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Master of Applied Science Thesis

Phosphorus Reclamation from Municipal Wastewater Sludge for Fertilizer Production

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Summary

The increasing population with its associated rising food demand requires more agricultural fertilizers to maintain the harvest for food security. However, the natural calcium carbonate phosphate mineral (carbonate apatite) used to produce phosphorus (P) fertilizer is a non-renewable ore. Therefore, in this study, the objective is to extract the inorganic polymeric phosphate (polyphosphate: polyP) P-component in waste activated sewage sludge (WAS) from Ottawa's municipal wastewater plant (ROPEC). Once extracted, the goal was to break down the polyP to inorganic phosphate (Pi) to produce carbonate apatite.

PolyP is a P-component in WAS because a group of wastewater microorganisms called polyphosphate-accumulating organisms uptake phosphate and generate intracellular polyP stores during aerobic digestion in municipal wastewater treatment plant processes. The total acidic, oxidative P content of WAS was measured. PolyP was extracted and quantified to estimate the fraction of total P as polyP in WAS. Different polyP extraction methods were undertaken. It was determined that the complicated composition and weight fractions of TWAS, including iron phosphate, complicated polyP extraction and Pi measurement.

Lessons learned were applied towards preliminary batch and continuous precipitation of carbonate apatite with the product slurry from the anaerobic digestion process at ROPEC. Limestone was tested as an inexpensive calcium carbonate source for carbonate apatite precipitation. The dissolution of calcium and carbonate from limestone was assessed, and it was determined that further optimization is required. Preliminary work indicated that the calcium-carbonate solution from limestone could precipitate synthetic carbonate apatite from anaerobic digester material. This synthetic carbonate apatite product may lead to mitigating the impending limitations on natural carbonate apatite availability for P-fertilizer production.

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Abbreviations

cAp	carbonate apatite
CCD	charge-coupled device
d-FISH	double-Fluorescence In Situ Hybridization
DAPI	4,6-Diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence In Situ Hybridization
HAp	hydroxyapatite
HPLC	high-performance liquid chromatography
ILEC	International Lake Environment Committee
NaOH	sodium hydroxide
ND filter	neutral density filters
NIR	near infrared reflectance
P	phosphorus
PAGE	polyacrylamide gel electrophoresis
PAOs	polyphosphate-accumulating-organisms
Pi	inorganic phosphate
polyP	polyphosphate
PR	phosphate rock
psig	pounds per square inch

ROPEC	Robert O. Pickard Environmental Centre
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
Si	silicon
SNR	signal-to-noise ratio
TBO	Toluidine blue-O
TEM	transmission electron microscopy
TWAS	thickened waste activated sludge
UV	ultraviolet
WAS	waste activated sludge
WWTPs	wastewater treatment plants

Phosphorus Reclamation from Municipal Wastewater Sludge for Fertilizer Production

1. Introduction of Thesis

1.1. General Background and Motivation

Phosphorus (P) is an essential element for any living being. It is a component of bones, proteins, DNA and RNA. P is also an indispensable element in the aquatic ecosystem and is found in terrestrial deposits. This natural P mineral – phosphate rock (PR) - is also the only meaningful P source of the raw material to produce phosphorus fertilizer (Jasinski, 1999). Phosphorus fertilizer is one of the three most common agricultural fertilizers, along with nitrogen (N) and potassium (K). This P-fertilizer supply is crucial for global agricultural productivity due to the rising food production (Daily et al., 1998). The increasing human population and its growing food demand require this non-renewable and currently irreplaceable input of PR. While PR is being depleted for P-fertilizer production, it becomes a relevant subject for global food security (Abelson, 1999a). 80 - 90% of PR is extracted for P-fertilizer production for agricultural use (Childers, Corman, Edwards, & Elser, 2011). Unfortunately, the overexploitation of PR for P-fertilizer production, and the overuse of P-fertilizer can result in excess P flow into the watershed; this causes eutrophication of natural waters. To avoid eutrophication near towns and cities, nutrient-rich municipal wastewater with human and other wastes is required by meet phosphorus (inorganic soluble P, PO_4^{3-}) emission limits. Municipal wastewater treatment plants collect and treat the wastewater, separating it into water that meets the emission limits, and a P-rich solid sludge.

Globally, the P problem is a complex and long-term problem. The public is largely unaware of the non-renewable resource that is required for meeting our current food demand. If they are aware, they may not understand the impending crisis. There are inherent risks and hazards from mining PR and for fertilizer production, including the radioactivity and/or heavy metals (e.g., cadmium, uranium) that are common PR impurities (Ajmal, Bhangare, Tiwari, Sahu, & Pandit,

2014). As the world has exhausted high-grade ores, lower-grade P-ores are being mined. At one threshold, the lower-grade P-ores cannot produce P-fertilizers with existing technologies (Kirkham, 1982). The shortfall of available PR will result in the shortage of food harvest. Before this shortfall, the number of countries that export PR will decrease. Currently, most of PR is located in only a few countries (Abelson, 1999b). P-fertilizer production in Canada completely depends on imported PR and phosphoric acid.

With the objective of long-term and sustainable P-availability, strategies for P-recovery from waste for agricultural application continue to be developed in recent years. Wastewater activated sludge has been identified as an important P-rich source (Barat & van Loosdrecht, 2006; Berg et al., 2005; Takiguchi, KISHINO, Kuroda, Kato, & Ohtake, 2004), because it contains P concentrated in the solid phase, as it is the product of P removal from municipal wastewater treatment. However, in wastewater sludge, the P is in a low solubility form, usually a metal phosphate, that is unavailable for plant uptake directly. Therefore processes for extracting P to recover it in a useful form is necessary (Donnert & Salecker, 1999; 2010). Discarding the municipal wastewater sludge occurs in landfills, and sometimes it is applied to agricultural fields directly for crop production, but for food not sold to humans (personal communication, Erik Apedaile). An efficient P-extraction process could enhance the options for P recovery from municipal wastewater treatment plants, and its reuse in agriculture.

1.2. Research Objectives

The overall objective of this study is to test and evaluate the possibility of reusing P from municipal wastewater sludge as a P-rich source to produce carbonate apatite (cAp) – a calcium and phosphate-rich rock which is the major mineral component of PR that is used to produce phosphorus fertilizer (Filippelli, 2011).

In this study, P-recovery was separated into two projects: P-extraction from thickened waste-activated sludge (TWAS), and P-precipitation, in order to study each process individually. TWAS samples were obtained from the aerobic section of the Ottawa municipal wastewater treatment plant, the Robert O. Pickard Environmental Centre (ROPEC). TWAS contains a significant population of polyphosphate-accumulating-organisms (PAOs). PAOs uptake

inorganic phosphate (PO_4^{3-} , Pi), and condense them to produce intracellular stores of polyphosphate (inorganic polymers of $(\text{PO}_3)^-$ units, polyP) (Hupfer, Gloess, & Grossart, 2007). The extraction process involved the important goal of extracting P as polyP, and subsequently breaking down the polyP into soluble Pi.

The P-precipitation project, the study aims to test the possibility if cAp could be precipitated first from Pi extracted from TWAS, then from the P-rich product from anaerobic digesters. Due to the presence of iron phosphate, precipitation from anaerobic digestate required a pre-treatment with ethylenediaminetetraacetic acid (EDTA). This precipitation was accomplished through mixing the Pi-rich solutions with a calcium and carbonate rich limestone solution with increased solubility generated by dissolving carbon dioxide gas in the limestone slurry.

1.3. Thesis Organization

This thesis is organized into six chapters that are described, and their relationships connected, in Figure 1-1.

