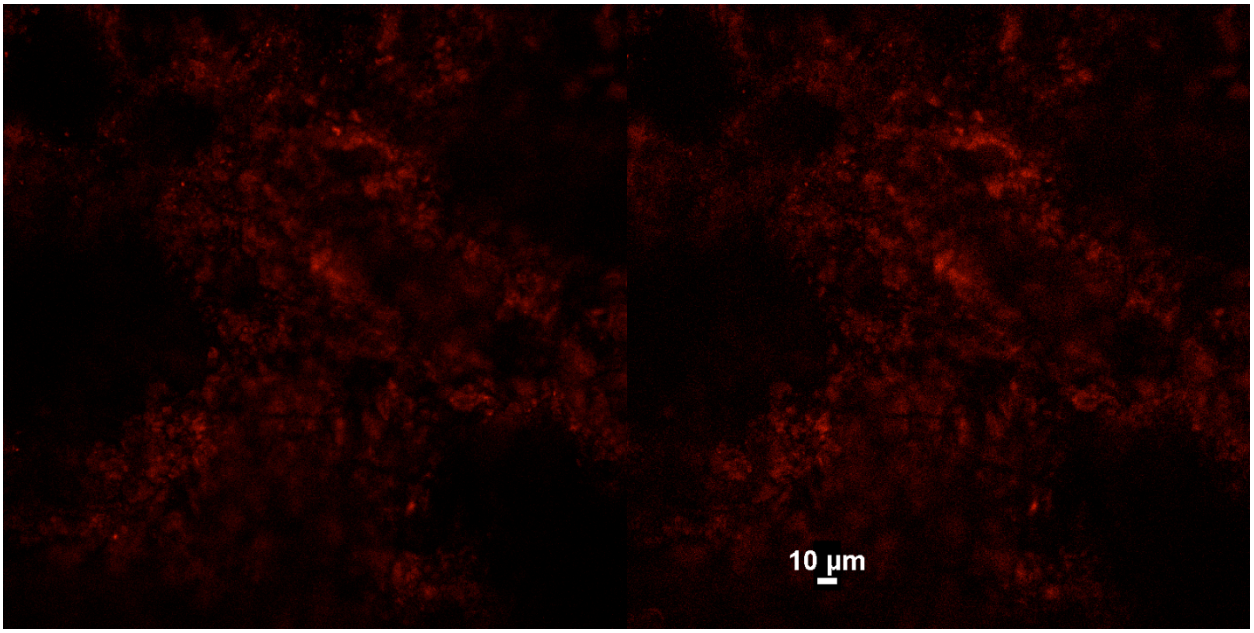

Figure 24 – Comparison of a FISH stained piece of concrete (without bacteria present) under the 488 (left) and 647 (right) laser being detected using a 650LP detector.

4. Conclusion

Based on all the information present the apparatus built to house the concrete during the testing did what it was designed to do. It was constructed in hopes of replicating, as best as possible, conditions found in a sewer system (moist environment with high nutrient content), promoting the colonization of the concrete by bacteria. From visual inspection alone this was achieved, as the chambers were consistently damp showing signs of moisture build up on the walls of the box and the concrete itself always appeared wet.

An oversight of the environmental conditions created, however, were their effects on the potentially extended curing time. The extended curing time is obvious based on visual inspection, consistent wet surface, and based on the concrete property results over time. This extra curing time led to a significant increase in the strength of concrete regardless of the mix characteristics and a significant decrease in the porosity. Absorption through full immersion as well showed a decline in absorption over time, following the trend of the other three tests. Although it was found not to be significant it should still be considered relevant to the trend seen in the other test methods.

The length of time allotted for the experiment was chosen based on studies that suggested it could take up to 6 months for sulphur oxidizing bacteria to colonize the surface of the concrete. Therefore, 8 months was chosen in hopes of seeing some degradation evident in the properties of the concrete. The most relevant of the physical properties for indicating biological degradation would be loss of strength. The time*metakaolin effect on strength may indicate that biological degradation was having some impact by reducing strength gain (with curing) in high metakaolin concretes relative to 0% metakaolin concrete. However, SEM images suggest greater crystal formation in concrete with 0% metakaolin. It is more likely that the concrete with no metakaolin, despite lower porosity, was more

effectively colonized by sulphur oxidizing bacteria. This might be due to pores that are larger and more accessible (as indicated by sorptivity), despite the decrease in total pore space. The interaction between time and metakaolin content may, instead, indicate that concrete with higher metakaolin content will cure and reach maximum strength more rapidly. An experiment would have to be run for much longer before effects of the bacteria would be apparent given the continued curing over the duration of this experiment.

While an increase in metakaolin content may increase resistance to sulphuric acid (Joorabchian, 2010) the increase in porosity had the potential to increase the colonization of sulphur oxidizing bacteria (hypothesis 2). With increased porosity the conventional testing of concrete resistance to sulphuric acid (standard test protocols with sulphuric acid immersion) may not be relevant, as concrete with higher porosity might be more susceptible to biological acid attack. As was expected, it would appear that bacteria colonize the pore structure of a concrete face. As a counter balance to that, however, the sorptivity of the concrete mixes suggests that an increase of metakaolin leads to a decrease in sorptivity. This could result in a concrete that is more porous, but the pores are less accessible for colonization. The apparent degradation patterns, based on crystal formation, suggest that this was the case. The results would then suggest that susceptibility to biogenic acid production attack is decreased by metakaolin, not enhanced as predicted (hypothesis 3). This further suggests that the approach to testing concrete resistance to sulphuric acid attack (immersion in dilute sulphuric acid), may be appropriate despite the lack of relevance with respect to the biological processes.

It is apparent from the observations of concrete staining, the observation of bacteria associated with those stained areas, and the formation of crystals on the surface of concrete exposed to the inoculum, that colonization by aerosols did, indeed, occur.

The direct ability to identify these bacteria on the surface of concrete while showing the topography of the concrete itself was inconclusive. Although spots that appeared to be bacteria were present, subsequent tests suggest that portions of the concrete (autofluorescing particles) could cause a false positive. Further studies are needed to determine whether or not these spots can be differentiated from the concrete itself for a confirmation of their presence. These results also suggest that a gene insert may be the best method for imaging as FISH products seem to increase autofluorescence.

4.1 *Future Work*

Considering the possibility that the image captured in figure 22 does indeed depict bacteria, specifically *E. coli*, then new ways to confirm the presence of bacteria need to be created. One possibility is through the use of double hybridization. Here one uses two different probes, labeled for the same RNA sequence, to hybridize in the same cell to produce a signal along two different emission wavelengths. This method, however, has cons as well. This is due to competition over the same target site which can lead to a reduction of signal by as much as ~ 50% less intense, assuming probes are added in equal quantities and have the same efficiency of hybridization (Behnam *et al*, 2012). This method has been used to differentiate mixed cultures in which different combinations of probes can be used to separate individuals (Pernthaler & Pernthaler, 2007; Valm *et al*, 2011). A slightly newer version of this idea, called double labelling of oligonucleotide probes (DOPE), has one individual probe with two different fluors. This was reported to have no reduction in signal intensity and allowed the researchers to separate up to 6 different species in a single pass (Behnam *et al*, 2012). In this case one could label the bacteria with two different fluors to allow for increased signal detection across multiple wave lengths.

The boxes were constructed in order to replicate, as best as possible, the conditions experienced by concrete in a sewer system. These boxes and the experimental design, however, will not

satisfy future work. As the concrete properties were found to be unaffected by bacterial colonization and would likely not show an effect for a longer period of time their use may be impractical. The need, however, to discover the colonization patterns of concrete still remains. This leaves an opening for the creation of better methods for realistic colonization patterns on concrete that would be similar to what was created in the boxes. The use of flow cells is a common method used for the examination of biofilms. Simple modifications of these methods could allow for the creation of a flow through that washes across the concrete face. This would replicate conditions similar to those found in sewer systems as the bacteria would be deposited on the surface similar to how they would be in a sewer. Through visual inspection one would hopefully be able to see colonies form over time as was seen in section 3.2.2, allowing for confirmation of the patterns seen above. Secondly, if methods are created for the visualization of bacteria on a concrete surface then the patterns of this colonization could be more easily examined. This could be run as a pure culture to see how a single individual colonizes, or be done as a succession of colonization through the use of mixed cultures.

As biogenic sulphuric acid has a microbial agent involved, it is hard to tell whether or not the sorptivity reduction in concrete with increased metakaolin would have an effect on its degradation. Studies should be conducted to differentiate damage based on pore volume and sorptivity. These could be done using a full immersion test to increase colonization speed. Here concrete cylinders could be placed in media containing the bacteria in question in which a reaction vessel could be created to bring the bacteria new media while clearing waste. These tests would have to be run until visual identification of damage on the concrete surface was visible. This would be to ensure colonization occurred. Tests of weight lost, change in strength, and then total porosity and sorptivity would allow for the indirect comparison of the effects of porosity and sorptivity.

5. Appendix

5.1 *Concrete Property Tests Raw Data*

5.1.1 *Sorptivity*

Table 7 – Raw data for sorptivity of 0% and 10% metakaolin mixes at initial time point

Time(min)/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
1	0.124	0.128	0.126	0.118	0.131	0.139
5	0.191	0.245	0.21	0.169	0.194	0.195
10	0.207	0.253	0.221	0.171	0.198	0.197
20	0.244	0.301	0.272	0.212	0.239	0.218
30	0.283	0.351	0.311	0.239	0.256	0.24
60	0.347	0.42	0.371	0.284	0.297	0.286
120	0.438	0.541	0.474	0.339	0.351	0.342
180	0.491	0.631	0.573	0.406	0.429	0.414
240	0.519	0.669	0.608	0.441	0.461	0.455
300	0.555	0.703	0.651	0.476	0.513	0.485
360	0.581	0.761	0.686	0.504	0.553	0.518

Table 8 – Raw data for sorptivity of 15% and 20% metakaolin mixes at initial time point

Time(min)/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
1	0.099	0.082	0.101	0.092	0.097	0.091
5	0.159	0.141	0.166	0.102	0.112	0.109
10	0.161	0.144	0.169	0.127	0.137	0.131
20	0.183	0.177	0.192	0.179	0.199	0.185
30	0.193	0.191	0.205	0.195	0.214	0.205
60	0.264	0.259	0.273	0.22	0.242	0.224
120	0.319	0.309	0.33	0.278	0.294	0.281
180	0.361	0.357	0.385	0.309	0.34	0.321
240	0.374	0.368	0.39	0.315	0.355	0.338
300	0.401	0.396	0.416	0.357	0.384	0.378
360	0.438	0.43	0.445	0.495	0.423	0.413

Table 9 – Raw data for sorptivity of 0% and 10% mixes at 7 months

Time(min)/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
1	0.124	0.139	0.128	0.073	0.132	0.111
5	0.255	0.283	0.214	0.164	0.24	0.216
10	0.266	0.305	0.245	0.204	0.289	0.246
20	0.285	0.341	0.265	0.228	0.336	0.27
30	0.321	0.438	0.302	0.243	0.325	0.305
60	0.385	0.507	0.372	0.27	0.351	0.379
120	0.493	0.61	0.483	0.335	0.398	0.429
180	0.524	0.656	0.522	0.388	0.444	0.445
240	0.566	0.702	0.562	0.422	0.486	0.452
300	0.606	0.747	0.603	0.469	0.493	0.482
360	0.622	0.767	0.619	0.478	0.507	0.495

Table 10 – Raw data for sorptivity of 15% and 20% mixes at 7 months

Time(min)/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
1	0.077	0.076	0.109	0.072	0.127	0.033
5	0.182	0.117	0.125	0.109	0.148	0.075
10	0.224	0.142	0.167	0.119	0.154	0.102
20	0.228	0.185	0.191	0.137	0.178	0.107
30	0.239	0.254	0.212	0.143	0.19	0.129
60	0.256	0.28	0.251	0.152	0.195	0.207
120	0.312	0.34	0.28	0.185	0.214	0.287
180	0.33	0.357	0.3	0.214	0.241	0.295
240	0.376	0.374	0.321	0.231	0.291	0.322
300	0.397	0.384	0.376	0.244	0.31	0.357
360	0.416	0.397	0.387	0.253	0.33	0.394

Table 11 – Raw data for sorptivity of 0% and 10% mixes at 8 months

Time(min)/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
1	0.131	0.129	0.135	0.119	0.133	0.131
5	0.227	0.218	0.239	0.216	0.228	0.229
10	0.291	0.288	0.295	0.255	0.266	0.271
20	0.319	0.311	0.33	0.28	0.29	0.298
30	0.35	0.349	0.359	0.299	0.311	0.314
60	0.389	0.381	0.393	0.326	0.338	0.342
120	0.489	0.482	0.499	0.359	0.366	0.371

180	0.539	0.538	0.555	0.398	0.411	0.422
240	0.578	0.579	0.594	0.437	0.457	0.466
300	0.623	0.619	0.636	0.479	0.491	0.505
360	0.649	0.649	0.661	0.499	0.519	0.521

Table 12 – Raw data for sorptivity of 15% and 20% mixes at 8 months

Time(min)/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
1	0.065	0.089	0.084	0.083	0.077	0.066
5	0.132	0.148	0.146	0.126	0.114	0.103
10	0.166	0.176	0.179	0.144	0.129	0.119
20	0.191	0.201	0.206	0.181	0.165	0.151
30	0.201	0.22	0.224	0.198	0.185	0.173
60	0.238	0.253	0.256	0.227	0.216	0.201
120	0.271	0.293	0.291	0.251	0.243	0.233
180	0.299	0.311	0.309	0.277	0.272	0.26
240	0.315	0.333	0.334	0.309	0.303	0.296
300	0.336	0.358	0.36	0.325	0.329	0.317
360	0.353	0.379	0.38	0.354	0.355	0.344

5.1.2 *Porosity*

Table 13 – Raw data for porosity of 0% and 10% mixes across all time points

Time/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
Initial	8.796451	5.29973	7.225236	10.13866	9.056381	8.547947
7 Months	5.256456	4.962468	7.13396	6.391721	6.813238	6.22869
8 Months	5.544526	5.190328	6.325398	6.570094	6.570649	6.584595

Table 14 – Raw data for porosity of 15% and 20% mixes across all time points

Time/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
Initial	9.187336	11.22157	10.21707	11.19136	9.932819	10.18886
7 Months	4.628229	6.473512	6.265753	6.867776	7.196796	6.871665
8 Months	7.607472	5.920598	6.742104	7.064158	6.931407	6.991554

5.1.3 *Absorption through Total Immersion*

Table 15 – Raw data for absorption of 0% and 10% mixes across all time points

Time/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
Initial	1.188926	1.212255	1.179694	1.543723	1.402146	0.951497
7 Months	1.085301	1.105955	1.195436	1.556275	1.045603	1.400936
8 Months	1.028223	1.389689	0.937573	1.133342	1.49903	1.345375

Table 16 – Raw data for absorption of 15% and 20% mixes across all time points

Time/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
Initial	1.667191	1.915211	1.920379	1.552009	2.371057	2.203978
7 Months	1.657974	1.67116	1.770639	1.788621	1.80616	1.908535
8 Months	1.408736	1.839574	1.842129	1.975713	1.661204	1.811507

5.1.4 *Strength*

Table 17 – Raw data for strength of 0% and 10% mixes across all time points

Time/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
Initial	32.065	35.984	34.819	40.273	45.997	48.76
7 Months	39.886	36.06	45.285	54.758	60.488	62.612
8 Months	54.469	53.366	53.934	66.542	57.296	56.213

Table 18 – Raw data for strength of 15% and 20% mixes across all time points

Time/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
Initial	54.29	49.215	51.084	61.147	62.232	57.124
7 Months	63.569	65.363	62.991	72.697	71.782	71.242
8 Months	65.88	69.651	66.662	76.518	68.145	73.858

5.1.5 *Rapid Chloride Permeability*

Table 19 – Raw data for rapid chloride permeability of 0% and 10% mixes across all time points

Time/Specimen	0%-1	0%-2	0%-3	10%-1	10%-2	10%-3
Initial	1621	1496	1489	640	583	449
7 Months	1008	950	967	545	522	497
8 Months	987	1011	890	409	473	414

Table 20 – Raw data for rapid chloride permeability of 15% and 20% mixes across all time points

Time/Specimen	15%-1	15%-2	15%-3	20%-1	20%-2	20%-3
Initial	371	407	291	221	153	220
7 Months	310	347	324	210	205	177
8 Months	299	348	304	201	199	222