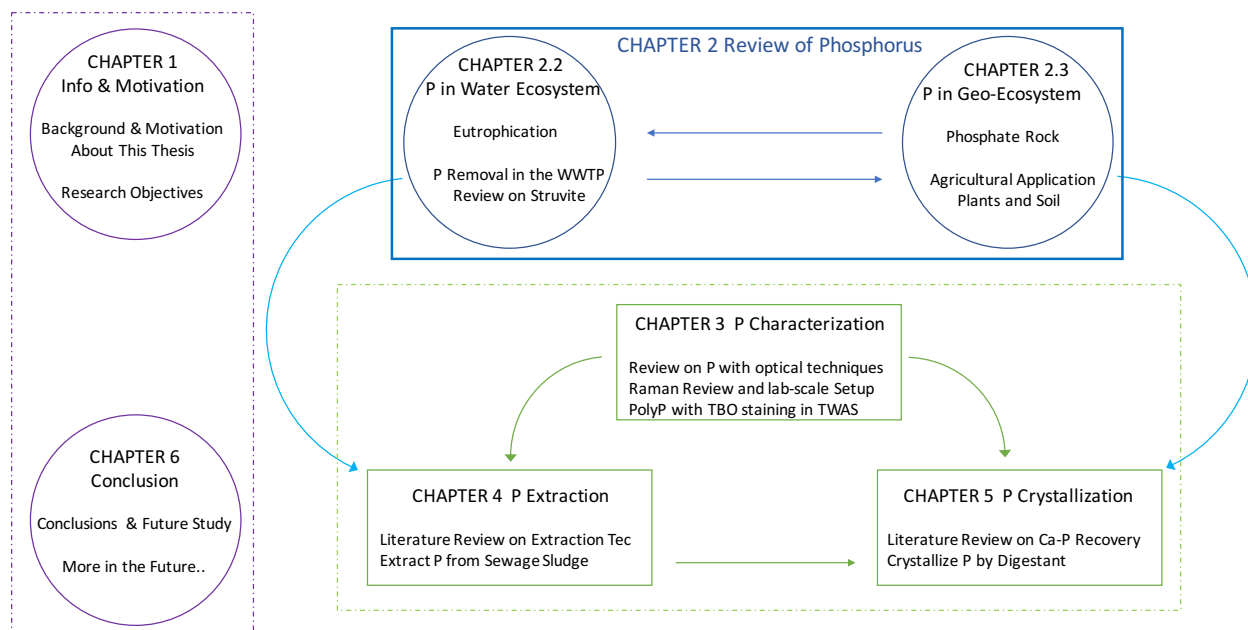


Figure 1-1 Schematic layout of this thesis based on the chapter contents.

In Chapter 2, the general background of the relevant P-subjects, including P in aquatic ecosystems, and P in agricultural ecosystems, is given without involving experimental details. It

is lamentable that future global food security will require more P fertilizer, available only from mined PR. The concept of net phosphorus is introduced, and its global accumulation explained, due to low agricultural efficiency (Bennett, Carpenter, & Caraco, 2001). Ironically, P loss on the ground diverts into the watershed, resulting in the eutrophic problem that harms the water quality, living systems and the surrounding environment.

Chapter 3 describes two related yet isolated projects in this thesis. PolyP as an inorganic polymeric phosphate is considered as a crucial P component in the waste activated sludge (WAS). Specific wastewater microorganisms called polyP-accumulating organisms (PAOs) can produce intracellular polyP in aerobic conditions. When transferred to an anaerobic environment such as anaerobic digesters found in larger municipal wastewater treatment plants, polyP is metabolized by PAO's, and the polyP breakdown product (P_i) is released from the PAO's, increasing the P_i concentration in the reactor. However, polyP identification is difficult. In the literature review for the first project, the different techniques visualizing polyP with optical microscopy are introduced. Metachromatic (turning blue to pink) Toluidine blue-O (TBO) staining was expected to be a simple tool to identify polyP in sludge extraction and crystallization processes. In the second project, the development of a lab-scale Raman spectrum setup is described. Raman spectroscopy allows for the simultaneous identification polyP and the target product of carbonated apatite during the desired reaction process (Kazanci, Fratzl, Klaushofer, & Paschalis, 2006). This setup was built in the Max Planck Centre of Extreme and Quantum Photonic (uOttawa) as one of the objectives for receiving an Eugen Lommel Scholarship.

The polyP extraction literature review and experiments are the subject of Chapter 4. The goal was to provide a preliminary study of the feasibility of utilizing P from TWAS to further produce cAp. The proposed process was to test polyP extraction from TWAS with different techniques. Once extracted, acid and heated hydrolysis would generate soluble P_i from polyP. To identify the fraction of polyP in the TWAS with respect to the total P available in the sludge, different total P (oxidative, acidic, and heated) measurements were made. Each experiment could be regarded as an isolated project to test different extraction methods under three subsections: introduction, materials and methodology, results and discussion. The comparison of the total P results from the different extracted polyP methods allows for evaluation of the polyP extraction

effectiveness despite to the uncertainly related to changes between different sewage sludge samples and storage time.

A P-recovery strategy from the anaerobic digestate from ROPEC will be introduced in Chapter 5.2. Struvite, an ammonium magnesium phosphate mineral, is one of the commercial P-recovery methods from anaerobic digestate. Unlike TWAS, where much of the P is in the form of polyP, in anaerobic digestate, most of the P is in the form of P_i . Struvite is a commercial N and P fertilizer, and its recovery from WWTPS achieved legal recognition as a P-recovered product (Chater 2.2.2.4.2). The last experimental section of this thesis explores a P-recovery strategy from ROPEC anaerobic digestate as calcium carbonate phosphate mineral. The application of cAp precipitation for P-recovery from municipal wastewater has not been studied or reported, except by Ross (Ross, 2017).

To precipitate calcium carbonate phosphate from a P-rich stream, a calcium and carbonate-containing solution is required. In Chapter 5, the same structure with Chapter 4 was used. In the experimental section, the process of mixing CO_2 -containing gases in a slurry of potable water and limestone was evaluated. The changes in pH and calcium concentration were measured, and the filtered, $Ca-CO_3$ -rich solution used in a series of precipitation experiments with EDTA-treated anaerobic digestate. EDTA treatment of anaerobic digestate was required to free the P_i from its associations with metal ions. Iron chloride is added at ROPEC to reduce the soluble P_i concentration as iron phosphate, and aluminum hydroxides are direct to ROPEC from the Lemieux potable water treatment plant. The main P- constituent of phosphate rock is cAp, which is also the main component of PR. Therefore, the desired cAp precipitate could mitigate the impending limitations on PR availability.

1.4. References

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2. Background and Literature Review of Phosphorus

2.1. Phosphorus

2.1.1. Introduction

Phosphorus (P) as a fundamental element, unlike carbon (C) and nitrogen (N), is commonly overlooked though it is essential to the life, soil and water ecosystem, food demand and waste management. Historically, attribution of identifying phosphorus is given to Hennig Brand in Hamburg. In 1669 he reported distilling P from urine (Krafft, 1969). Inorganic phosphate (Pi : PO_4^{3-}) is the mainly recognized phosphorous molecule. It circulates in the terrestrial ecosystem after dissolution from geological phosphate rock (PR). The critical use of PR is as the raw material to produce phosphorous fertilizer to maintain the harvest for our food security. Among variable PRs, the most soluble one is carbonate apatite (cAp), with an increased solubility due to its carbonate content (Easterwood, Sartain, & Street, 1989).

Sustainable phosphorus availability for food is a long-term, global problem, which requires a closing of P cycle to solve. Currently, the P loop starts from mining the natural PR for the anthropogenic demand for P fertilizer. Along the P cycle, a large amount of waste phosphorus is temporarily accumulated in municipal wastewater treatment plants (WWTPs). Instead of P loss by disposal of WWTP sludge, P recycling could be achieved by recovering P at municipal WWTPs as a usable P product. This is the main subject of this thesis. Only limited commentary on current P loss issue will be mentioned.