5.2 *Images*

5.2.1 *SEM images of Concrete Surface at Initial Time Point*

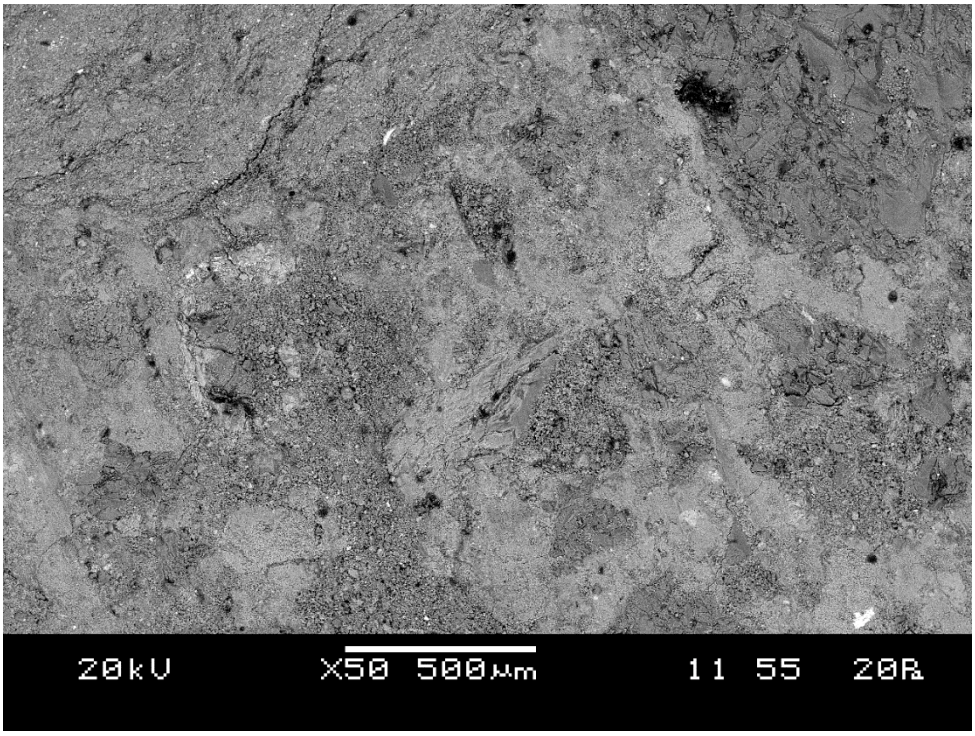

Figure 25 –Image of 0% metakaolin mix surface at 50x magnification

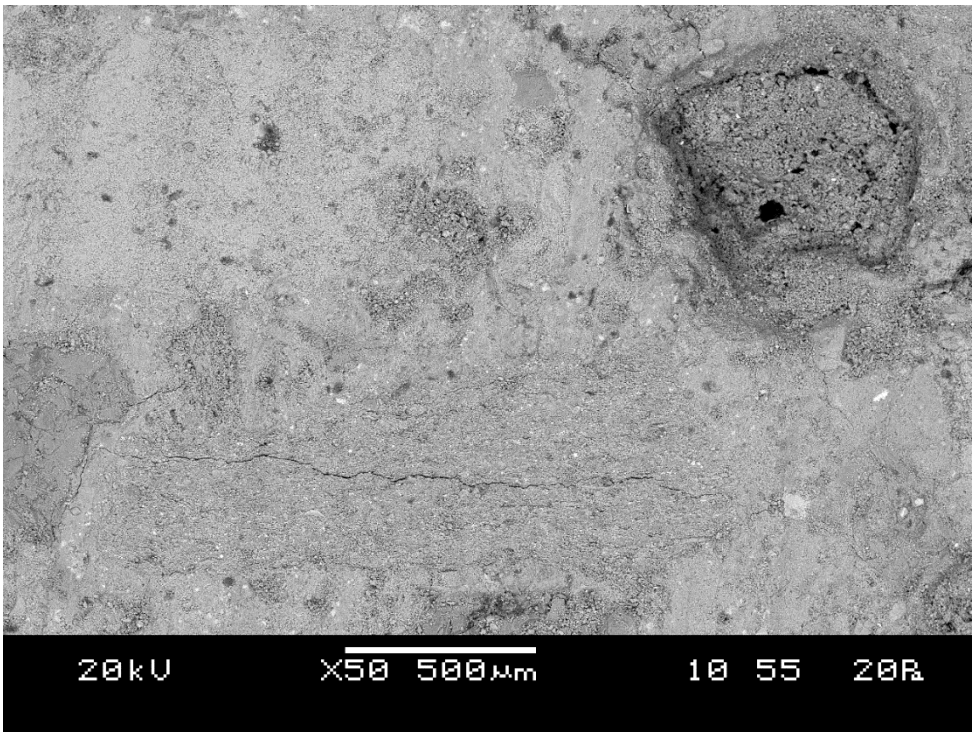

Figure 26 – Image of 10% metakaolin mix surface at 50x magnification

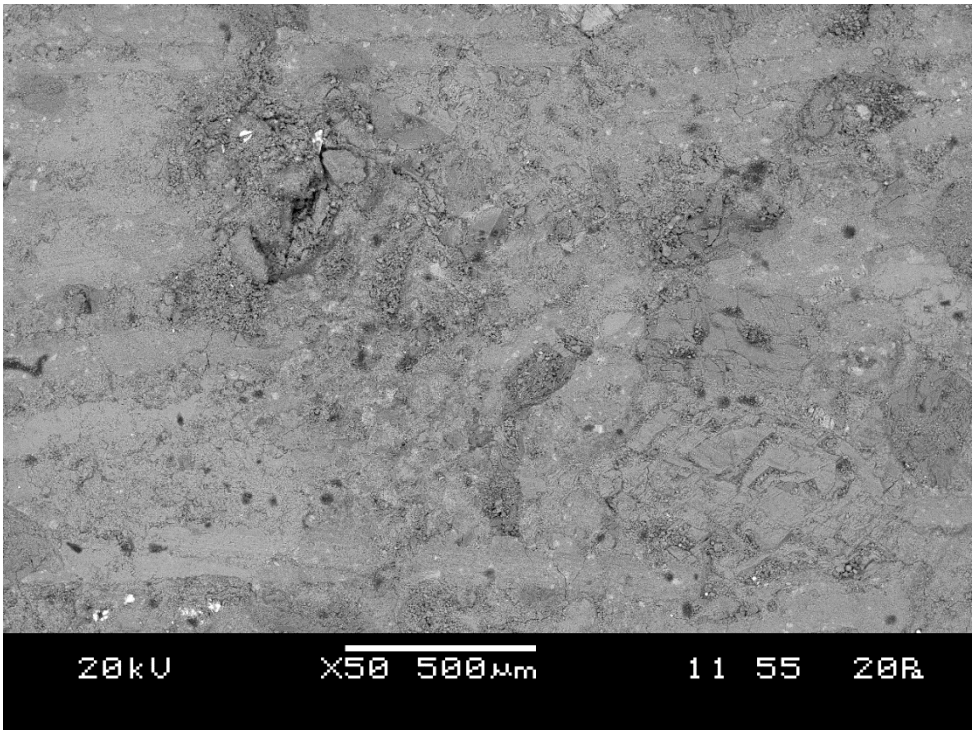

Figure 27 – Image of 15% metakaolin mix surface at 50x magnification

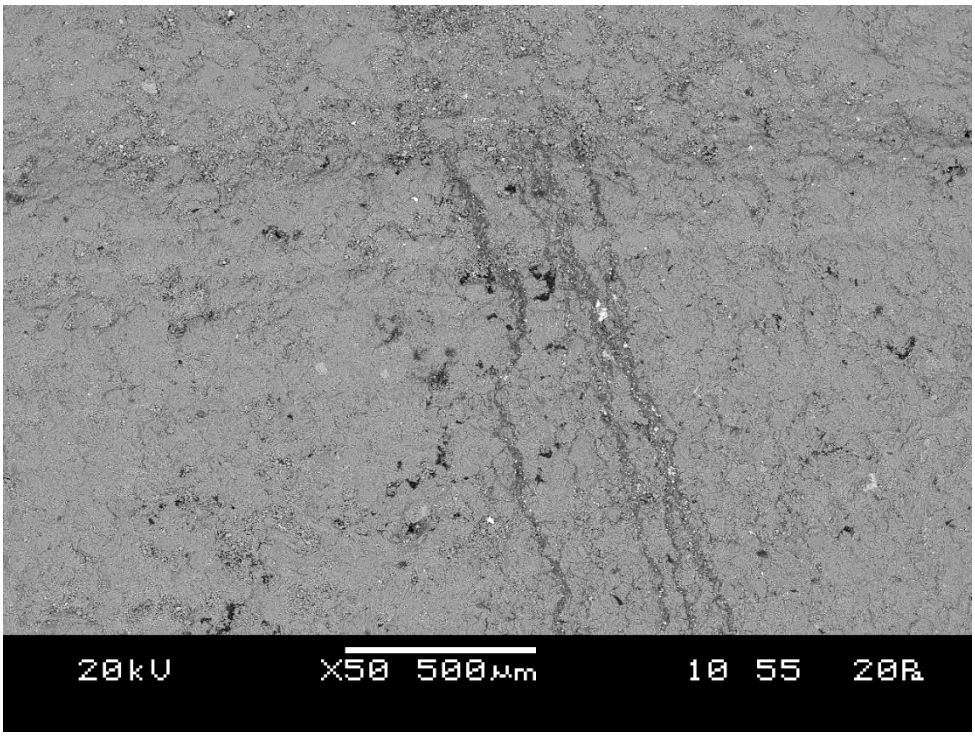

Figure 28 – Image of 20% metakaolin mix surface at 50x magnification

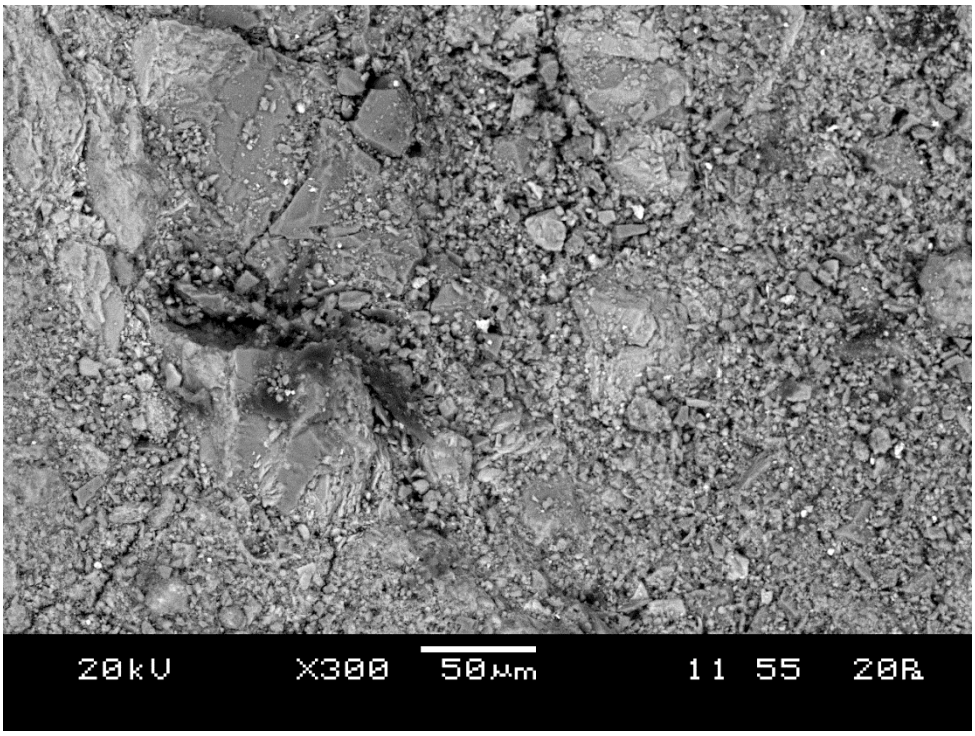

Figure 29 – Image of 0% metakaolin mix surface at 300x magnification

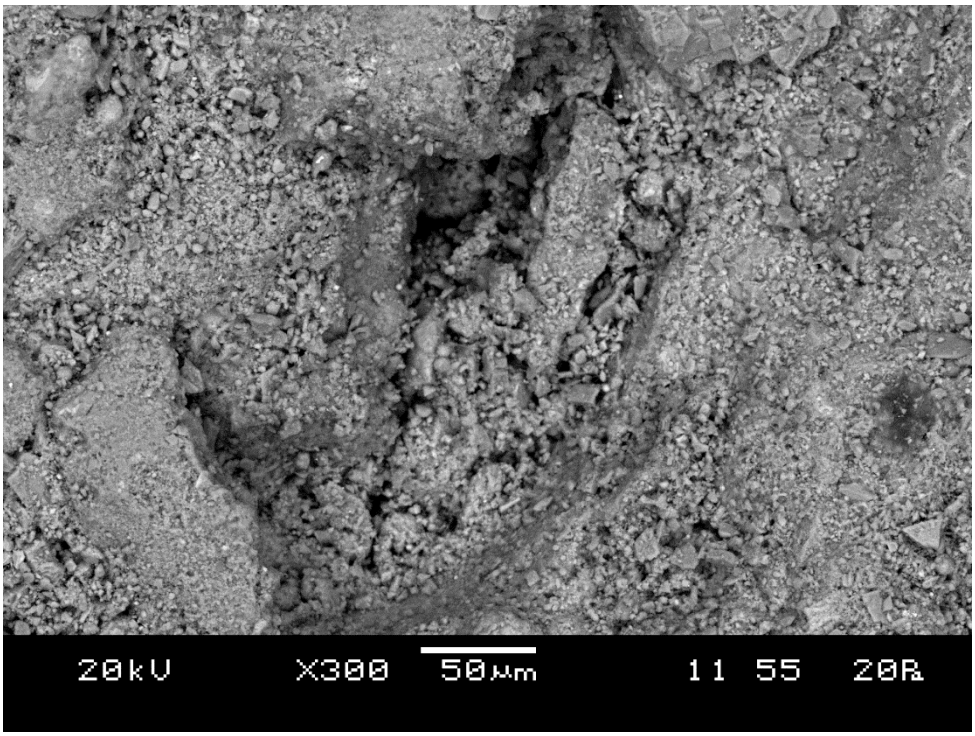

Figure 30 – Image of 10% metakaolin mix surface at 300x magnification

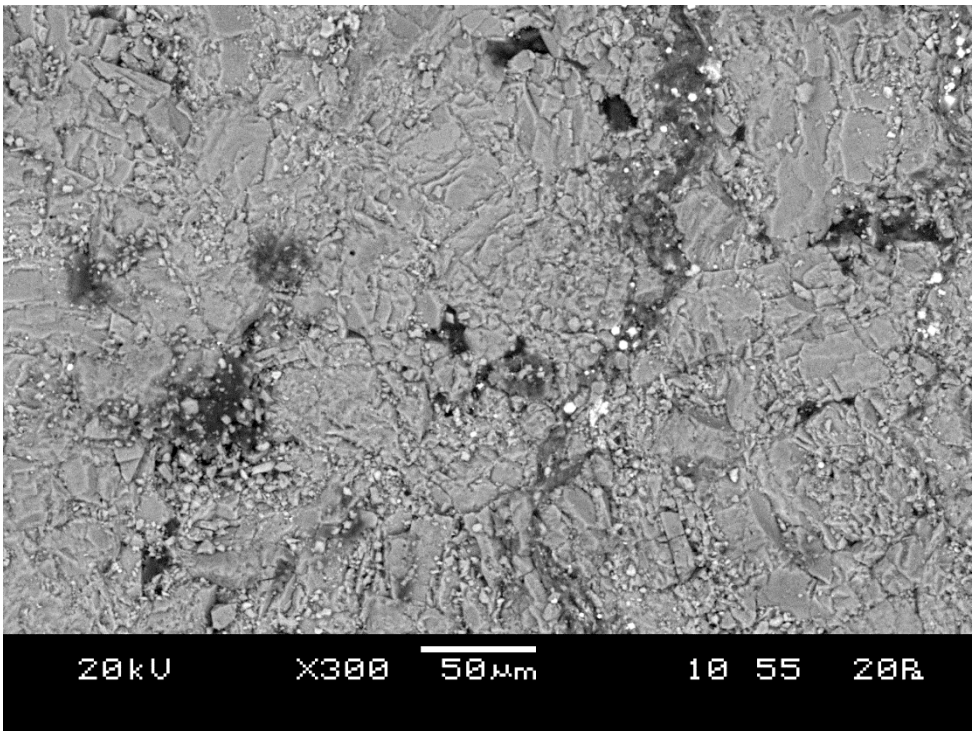

Figure 31 – Image of 15% metakaolin mix surface at 300x magnification

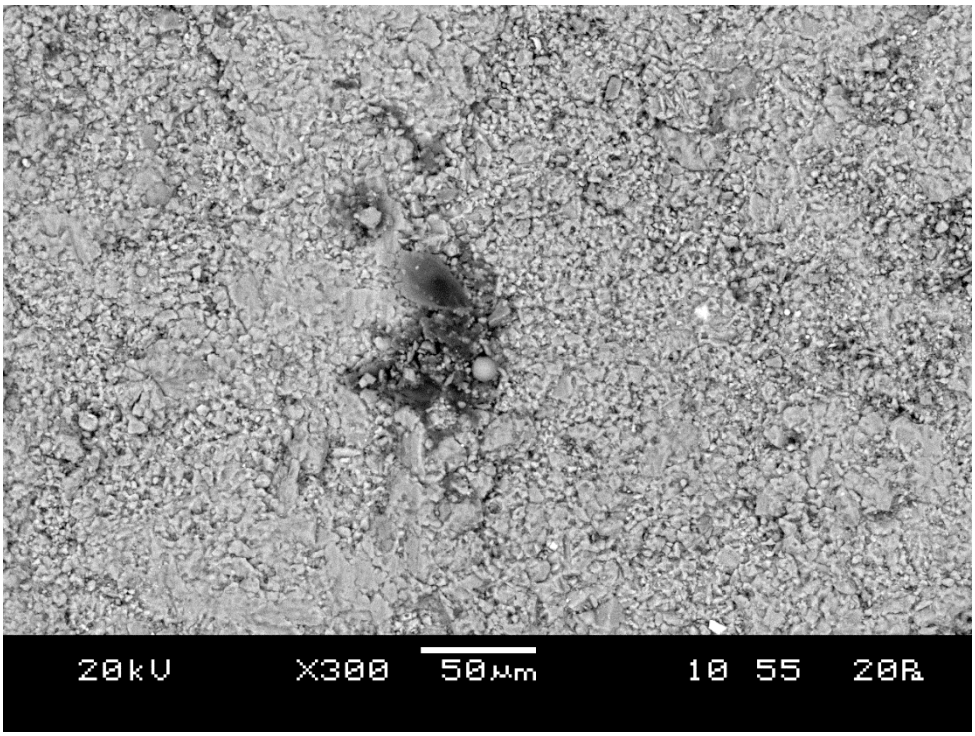

Figure 32 – Image of 20% metakaolin mix surface at 300x magnification

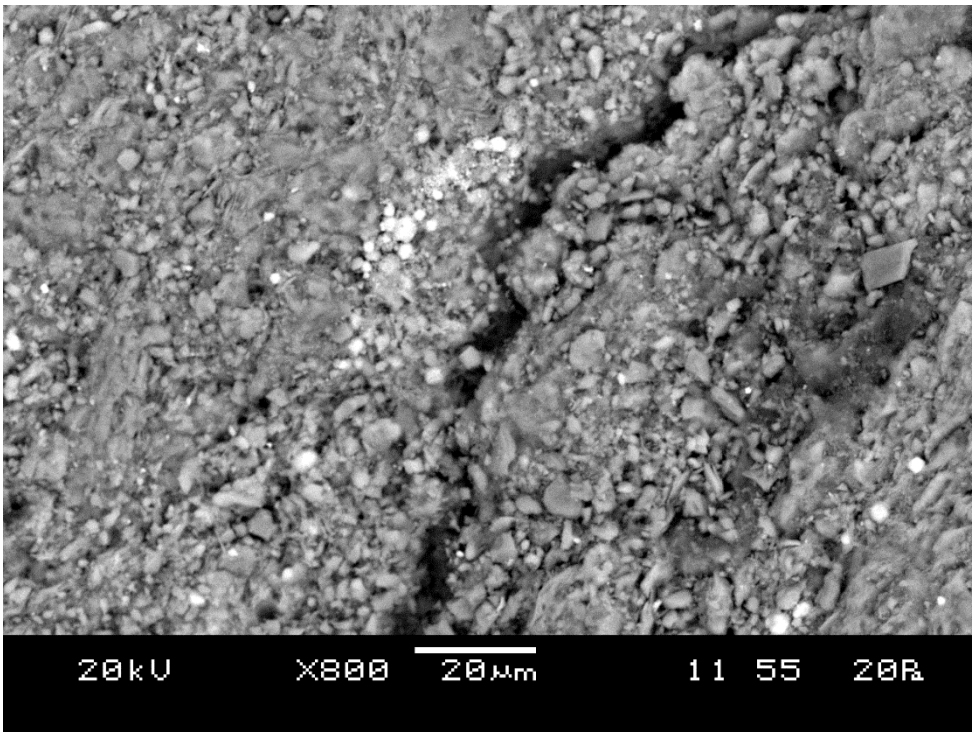

Figure 33 – Image of 0% metakaolin mix surface at 800x magnification

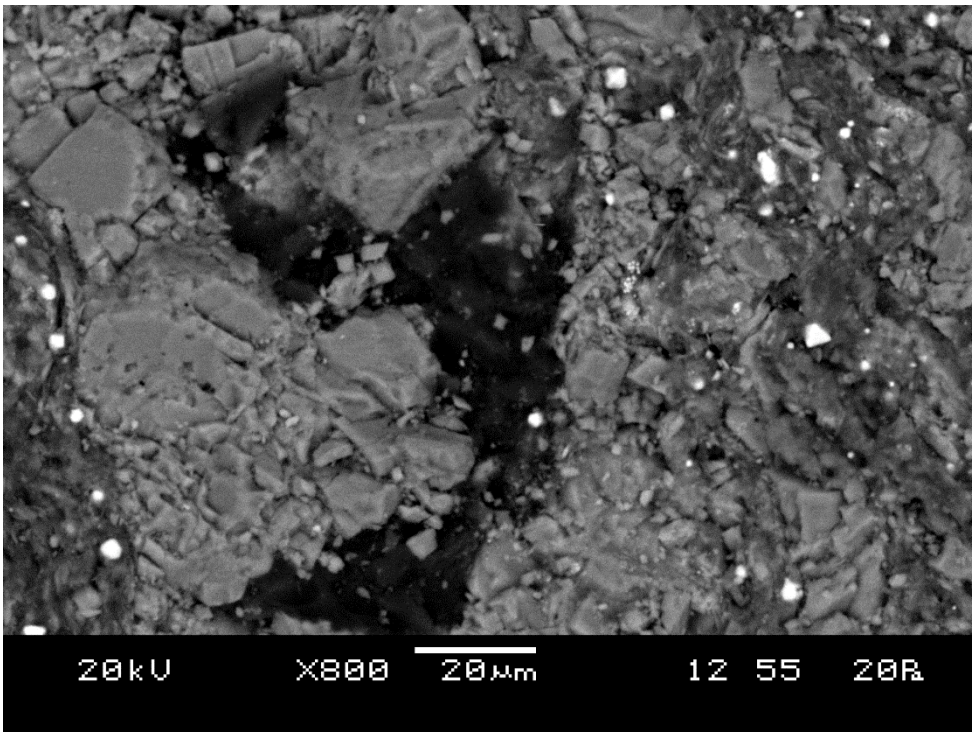

Figure 34 – Image of 10% metakaolin mix surface at 800x magnification