The achievement of P recycle by a closure of a P cycle is the final goal for sustainable P management. However, P cycles are dependent on the different circumstances of the different P states, and the anthropogenic impact. It is possible that no single schematic can represent the global phosphorous cycle. In Figure 2-1, an example of the phosphorus interconnections between the environment and humans is partially represented based on the literature. A considerable amount of PR mineral is extracted to begin this phosphorous cycle, with a low efficiency in its application and utility. This result is more clear in the review paper of global P over the last five decades, which concluded that only 45%_w of extracted PR entered the market (Minpeng Chen & Graedel, 2016).

Due to the societal and commercial pressure for efficient agricultural output, the imbalance of phosphorus between the depleted natural P-mineral resources and the excess P accumulation in the soil might be inevitable (statistic in e.g., (Bennett, Carpenter, & Caraco, 2001)). The undesirable pollution of eutrophication, caused by the emission of excess phosphorus to the watershed, results in global phosphorus emission regulations for WWTPs in the 1960s (de Haas, Wentzel, & Ekama, 2000a). The strategy for phosphorus reduction and the effects of this strategy on potential phosphorus recovery strategies will be discussed in the section 2.2.2.2. and 2.2.2.

Figure 2-2 shows a simplified historical evolution of phosphorus use in the development of P re-use and recycling. In the section of 2.2.2.4, the mineral struvite ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$) will be discussed, as it is currently the most promising P-rich, fertilizer product that is recovered from WWTPs. The general application of sewage sludge, biosolids, and other less competitive options for P recovery are presented in Chapter 5.



Figure 2-2 The schematic phosphorous evolution by the sanitation system. Source from (Gumbo, 2005) redrawn from (Ashley, Cordell, & Mavinic, 2011).

2.2. Phosphorus in Water Ecosystem

2.2.1. Phosphorus, Its Current Flows, Eutrophication, and P-Capture

Although phosphorus is a nutrient for all life forms, an excess of P in the aquatic environment also results in some visible harm to the aquatic ecosystem. Eutrophication is the reduction of dissolved oxygen in the water by the rapid growth of algal blooms (Duce et al., 2008; Hammond, 1971), reducing the water quality for other species that require this dissolved oxygen (Schindler, 1977). The International Lake Environment Committee (ILEC) estimated that 48% of lakes in North America were damaged by eutrophication (with the exception of local, near shore events) in 1994 (Günther, 2012). The uncontrolled fast-growth of phytoplankton or similar aquatic plants in the water body is the most obvious, measurable phenomenon (Smith, 1998). Marine contamination by the continental coastlines caused by fugitive phosphorus can lead to poisoning of coral reefs (Smith, 1998) and amnesic shellfish poisoning (Anderson, 1994; 2007).

Eutrophication is triggered by the concentrated disposal of inorganic phosphorus-containing molecules into the aquatic ecosystem. Unfortunately, human impact accelerates this negative process (US EPA, 2010). Many aquatic researchers and government environmental agencies have worked to reduce eutrophication events by limiting the excess input of phosphorus for several years. Moreover, as a strategy to mitigate the eutrophication, phosphorus emission limits have been mandated to WWTPs to control the effluent to the lake or sea with legislation (details presented in Section 2.2.2.2).

In Figure 2-3¹, it implied that the eutrophication might be caused by the direct and even indirect disposal of phosphorus-containing effluents from waste treatment processes. Concentrated phosphorus in municipal waste sludge that was previously emitted directly to water bodies is now concentrated in a solid form after a phosphorus precipitation processes such as insoluble iron and/or aluminum phosphate in WWTPs. However, P runoff and leachate from the soils to the aquatic environment still continue to contribute to eutrophication processes. Application of the phosphorus precipitation products from WWTPs to the ground (usually as iron phosphate) does not contribute to bioavailable phosphorus for the soil because iron and aluminum

¹ This figure was created by personal and simplistic understanding of the relationships based on the literature; the practical conditions are much more complex.

phosphates are insoluble. The creation of an insoluble phosphate mineral reduces the effluent phosphorus concentration but generates a bio-unavailable phosphorus product. This process contributes to a phosphorus-cycle imbalance. Therefore, it is necessary to design a phosphorus recovery process to guide the phosphorus in municipal waste sludge towards a useful application, such as fertilizer production.

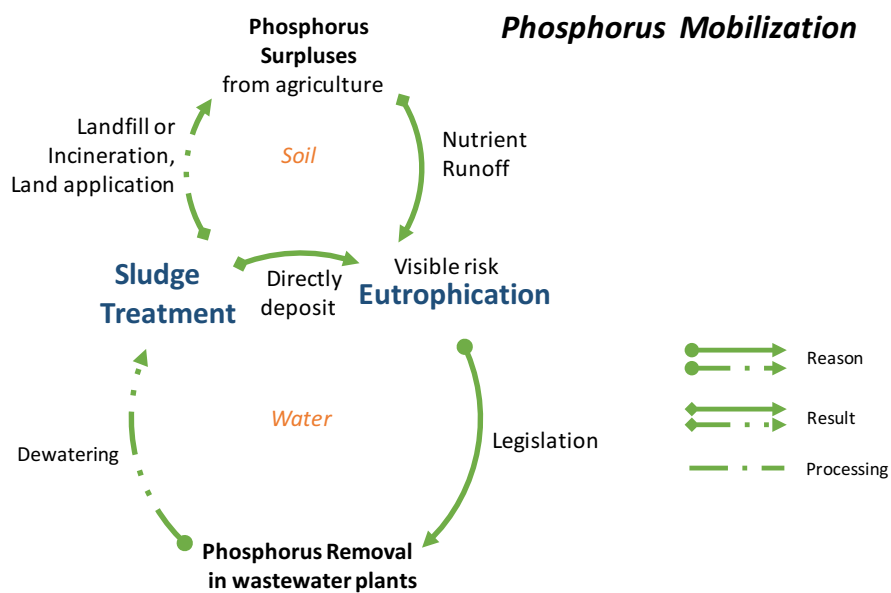


Figure 2-3 Causal relationship diagram between eutrophication and sludge treatment in the water and soil systems.

2.2.2. Phosphorus in Municipal Wastewater Treatment Plants

2.2.2.1. Introduction

One goal for the municipal wastewater treatment plants is to separate the undesirable components from wastewater, purify the water before it is recycled to the natural watershed, and to collect the undesirable materials for disposal or recycling (Shammas, Idris, Saed, Hung, & Wang, 2008). Although sewage sludge is the phosphorus-rich byproduct from wastewater treatment plants, its disposal is the most expensive and difficult task (United States.

Environmental Protection Agency, Joint US-USSR Committee on Cooperation in the Field of Environmental Protection, 1975). The sludge treatments (Figure 2-3) are usually followed by (1) disposal of the untreated sludge directly into a lake or ocean through shipping or pipeline transfer; (2) landfilling a dewatered, treated sludge, or incinerated sludge ash; or (3) other land

applications. Due to the high cost of sludge dewatering, land application is often considered to be an economical solution for wet sludge management (Kirkham, 1982).

The origins of phosphorus in municipal wastewater range from human excrement, domestic food waste, industrial and commercial waste, and synthetic household cleaners and some detergents, although some domestic detergents are currently phosphorus-free. Estimates of individual phosphorus contribution to municipal wastewater are 1.3 g P per day (Jönsson, Baky, Jeppsson, Hellstrom, & Karrman, 2005) or 2 g organic bound P per day (Günther, 2012). In WWTPs, soluble phosphate is often mixed with soluble iron chloride to precipitate the phosphate as insoluble iron phosphate. This product is a bio-unavailable, insoluble P-compound, which can be applied to soils. However, due to its insolubility, it will not re-enter the phosphorus cycle.

2.2.2.2. The History of Phosphate Removal from, and Its Characterization in Municipal Wastewaters

Excessive phosphorus is emitted to the aquatic environment due to human activity. Since the early 19th century, as the result of increasing phosphorus runoff from the soil, due to the increasing population and excessive fertilizer application, the average the natural soluble phosphorus concentration in rivers has increased approximately 4.8 times (Günther, 2012). To reduce this emission of phosphorus to the environment, phosphorus removal in wastewater treatment plants was legislated (Drynan & Commission, 1978). In 1991, the Waste Water Treatment Directive was issued in European Union countries to outline the WWTP sludge production strategies (HELCOM, 2015). The legislated goal of reducing effluent total phosphorus concentration is required to less than 1 mg/L in the P removal process (Gaterell, Gay, Wilson, Gochin, & Lester, 2000; Stratful, Brett, & Scrimshaw, 1999).

For the last several decades, engineers have continually improved the process of phosphorus removal in the WWTPs with phosphorus fixation in the sludge to restrict the amount of soluble inorganic phosphate that is released to the environment (Rittmann, Mayer, Westerhoff, & Edwards, 2011). Among these strategies, soluble P is transformed into a component of the solid sludge produced by the aerobic process². However, this phosphorus-accumulation process also

² The aerobic process at Ottawa's municipal sewage plant (ROPEC), defined it as an uncovered and deep cuboid with air sparging to settle the solids and to allow the natural wastewater bacteria, without any addition, to break down the particles. Detailed

increases the sludge volume in the solid stream, and therefore the amount of solid sludge to be disposed of.

Phosphorus is present in a range of diverse chemical compounds in municipal wastewater. The primary goal for phosphate removal is to incorporate soluble phosphate into insoluble P-containing solids and then to isolate the insoluble P-solids into wastewater sludge³. The main phosphorus-removal processing options are either chemical precipitation by adding soluble metal salt, or biological P removal by certain bacteria, such as biological nutrient removal (BNR) and enhanced biological phosphorus removal (EBPR). Moreover, the biological P removal process may be supported by the extra chemical (mainly iron salt) to achieve the higher P removed efficiency (e.g., (de Haas, Wentzel, & Ekama, 2000b; 2001b; 2001a). As a result, these processes accumulate phosphorus in the solid phase as wastewater sludge (Heinzmann, 2005).

In the chemical precipitation process, soluble phosphates in the wastewater are precipitated by the addition of soluble aluminum or iron ions (Berg et al., 2005; Donnert & Salecker, 1999; 2010). The use of iron salts (e.g., FeCl_2) is widespread because it is an economical process for reducing soluble phosphate concentration. The phosphorus-rich precipitates can be separated from the aqueous solution by flotation, filtration, or sedimentation (Valsami-Jones, 2005).

Motivated by environmental protection, biochemical P removal has attracted more recent research interest than chemical precipitation. P removal is accomplished by the generation of inorganic polyphosphate (polyP), also known as “condensed phosphates” by a series of organisms in wastewater treatment plants (BAO et al., 2007; Satoh, Mino, & Matsuo, 1992). The group of microorganisms that can accumulate polyP is called “polyphosphate accumulating organisms” (PAOs) (Hirota, Kuroda, Kato, & Ohtake, 2010; Mino, van Loosdrecht, & Heijnen, 1998). It is an impressive fact that biological P removal processes are built on the observation of biological phosphorus reduction at the scale of WWTPs, instead of research performed on pure culture tests (Serafim et al., 2002). Generally speaking, there are many microorganisms,

information is available at <http://ottawa.ca/en/residents/water-and-environment/wastewater-and-sewers/wastewater-collection-and-treatment>

³ Wastewater sludge refers to the residual and undesired semisolid after treating wastewater, it usually is considered as the byproduct from wastewater plants.

including bacteria, microalgae, and fungi that have the ability to accumulate polyP as PAOs (Hirota et al., 2010; Powell, 2009; Ye, Gan, & Hu, 2015).

Unfortunately, as previously mentioned, the P-removal efficiency of biological removal of P is still too unreliable for consistently meeting emission regulations. Many biological phosphorus removal systems must be assisted by the addition of soluble metal salts to precipitate⁴ phosphate to attain the legal effluent P-limits (de Haas, Wentzel, & Ekama, 2000b; 2001a; 2001b).

According to a German survey of municipal WWTPs of more than 100,000 population equivalent (PE), only 8.92% of EBPR processes did not require chemical precipitation as an aid in the 100 WWTPs with ERPR (182 total WWTPs) (Günther, 2012).

2.2.2.3. Phosphorus Speciation

Waste activated sludge can contain almost 12% w/w phosphorus after drying (Xie et al. 2011; Liao et al. 2005). Among them, the natural phosphorous compounds in the municipal wastewater treatment plants are orthophosphate, polyP and organic phosphate species. Figure 2-4 introduces the analytical techniques to measure each species of phosphorus.

Total Phosphorus

Total phosphorus is the sum of orthophosphate, polyP and organic phosphate components in a sample. In the experimental section of Chapter 4, viable methods were induced to measure the total phosphorus in the wastewater sludge sample based on converting all phosphorus species to inorganic phosphate via hydrolysis.

⁴ In some literature, this kind of sludge in these plants is called combined (chem-bio-) sludge.

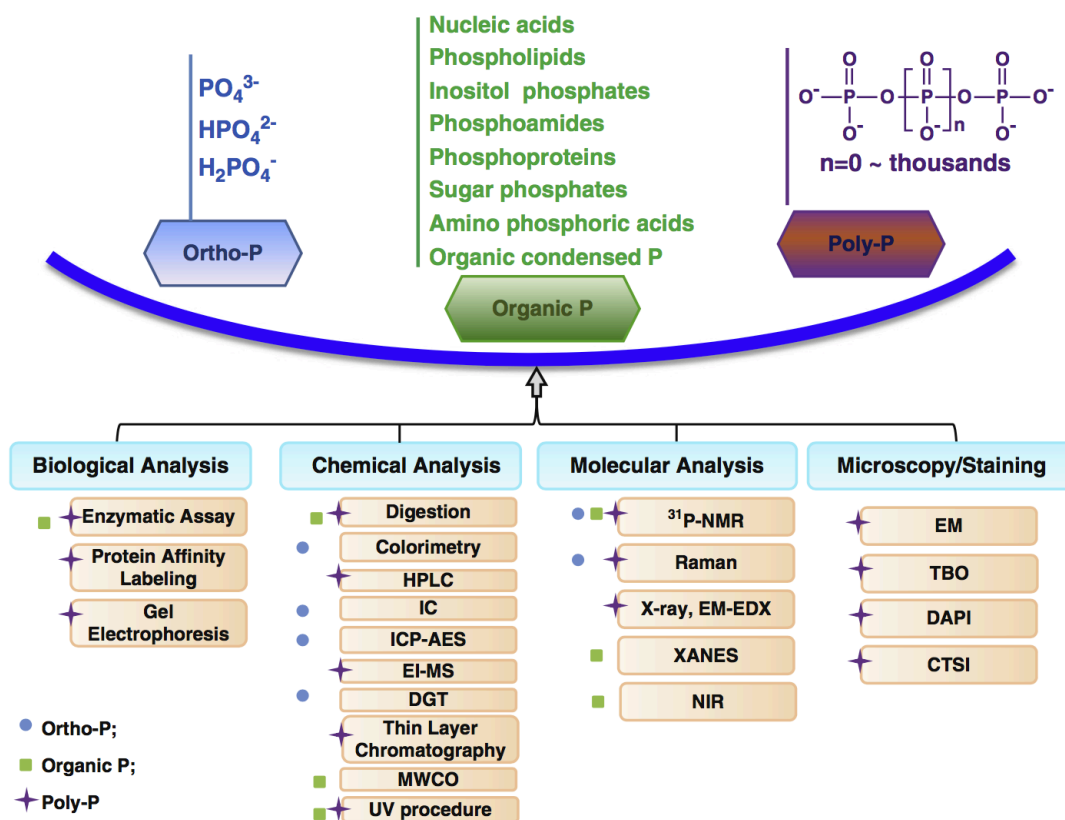


Figure 2-4 Schemes of phosphorus analytical techniques. A detailed summary table is included in Table 1 of this review paper (Majed, Li, & Gu, 2012).