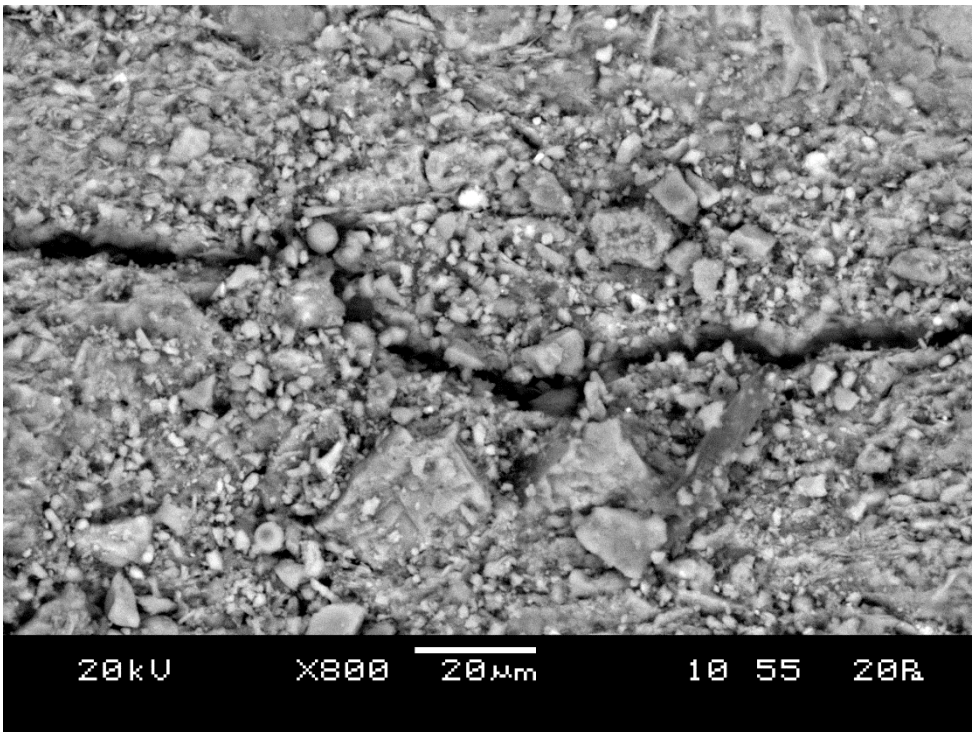

Figure 35 – Image of 15% metakaolin mix surface at 800x magnification

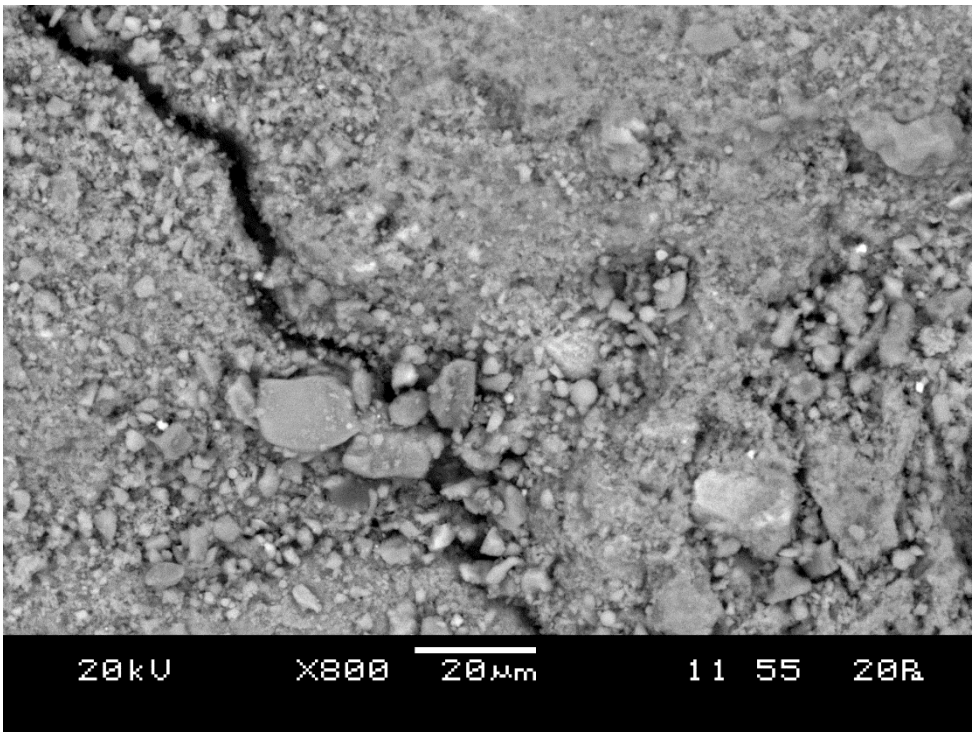

Figure 36 – Image of 20% metakaolin mix surface at 800x magnification

5.2.2 *SEM Images of Concrete Surface at 8 Months*

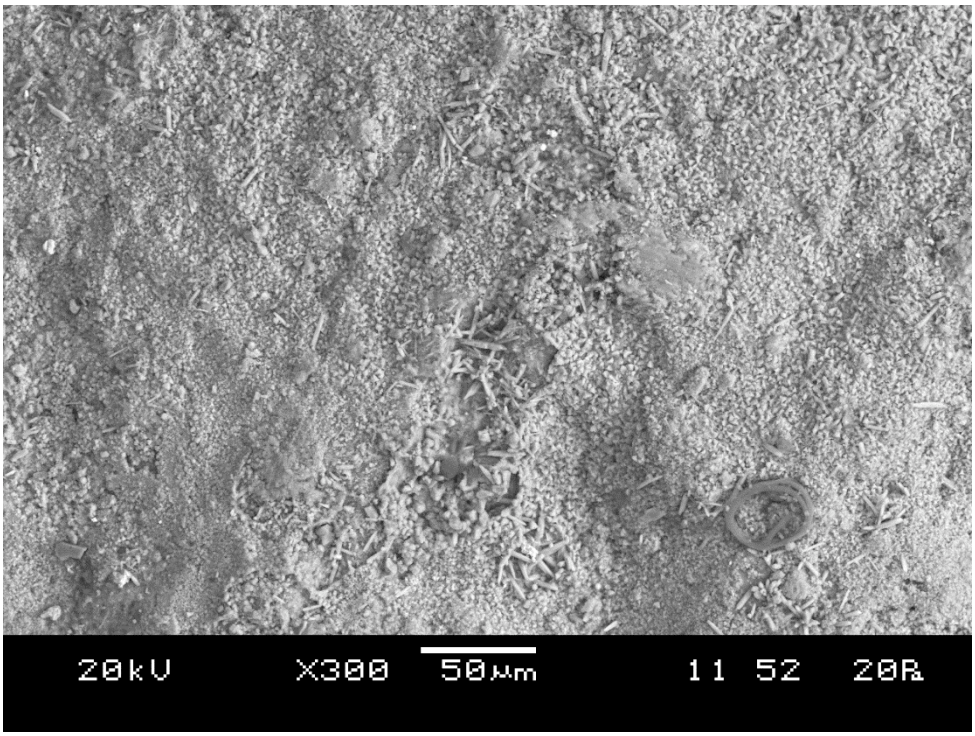

Figure 37 – Image of 0% metakaolin mix surface at 300x magnification showing signs of crystal formation

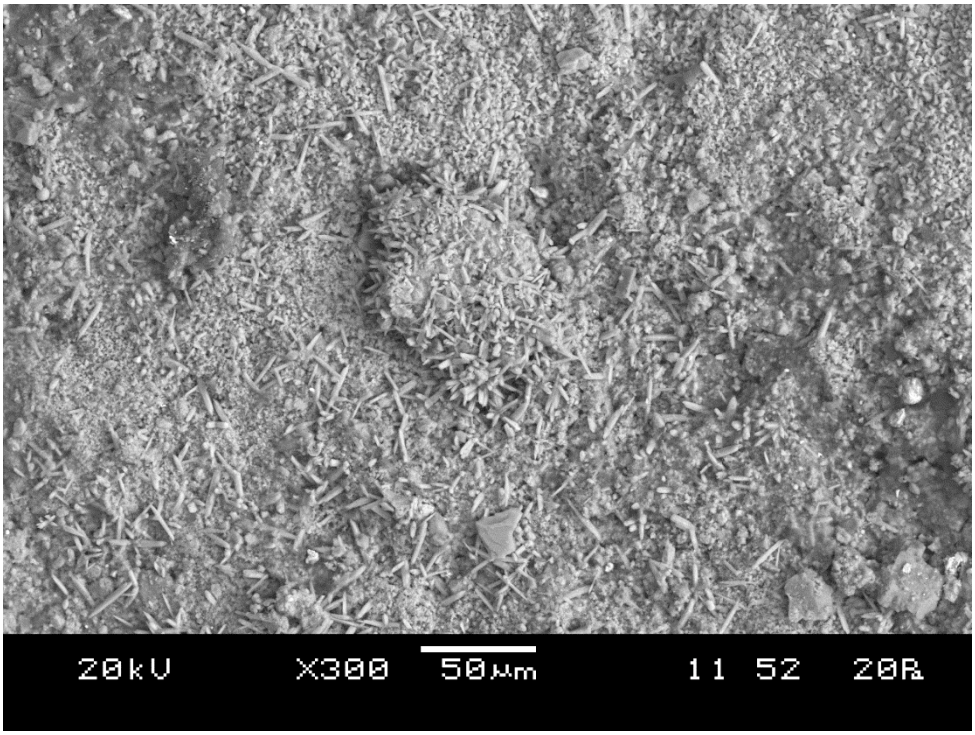

Figure 38 – Image of 0% metakaolin mix surface at 300x magnification showing signs of crystal formation