Orthophosphate (Ortho-Pi)

The definition that segregates dissolved phosphate and particulate P fractions is based on the separation by filtration through a 0.45-micron membrane filter (Sedlak, 1991). Once separated, the soluble and particulate P-compounds can be defined by their response to acid hydrolysis and oxidative and acidic digestion. In this thesis, orthophosphate (Pi) is considered to be soluble phosphate, which can be measured by the colorimetric Pi assay. There are three standard protocols for orthophosphate measurement by the colorimetric assay (American Public Health Association, 2012). However, in this thesis, the measurement of the soluble phosphate concentration only is measured by the vanadomolybdate colorimetric assay (Robinson, Roughan, & Wagstaff, 1971) with modified Tomson (Tomson, Barone, & Nancollas, 1977). This is a spectrophotometric technique in which the intensity of the color in solution is proportional to the soluble inorganic phosphate concentration up to 5 mM phosphate. Triton X-100 was included as one of the reagents to solubilize proteins, and aid in the colorimetric assay (Kyaw, Maung-U, & Toe, 1985).

Orthophosphate in the Fe-P/Al-P

The most common extracellular phosphorus components in waste activated sludge is insoluble precipitates produced by the addition of soluble metal salts and sometimes precipitated without metal salt addition. Soluble aluminum or iron salts are favored for precipitating phosphorus in all chemical, and some biological phosphorus removal WWTP processes to generate insoluble iron- and/or aluminum phosphate salts. Another mechanism for Pi removal to the solid phase is complexation of Pi with iron hydroxide polymers in the sludge, as identified by X-ray coupled transmission electron microscopy (TEM) (He, Leppard, Paige, & Snodgrass, 1996). 1,200 kg of ferric chloride (FeCl_3) per day is added into the open aerobic pool to conduct chemical precipitation with soluble phosphate in the ROPEC WWTP (2017 field trip, Ottawa, ON). Iron phosphate is suspected to be the major phosphorus species in wastewater sludge (Ross, 2017).

Regarding the financial feasibility and improved phosphorus removal efficiency, chemical precipitation of phosphorus by the addition of metal salts is preferred for the wastewater plants instead of the biological process of P removal. However, due to their insolubility (Figure 2-5), P precipitates produced in WWTPs with soluble metal (Fe, Al) addition are not effective phosphorus fertilizers for plant uptake (Morse, Brett, Guy, & Lester, 1998; Weimberg & Orton, 1965). This low solubility restricts the working efficiency for P re-use from sludge with Fe-P or Al-P components. Aluminum toxicity also limits the volume of Al-based precipitation products that can be applied to agricultural soils (Hue, Craddock, & Adams, 1986; Matsumoto, 2000). The literature suggests that plants may be able to acquire phosphate from iron phosphate, such as insoluble iron-containing triple superphosphate ($(\text{Ca}(\text{Fe})\text{H}(\text{PO}_4)_2 \cdot 4(\text{H}_2\text{O}))$) (Frazier & Lehr, 1967), by their particular biological and enzymatic processes, instead of the passive iron phosphate solubility in the soil (Johnston A E & Richards, 2003a).

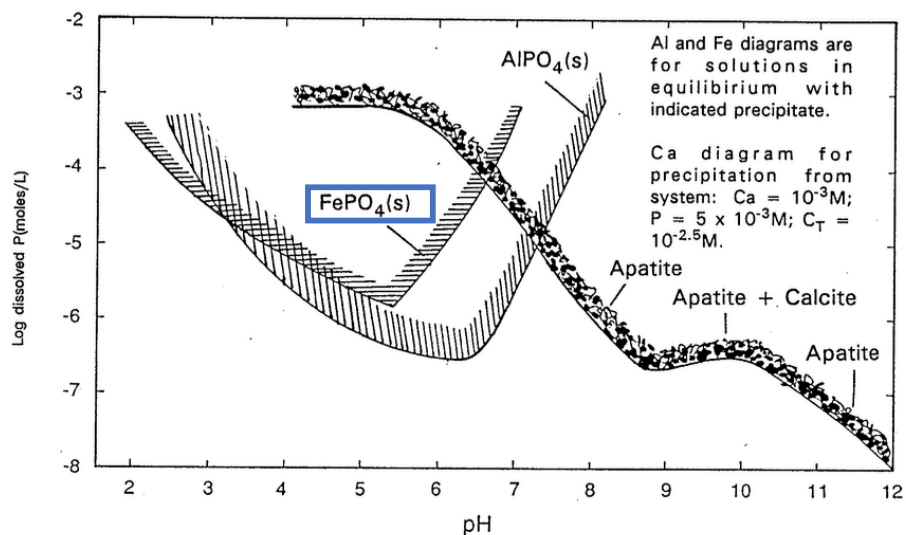


Figure 2-5 The principle of Fe-P and Al-P formation in the chemical phosphate removal process (Sedlak, 1991).

Polyphosphate and PAOs

PolyPs are multiples orthophosphate monomers bound with a phosphoanhydride bond (Keefe & Miller, 1995), as a linear or ring shape (Schlegel, 1993), and with a variable degree of polymerization (Kulaev, 1975; Kulaev, Vagabov, & Kulakovskaya, 2005). Intracellular polyP is also known as “volutin” or “metachromatic” granules (Harold, 1966) due to its first discovery in *Spirillum* solutions (Schlegel, 1993).

Polyphosphate accumulating organisms (PAOs) in WWTPS absorb and accumulate inorganic phosphate within their biomass by condensing P_i to polyP as an energy storage at the aerobic zone; they incorporate large amounts of inorganic phosphates as intracellular condensed polyPs to survive under the oxygen-lean, anaerobic conditions in some WWTPs (Driver, Lijmbach, & Steen, 1999; Mulkerrins, Dobson, & Colleran, 2004; Serafim et al., 2002). This phosphate accumulation as polyP in wastewater activated sludge generates its largest phosphorus pool (Röske, 1994; Serafim et al., 2002; Uhlmann, Röske, Hupfer, & Ohms, 1990).

The models of biological P removal are based on efficient operational observations and the assumption that PAOs are responsible for the reduction of dissolved phosphate concentration. Researchers have isolated the hypothetical PAOs strains to analyze their metabolism for understanding the biological P removal process. *Acinetobacter spp.* is accepted as one of the

PAO significant strains in the sludge (Fuhs & Chen, 1975; Van Groenestijn, Bentvelsen, Deinema, & Zehnder, 1989). Some publications reported that *Acinetobacter spp.* have not exhibited the essential characteristics that correspond with those assumed to be crucial for PAOs, and they might act on behalf of the EBPR sludge (Cloete & Steyn, 1987). Other microbial strains that have been separated from bio-sludge of EBPR (Kawaharasaki, Tanaka, Kanagawa, & Nakamura, 1999) have not displayed the complete functionality of the ideally proposed PAOs, such as *Lamproedia spp.* or *Microthrix phosphovorans* (M. M. Santos, Lemos, Reis, & Santos, 1999).

Organic Phosphorus

Organic phosphorus is assumed to be the minor phosphorus species in wastewater activated sludge, though it is considered to be a significant phosphorus component in the phosphorous wastewater pool (Rittmann et al., 2011). Generally, organic phosphorus can be categorized as “non-reactive” and/or “non-bioavailable”, even though its definition and the associated organic forms can be complicated (McKelvie, 2005). The roles of organic phosphorus are commonly underrated due to the difficulty in their characterization, largely because analytical identification is not possible with the colorimetric or chromatographic assay. It has been reported to as soluble reactive P as the result of overestimating of soluble P_i by colorimetric assay (Brandes, Ingall, & Paterson, 2007). The possible transformation reactions in the sewage sludge that would generate inorganic phosphate from organic phosphate species are also not generally considered.

2.2.2.4. P Recovery for Agricultural Production from Municipal Wastewater Treatment

2.2.2.4.1. Introduction

Struvite (magnesium ammonium phosphate, $MgNH_4PO_4 \cdot 6H_2O$) is the most promising technology to produce P-recovered fertilizer from municipal WWTPs (Durrant, Scrimshaw, Stratful, & Lester, 1999; Schröder, Cordell, & Smit, 2010). Currently, struvite spontaneously precipitates in the anaerobic systems at some WWTPs (Booker, Priestley, & Fraser, 2010; Ohlinger, P E, Young, & Schroeder, 1999; Stratful, Scrimshaw, & Lester, 2001; Williams, 2010), especially if there is a sufficient divalent magnesium ion (Mg^{2+}) content (Bouropoulos & Koutsoukos, 2000; Doyle & Parsons, 2002). Although it has a relatively recent research and

production history, struvite production from WWTPs has gained interest as a P-recovery option over the last decade.

Struvite offers a possible, economically available P-recovered product with a potential market value, as its efficiency equates with monocalcium phosphates (MCP, $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$) as a fertilizer (Johnston A E & Richards, 2003b). Researchers have also worked to prove the effectiveness of struvite as a fertilizer in comparison with conventional phosphorus fertilizers (Ghosh, Mohan, & Sarkar, 1996; Johnston A E & Richards, 2003a; Münch & Barr, 2001; Rothbaum & Rohde, 1976), such as di-ammonium phosphate (de-Bashan & Bashan, 2004) or superphosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$). As a slow-releasing N/P-fertilizer, struvite should not damage plants, and should not dissolve so rapidly as to contribute to P- and N-rich runoff to the watershed even if its application is overdosed (Johnston A E & Richards, 2003a; X. Z. Li & Zhao, 2003; Münch & Barr, 2001).

Struvite also offers other potential uses, such as a component in fire-resistant panels, in cement (Sarkar, 1990; Schuiling & Andrade, 2010), detergents (A. Kumar, Das, Goel, & Kumar, 2013) and cosmetics (Zhang, Bowers, Harrison, & Chen, 2010), and animal feed (Gaterell et al., 2000). Even if the acceptable feasibility of P-recovered fertilizer from WWTPs is still in question, struvite and other two phosphorus-recovered fertilizers (magnesium phosphate and dicalcium phosphate (DCP)) have been recognized by a Dutch fertilizer law ⁵. This may imply that other countries might follow, and accept P-recovered fertilizer, and possibly other products from WWTPs for re-use. Struvite precipitation from anaerobic digester systems has been conducted on the bench and pilot scale, and it was also brought to full-scale trials more than a decade ago in the Netherlands (Giesen, 2010) and Italy (Battistoni, Boccadoro, Fatone, & Pavan, 2005). At that time, Japan allowed the use of struvite produced from WWTP processes by their agricultural industries (Gaterell et al., 2000; Ueno & Fujii, 2001). Details with input/product data and global P recovery overviews are available in the comprehensive review paper by Desmidt et al., (Desmidt et al., 2014) and a summary table by P-REX (Kabbe, 2017).

⁵ News available at Overheid.nl (the central access point to all information about government organizations of the Netherlands) <https://zoek.officielebekendmakingen.nl/stb-2014-543.html>

2.2.2.4.2. Struvite Formation

When its solubility is exceeded, struvite forms as scale deposits in the pipes that transfer liquids in anaerobic digestion processes (Aage, Andersen, Blom, & Jensen, 2005; JR & B, 1982; Taylor, Frazier, & Gurney, 1963; Webb & Ho, 1992). The higher pH values and the higher Pi concentrations in the anaerobic digester processes can produce the struvite by increasing its supersaturation (Lim, Kim, Shin, Okuda, & Okada, 2004). As the higher phosphate levels and ammonia generation from urea and other organic nitrogen sources usually occur in the anaerobic digestion process (Ward, 2013), the pipes which transport the sludge from this process and the reactor surfaces, accumulate struvite scale.

Unfortunately, these struvite deposits on the pipes and reactor surfaces can be removed only by shutting down the process and physically removing the scale, or by its dissolution with sulfuric acid (Durrant et al., 1999; Stratful et al., 2001; Williams, 2010). Struvite precipitated as undesirable scale deposits on the inside of anaerobic operations for 35 years (Dhir, Limbachiya, & McCarthy, 2001), before it was considered to be a potentially valuable commodity produced by the WWTP process (Mohajit, Bhattarai, Taiganides, & Yap, 1989). Struvite can spontaneously precipitate under basic pH conditions if dissolved phosphate, magnesium, and ammonium ion are present in municipal wastewater above its saturation limit. With a dedicated struvite crystallization reactor, a higher purity struvite can be generated by the products of the anaerobic digestion process.

During struvite precipitation, equivalent dissolved molar ratios of Mg^{2+} , NH_4^+ and PO_4^{3-} are reduced. Struvite precipitation process also reduces the amount of total suspended solids, and the solution pH if the struvite precipitation occurs at a $pH > 7.5$ (Booker et al., 2010; Doyle & Parsons, 2002; Williams, 2010). Sodium hydroxide (NaOH) can be added to control the pH (Stratful et al., 2001). As many municipal wastewater streams do not have a magnesium ion concentration high enough for struvite precipitation, low-priced, soluble Mg^{2+} salts are usually added into the ammonium-rich solutions in anaerobic digesters to maintain the molecular ratios and exceed the struvite solubility product as required (Chimenos et al., 2003; Lee et al., 2003; Münch & Barr, 2001; Nelson, Mikkelsen, & Hesterberg, 2003; Van Rensburg, Musvoto, Wentzel, & Ekama, 2003). Air stripping removes CO_2 from the aerobic digestion process fluids and elevates the pH; this can also induce struvite crystallization (Battistoni et al., 2005;

Battistoni, Fava, Pavan, Musacco, & Cecchi, 1997; Kalyuzhnyi et al., 2003). By selecting for certain adsorbents, ion exchange can also be used to form struvite (Petrizzelli et al., 2003). A review of a comparatively complete design for the chemical equilibrium models and optimal conditions of the struvite precipitation process is discussed for both synthetic and practical wastewaters by Gaddekar and Pullammanappallil (Gaddekar & Pullammanappallil, 2010).

2.2.2.4.3. The Problems Associated with Struvite Formation

The production of struvite as a phosphorus-recycled mineral from WWTPs has some limitations. There are additional maintenance and energy costs for the additional crystallization process, the operational cost of the magnesium chloride reagent and the uncertain market value of struvite fertilizer adds uncertainty to the status of struvite as an optimal product for recycling phosphorus from WWTPs. Recent publications voice doubt if struvite produced by WWTPs can disseminate globally as a fertilizer, due to constraints with its practical availability and economic feasibility (Shu, Schneider, Jegatheesan, & Johnson, 2006). Currently, struvite precipitation is available as a commercial process to remove phosphorus from WWTPs, which also produces a N/P-fertilizer as a product.

Struvite production removes nitrogen as well as phosphorus from solution. The optimal mole ratio of N/P is about 5/1 - 10/1 in wastewater plants (Gaterell et al., 2000; Winkler, 2005), but the observed ratio is usually slightly lower in operational WWTPs (Ammery, 2004). Even if an increasing number of researchers promote the biological phosphorus removal process as well as struvite production for P- removal and recovery WWTPs, the full-scale struvite production processes are also not widespread is that the most widely applied, simple, and the inexpensive P-removal process is chemical precipitation by iron (Donnert & Salecker, 1999; 2010). The soluble iron salts added to the WWTP process can inhibit the precipitation of struvite (Mamais, Pitt, Cheng, Loiacono, & Jenkins, 1994) through the removal of free phosphate as insoluble iron phosphate (Figure 2-5). However, even without the addition of soluble iron salts, the dissolved calcium in the wastewater can also inhibit the crystallization of struvite (Kabdaszli, Parsons, & Tünaya, 2006; Le Corre, Valsami-Jones, Hobbs, & Parsons, 2005).