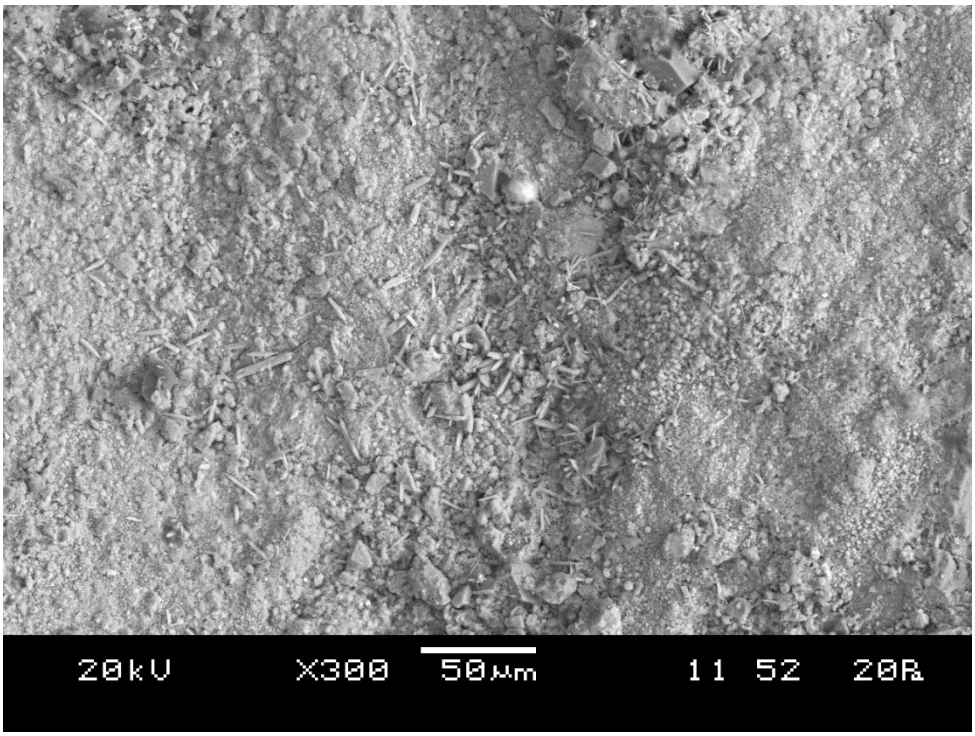

Figure 39 – Image of 0% metakaolin mix surface at 300x magnification showing signs of crystal formation

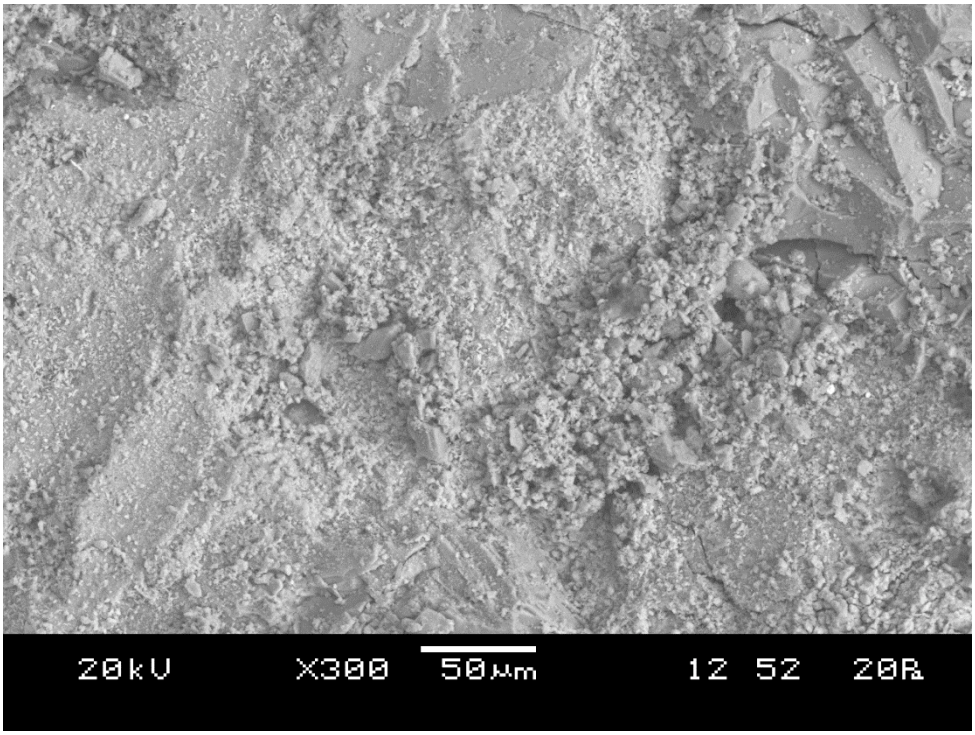

Figure 40 – Image of 20% metakaolin mix surface at 300x magnification showing signs of crystal formation

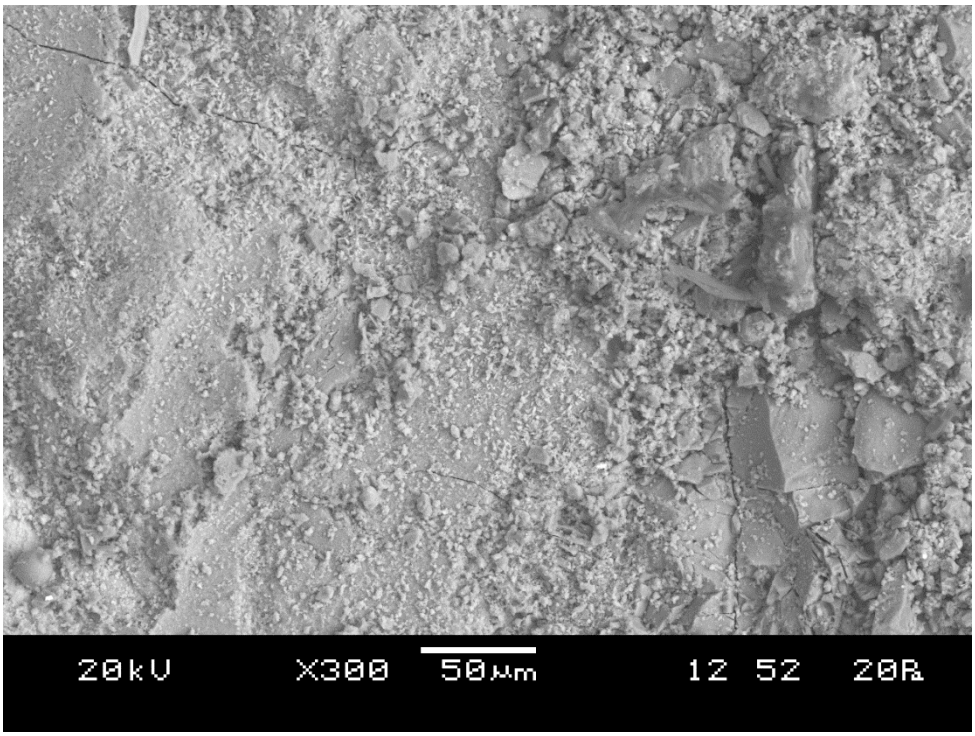

Figure 41 – Image of 20% metakaolin mix surface at 300x magnification showing signs of crystal formation

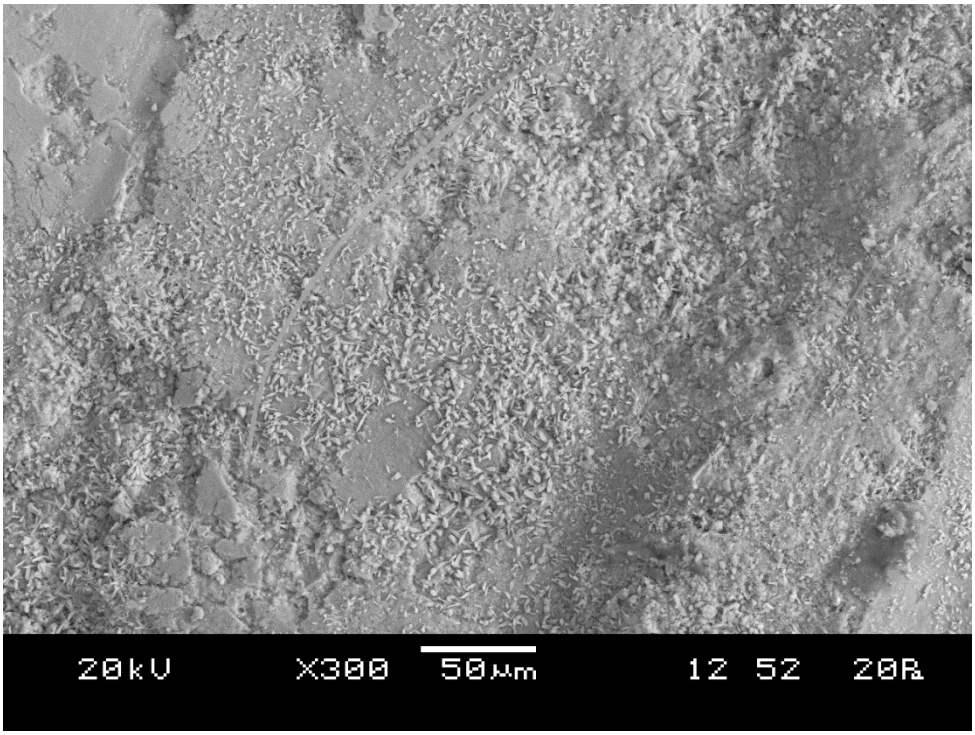

Figure 42 – Image of 20% metakaolin mix surface at 300x magnification showing signs of crystal formation

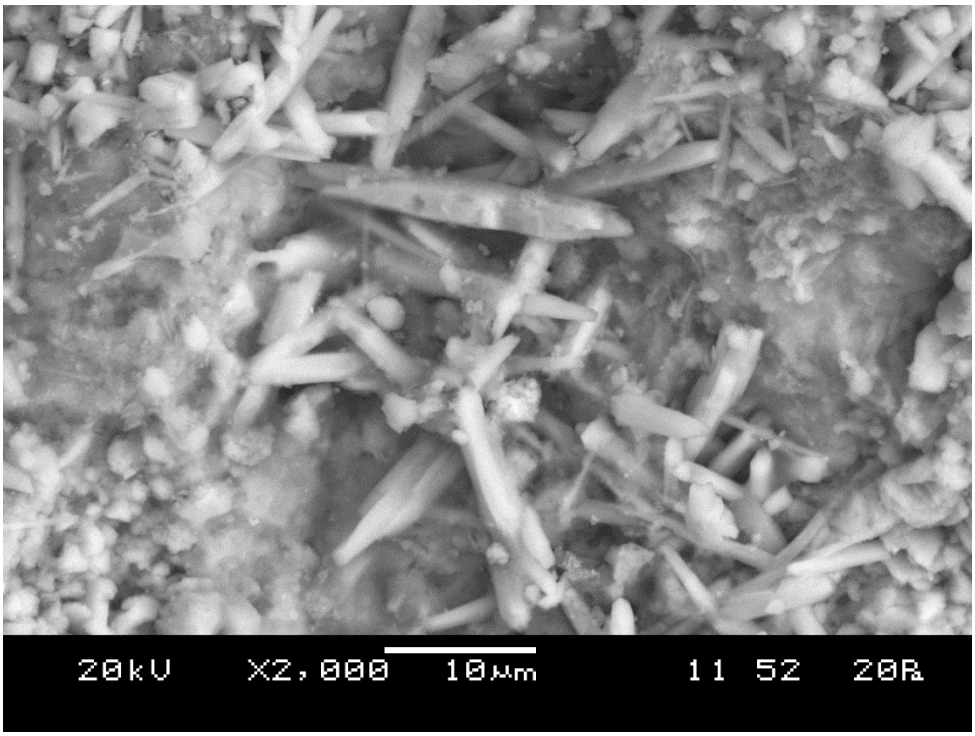

Figure 43 – Image of 0% metakaolin mix surface at 2000x magnification showing signs of crystal formation