2.3. Phosphorus in Agricultural Ecosystems

2.3.1. Introduction

With the increase of global population, phosphorus-rich mineral extraction is increasing to meet the demands for phosphorous fertilizer and animal feed supplement production. Phosphate rock (phosphorite) reserves are dwindling as a non-renewable, natural resource (Cordell & White, 2014; Herring & Fantel, 1993; Kirkham, 1982; Mayer et al., 2016; Schröder et al., 2010; Villalba et al., 2008). Without any alternative option for phosphorus fertilizer production, these geological phosphate rock deposits are recognized as the only significant reserves as the exclusive feed material of phosphorous fertilizer production (Jasinski, 1999). After mining, concentration, and shipping to the phosphorus fertilizer production facilities, phosphate rock is dissolved in an acid treatment to produce P-fertilizer (Figure 2-6).

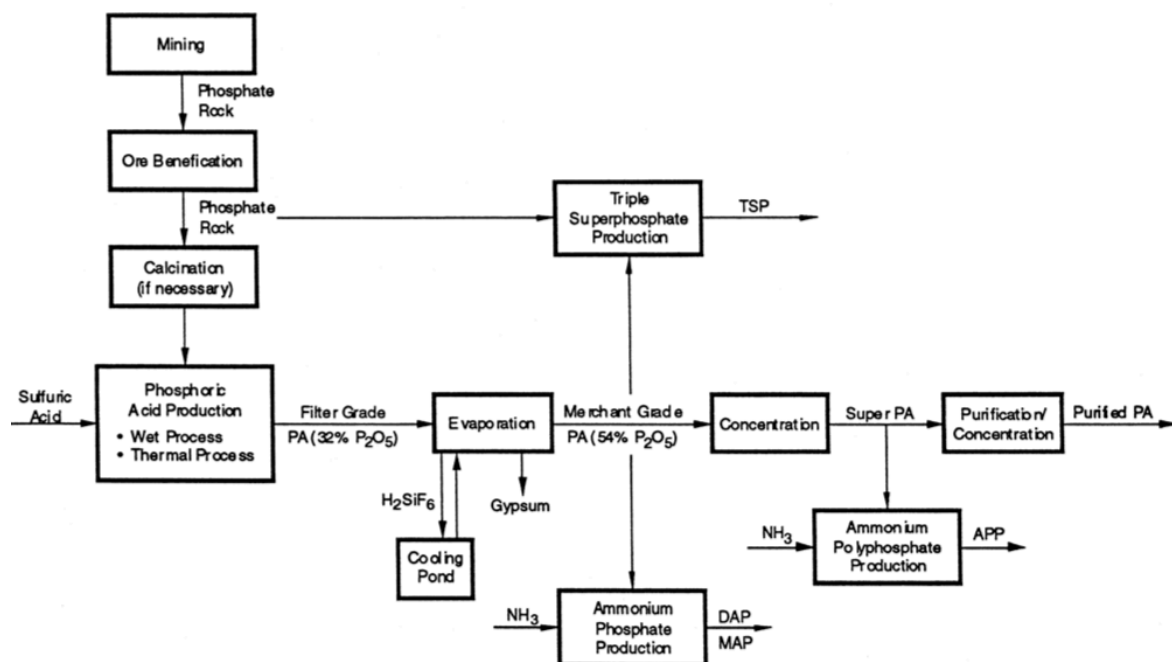


Figure 2-6 The process of phosphorous fertilizer production after phosphoric acid manufacturing (U.S. Environmental Protection Agency 1993).

The rising demand for phosphate rock and the concurrent accumulation of P in the soil sound paradoxical, but this relationship has been measured and reported as a mass balance estimate of total global phosphorus. The driver of this phenomenon is the application of phosphorus fertilizer to increase the crop production. Because of declining availability of higher grade natural

phosphate-rich ores ($>22\%$ P_2O_5 (Government of India, Ministry of Mines, Indian Bureau of Mines, 2015)) for fertilizer production, the lower grade minerals ($10\%-12\%$ P_2O_5 (Government of India, Ministry of Mines, Indian Bureau of Mines, 2015)) are also extracted to meet the market demand. Not all of phosphorus-containing minerals are available for fertilizer production because, below a given grade of (5% P_2O_5) phosphorus content, current industrial techniques to produce phosphorus fertilizer are insufficient (Kirkham, 1982). Lower grade ore also requires that larger ore volumes are mined, more energy is used, and more waste is produced to concentrate the ore.

Careful management of phosphorus fertilizer application is required not to have an excess of phosphorus build up in soils. The problems of phosphorus accumulation in soils and concomitant reduction of phosphate rock ore could be mitigated by better management of reducing the supply and consumption of this natural resource. However, another useful feedstock material for phosphorus-fertilizer production could be produced with waste phosphorus to continue to supply of phosphorus for food production.

While the total measured soil phosphorus may be very high, the availability of phosphorus for plant absorption depends on the solubility of P species in the soils (Richardson, Hocking, Simpson, & George, 2009). The phosphorus transportation between soils and plants and soils is very complicated, so it is not reviewed in this thesis. A recent book reviews the literature on this important subject (Selim, 2015). Improving the “phosphorus use efficiency” (PUE) should be prudently evaluated for high-efficiency agricultural management in the future (Cordell & White, 2014; Karunanithi et al., 2015). PUE refers to the yield of grain per available soil-P (Parentoni & Souza Júnior, 2008)⁶ or the weight of dry matter per P-uptake (Payne, Hossner, Onken, & Wendt, 1995). To achieve the target of increased PUE, phosphorus recovery from waste and recycling it back into the phosphorus use chain to close the P cycle is an option to increase phosphorus sustainability. The annual PUE is currently thought to be less than 50% for crop production, and approximately 20-30% for grassland (Richardson et al., 2011).

⁶ In this paper, the crop production of PUE is noted to be equal to available plant exports per total P input.

2.3.2. The Raw Material of P-fertilizer: Phosphate Rock

In general, the most of the natural phosphorus minerals on Earth are formed as calcium phosphate, of the “apatite” mineral group (Quade, 2003). Among the large and complex apatite group, $(\text{Ca}_5(\text{PO}_4)_3(\text{F}, \text{OH}, \text{Cl}))$ represents the most common formula because either one or more of F-, Cl- or OH- could be the general anion(s) substitutions (McConnell & Howie, 1974; Omelon, Ariganello, Bonucci, Grynpas, & Nanci, 2013). Another common substitute is carbonate, which is common in carbonate fluorapatite (CFA) (Crosby & Bailey, 2012). The general definition of apatite allows for other cation substitutions as well, but this work will focus on apatite with a majority of calcium cation content. Unfortunately, the variable chemical forms apatites often result in difficulty analyzing and identifying this important mineral group (Omelson et al., 2013).

Specifically, there are two categories of natural phosphorus reserves: apatite and phosphorite, which vary in chemistry and geology (Knudsen, Ewing, Gunter, & Wang, 2002; Omelon & Grynpas, 2008; Piccoli & Candela, 2002). Apatite, as the low-solubility igneous rock on the terrestrial lithosphere, is a low-grade P-deposit (Horn & Adams, 1966), which can be concentrated by mineral beneficiation techniques. The other, more desirable phosphorus ore is high-grade phosphorite, which has a sedimentary origin (Filippelli, 2011; Horn & Adams, 1966; J. Wazer, Wazer, Campanella, & Campanella, 1950), and a minimal 9 weight percent phosphate (Bailey et al., 2013).