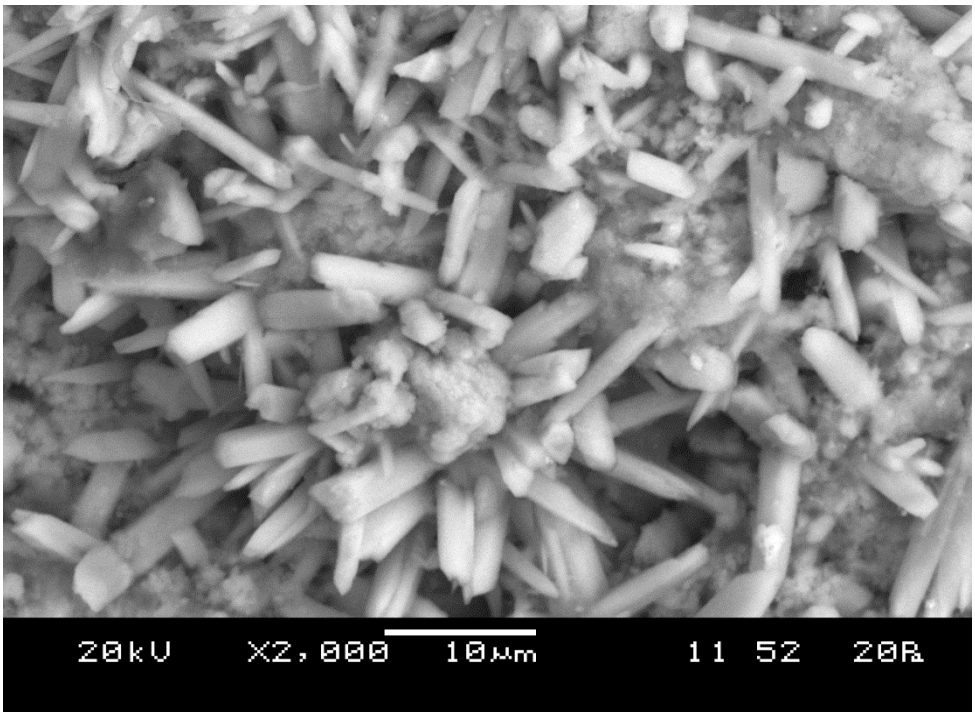

Figure 44 – Image of 0% metakaolin mix surface at 2000x magnification showing signs of crystal formation

Figure 45 – Image of 0% metakaolin mix surface at 2000x magnification showing signs of crystal formation

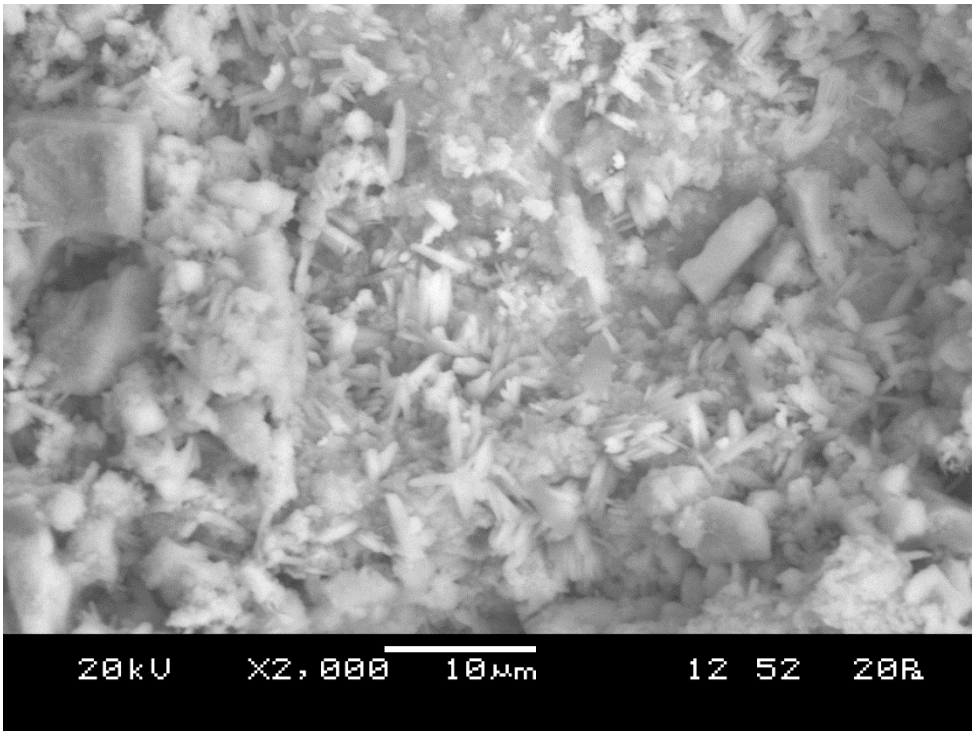

Figure 46 – Image of 20% metakaolin mix surface at 2000x magnification showing signs of crystal formation

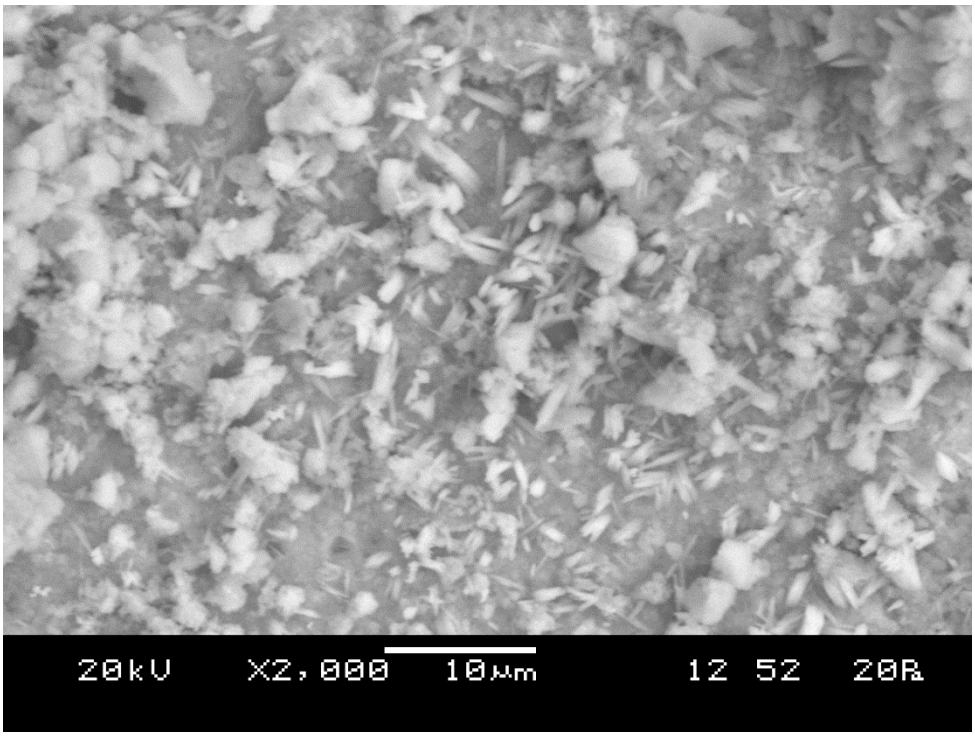

Figure 47 – Image of 20% metakaolin mix surface at 2000x magnification showing signs of crystal formation

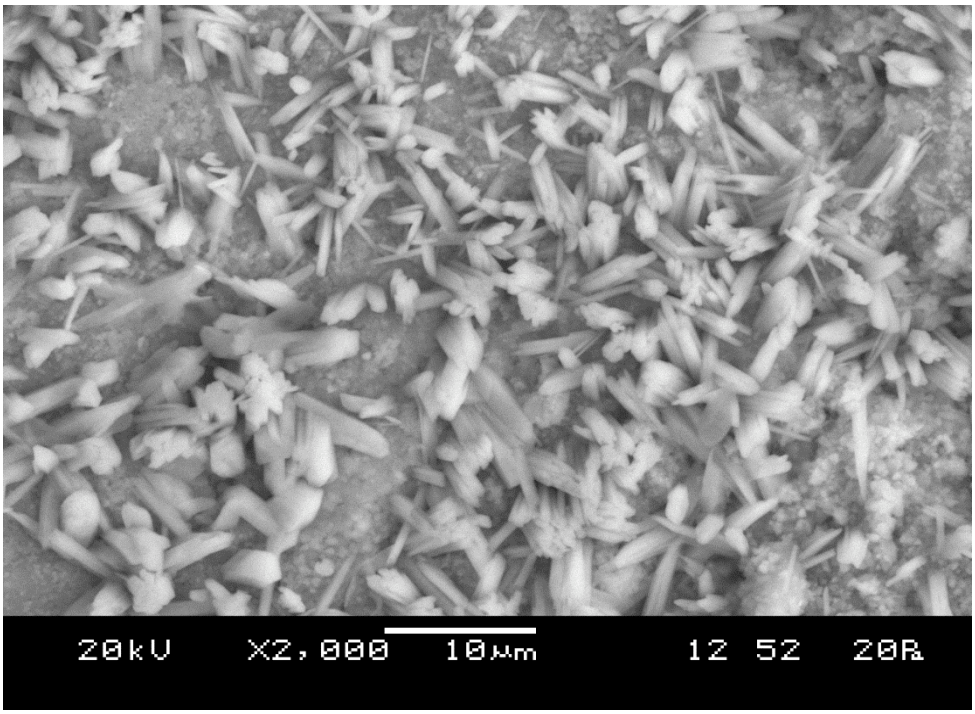

Figure 48 – Image of 20% metakaolin mix surface at 2000x magnification showing signs of crystal formation

5.3 *Growth Mediums for Halothiobacillus neapolitanus* (ATCC – 23638) (where * indicates added ingredients or modified ingredient quantity not included in original instructed medium)

5.3.1 *ATCC Medium: 290 S-6 Medium for Thiobacilli*

Ingredient	Quantity
Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgSO_4 \times 7H_2O$	0.1 g
$(NH_4)_2SO_4$	0.1 g
$CaCl_2$	0.03 g
$FeCl_3$	0.02 g
$MnSO_4$	0.02 g
$Na_2S_2O_3$	10.0 g
Agar (if needed)	15.0 g
Millipore water	1000 mL

Autoclaved at 121 degrees Celsius and remaining precipitate was shaken each day for two days to reduce precipitate amount.

5.3.2 *ATCC Medium: 290 S-6 Medium for Thiobacilli modified with LB Broth*

Ingredient	Quantity
Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgSO_4 \times 7H_2O$	0.1 g
$(NH_4)_2SO_4$	0.1 g
$CaCl_2$	0.03 g
$FeCl_3$	0.02 g
$MnSO_4$	0.02 g
$Na_2S_2O_3$	10.0 g
Agar (if needed)	15.0 g
Millipore water	1000 mL
*LB Broth	25 g

Autoclave at 121ºC. Medium will have a slight precipitate. Agitating medium a little every day for two consecutive days should diminish the amount of precipitate.

5.3.3 *ATCC Medium: 290 S-6 Medium for Thiobacilli adjusted to 1.5X concentration*

Ingredient	Quantity
*Na_2HPO_4	1.8 g
*KH_2PO_4	2.7 g
*$MgSO_4$ x $7H_2O$	0.15 g
*$(NH_4)_2SO_4$	0.15 g
*$CaCl_2$	0.045 g
*$FeCl_3$	0.03 g
*$MnSO_4$	0.03 g
*$Na_2S_2O_3$	15.0 g
Agar (if needed)	15.0 g
Millipore water	1000 mL

Autoclave at 121ºC. Medium will have a slight precipitate. Agitating medium a little every day for two consecutive days should diminish the amount of precipitate.

5.3.4 *DSMZ Medium 68: Thiobacillus neapolitanus Medium*

Ingredient	Quantity
K_2HPO_4	4.0 g
KH_2PO_4	4.0 g
$MgSO_4$ x $7H_2O$	0.8 g
NH_4Cl	0.4 g
$Na_2S_2O_3$ x $5H_2O$	10.0 g
Trace Element Solution	5.0 mL
*Chlorophenol Red	0.08 g
Agar (if needed)	15.0 g
Millipore water	1000 mL

pH was adjusted to 6.6-7.0 and $Na_2S_2O_3$ x $5H_2O$ was autoclaved at 121 degrees Celsius separately in 100 mL of Millipore water before being added to final solution and again being autoclaved at 121 degrees Celsius.

* Chlorophenol Red was used in substitution to bromocresol purple as bromocresol purple is used strictly as an indicator for pH which chlorophenol red is as well.

Trace Element Solution Ingredients	Quantity
Na_2-EDTA	50.00 g
$ZnSO_4$ x $7H_2O$	22.00 g
$CaCl_2$ x $2H_2O$	5.54 g
$MnCl_2$ x $4H_2O$	5.06 g
$FeSO_4$ x $7H_2O$	5.00 g
$(NH_4)_6Mo_7O_{24}$ x $4H_2O$	1.10 g
$CuSO_4$ x $5H_2O$	1.57 g
CoCl x $6H_2O$	1.61 g
Millipore water	1000 mL

pH was adjusted to 6.0.

5.3.5 *Thioglycollate Medium*

Ingredients	Quantity
Fluid Thioglycollate Medium	29.8 g
Millipore water	1000 mL

Powder was placed in 1L of water and boiled until completely dissolved. This solution was then autoclaved at 121 degrees Celsius. When medium was to be used if more than 30% of the solution appeared pink then solution was boiled to release absorbed oxygen.

5.4 *Growth Medium for Thiomonas intermedia* (ATCC – 15466) (where * indicates added ingredients or modified ingredient quantity not included in original instructed medium)

5.4.1 *ATCC Medium 152: Thiobacillus Medium*

Ingredients	Quantity
$Na_2S_2O_3$ x $5H_2O$	10.0 g
NH_4Cl	1.0 g
$MgCl_2$	0.5 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
$FeCl_3$	0.02 g
Yeast Extract	1.0 g
Chlorophenol Red	0.08 g
Agar (if needed)	15.0 g
Millipore Water	1000 mL

Autoclaved at 121 degrees Celsius.

5.4.2 *ATCC Medium 152: Thiobacillus Medium with LB Broth*

Ingredients	Quantity
$Na_2S_2O_3$ x $5H_2O$	10.0 g
NH_4Cl	1.0 g
$MgCl_2$	0.5 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
$FeCl_3$	0.02 g
Yeast Extract	1.0 g
Chlorophenol Red	0.08 g
Agar (if needed)	15.0 g
Millipore Water	1000 mL
*LB Broth	25 g

Autoclaved at 121 degrees Celsius.

5.4.3 *ATCC Medium 152: Thiobacillus Medium adjusted to 1.5X concentration*

Ingredients	Quantity
*$Na_2S_2O_3$ x $5H_2O$	15.0 g
*NH_4Cl	1.5 g
*$MgCl_2$	0.75 g
*K_2HPO_4	0.9 g
*KH_2PO_4	0.6 g
*$FeCl_3$	0.03 g
*Yeast Extract	1.5 g
Chlorophenol Red	0.08 g
Agar (if needed)	15.0 g
Millipore Water	1000 mL

Autoclaved at 121 degrees Celsius.

5.4.4 *Thioglycollate Medium*

Ingredients	Quantity
Fluid Thioglycollate Medium	29.8 g
Millipore water	1000 mL

Powder was placed in 1L of water and boiled until completely dissolved. This solution was then autoclaved at 121 degrees Celsius. When medium was to be used if more than 30% of the solution appeared pink then solution was boiled to release absorbed oxygen.

5.5 *Growth Medium for Thiobacillus thiooxidans* (ATCC – 8085)

5.5.1 *ATCC Medium 125: Thiobacillus Medium*

Ingredients	Quantity
$(NH_4)_2SO_4$	0.2 g
$MgSO_4$ x $7H_2O$	0.5 g
$CaCl_2$	0.25 g
KH_2PO_4	3.0 g
$FeSO_4$	5 mg
Tap Water	1000 mL

Due to sulphurs low melting point of 106.8 degrees Celsius, it was processed separately following the instructions bellow.

Step 1 – a dry 100 mL flask was obtained where 1.0 g of sulphur powder was placed. This flask was covered and autoclaved at 100 degrees Celsius for 30 minutes on 3 consecutive days. 1.0g of autoclaved sulphur powder was added to 100mL of salt solution. The salt solution was filter sterilized and poured gently down the side of the flask to allow the sulphur powder to float on the surface of the water.

5.6 *Growth Medium for Thiomonas intermedia* (DSM – 18155)

5.6.1 DSMZ Medium 35a: *Thiomonas intermedia* Medium

Ingredients	Quantity
$Na_2S_2O_3$ x $5H_2O$	5.0 g
NH_4Cl	0.1 g
$MgCl_2$ x $5H_2O$	0.1 g
KH_2PO_4	3 g
Yeast Extract	1.5 g
Agar (if needed)	15.0 g
Millipore Water	1000 mL

pH was adjusted to 5.5 – 6.0 and autoclaved. Sodium thiosulfate was added after autoclaving from a sterile stock solution that was sterilized through filtration.

5.7 *FISH Solutions*

Solutions for FISH were dependant on the bacteria used and therefore are separated into subsections below.

5.7.1 *Hybridization and Wash Buffers for Halothiobacillus neapolitanus*

Hybridization Buffer:

Ingredients	Quantity (µl)
NaCl	270
Tris/HCl	30
ddH_2O	675
Foramide	525
10% SDS	1.5

Wash Buffer:

Ingredients	Quantity (µl)
NaCl	700
Tris/HCl	1000
ddH_2O	47750
EDTA	500
10% SDS	50

5.7.2 *Hybridization and Wash Buffers for Thiobacillus intermedia*

Hybridization Buffer:

Ingredients	Quantity (µl)
NaCl	270
Tris/HCl	30
ddH_2O	1050
Foramide	150
10% SDS	1.5

Wash Buffer:

Ingredients	Quantity (µl)
NaCl	4500
Tris/HCl	1000
ddH_2O	44450
EDTA	0
10% SDS	50

5.7.3 Hybridization and Wash Buffers for *Escherichia coli*

Hybridization Buffer:

Ingredients	Quantity (µl)
NaCl	270
Tris/HCl	30
ddH_2O	600
Foramide	600
10% SDS	1.5

Wash Buffer:

Ingredients	Quantity (µl)
NaCl	460
Tris/HCl	1000
ddH_2O	47990
EDTA	500
10% SDS	50

5.8 *Email from Cedarlanes*

From: "Ryan Bratkovich (Purchasing)" <Ryan.Bratkovich@cedarlanelabs.com>
Date: 12 June, 2012 11:47:21 AM EDT
To: "lvictori@ryerson.ca" <lvictori@ryerson.ca>
Cc: "Alice Achankunju (Technical)" <Alice.Achankunju@cedarlanelabs.com>
Subject: FW: Cedarlane Order - 369648

Good Day Liberty,

Unfortunately ATCC item # 23638 has failed QC testing and has also been pushed back to late August . I have copied my colleague Alice who is our ATCC Tech. Specialist and can conduct a search for possible alternative solutions. We apologize for this inconvenience.

Regards,

RYAN BRATKOVICH

Purchasing Department, Extension 228

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