The exploration for unknown phosphate rock mines continues, as the natural geologic phosphorous rock is in demand, and is a non-renewable resource with unequal global distribution (Figure 2-8). The known global phosphate rock reserves are predicted to be consumed within the next 100 years if there are no new substitutes or another process for capturing and reusing phosphorus (D. F. Andrade & Pereira-Filho, 2016; Zapata, Roy, Food and Agriculture Organization of the United Nations. Land and Water Development Division, International Atomic Energy Agency, 2004). The statistical details of the phosphate rock distribution, the different natural apatites ores, and their production are subject to many reviews (Cook, Cook, & Shergold, 2005; Notholt, Sheldon, & Davidson, 2005). According to the industry statistics (Cordell et al., 2009; Jasinski, 2013), it is conservatively estimated that global “peak

phosphorus”⁷ may occur in 2033 (Ziadi, Whalen, Messiga, & Morel, 2013) or 2035 (Cordell et al., 2009). These estimates assume that the demand for phosphorus fertilizer will be achieved by processing natural phosphate rock of lower phosphorus concentration. However, this prediction of P-peak is disputed based on the estimation method and over-simplifications (Jackson & Mew, 2015; Vaccari & Strigul, 2011).

Regardless of precision, the studies of history (Ulrich & Frossard, 2014), and the published statistics and predictions on phosphate rock availability would encourage and reinforce attention to and action for phosphorus sustainability. The predictions for “peak phosphorus” are not in the very short-term (Scholz & Wellmer, 2013). However, it is clear that without sustainable P management, the global food supply will be vulnerable in the long-term because of the scarcity of phosphate rock mineral, the low efficiency of its use, as the loss to the environment (Scholz & Wellmer, 2013).

Regardless of the approaching estimated “peak phosphorus”, phosphorite reserves have depleted in Canada after the closing down of phosphate rock mine in Kapuskasing (ON, CA) in 2013 (Ednie, 2000). With this mine closure, phosphate rock is now shipped to Canada from Western Sahara. This causes an increased cost and negative environmental impact by this international transportation of phosphate rock for Canadian P-fertilizer production.

For a perspective of the numbers, phosphate rock production and reserves are represented in Figures 2-7 and 2-8. They suggest that it would be worthwhile to direct attention to the possibility of recovering waste phosphate in a form that would be useful to phosphorus fertilizer producers, to ease their reliance on phosphate rock as the only feedstock material.

⁷ “Peak resource theory (based on empirical evidence) postulates that a critical period (the so-called ‘peak’ in the production of the commodity) will occur long before 100% of the reserve is theoretically depleted.” (Cordell and White, 2011).

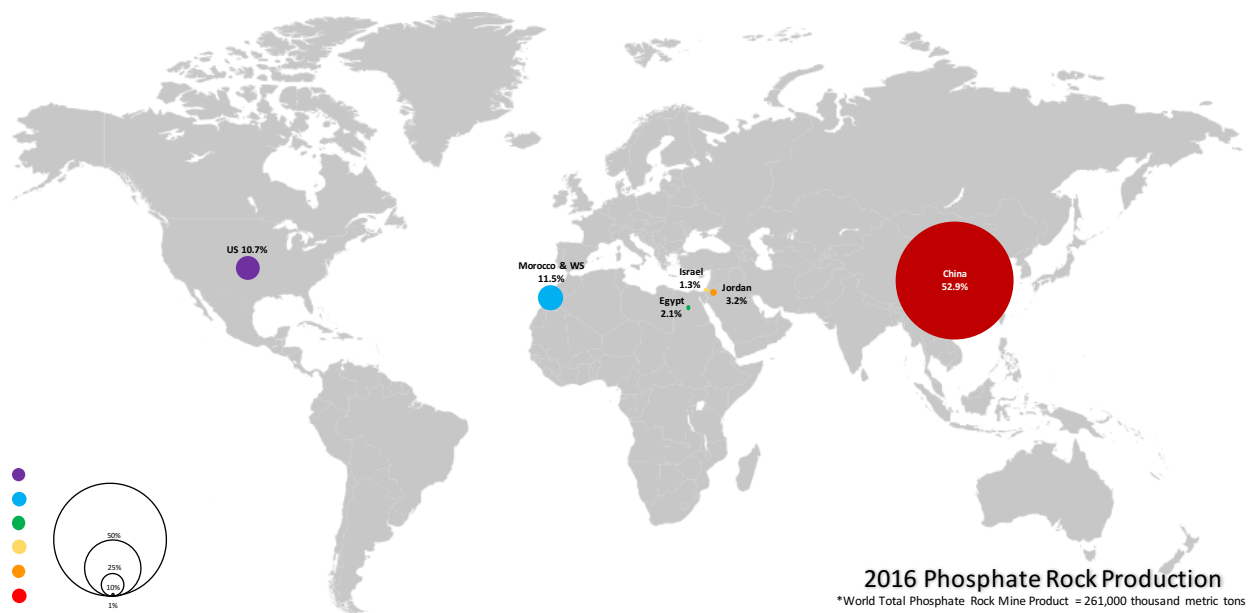


Figure 2-7 The schematic global map with phosphate rock production in 2016 (Data: (USGS National Minerals Information Center, 2017)).

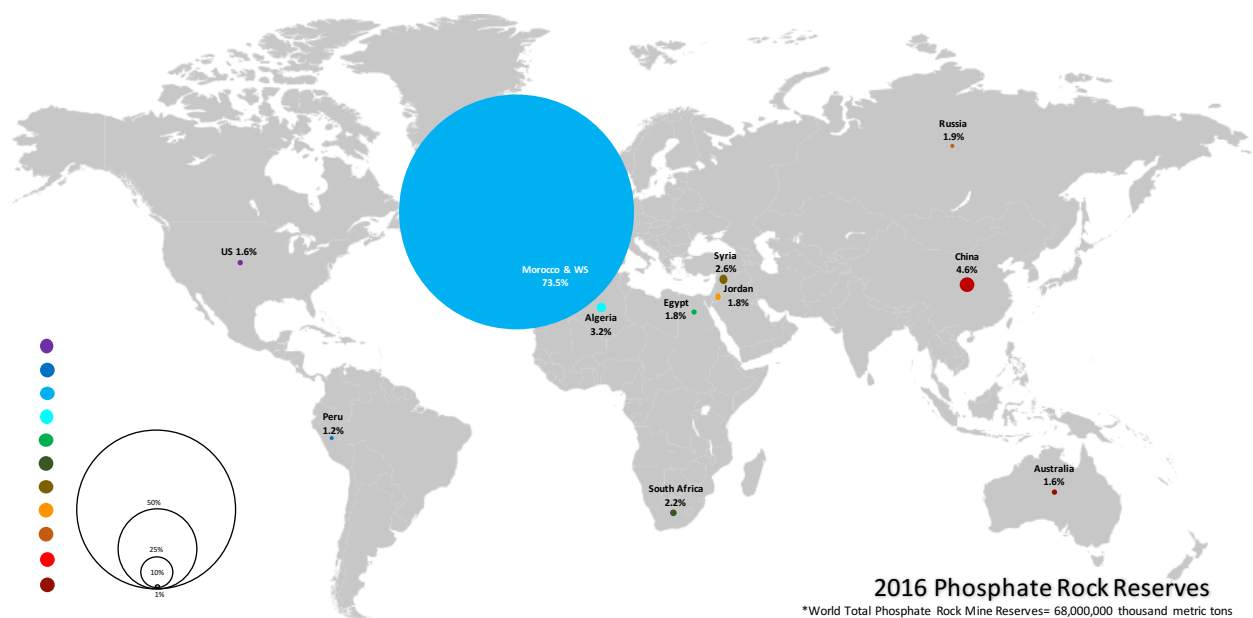


Figure 2-8 The schematic global map with phosphate rock reserves in 2016 (Data: (USGS National Minerals Information Center, 2017)).

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3. Polyphosphate Identification by Optical Microscopy and Raman Spectrometry

3.1. Introduction

It is challenging to identify polyP in samples. One technique is to stain polyP with a dye and use optical microscopy as a visual identification tool. This technique is used to identify and qualitatively measure intracellular polyP granules in life forms, and in PAOs (Hupfer, Glöss, Schmieder, & Grossart, 2008; Majed, Li, & Gu, 2012b; Serafim et al., 2002). Section 3.2 is a review of the microscopic polyP identification techniques. The use of one of the polyP stains (Toluidine blue-O: TBO) to identify polyPs in ROPEC municipal waste activated sludge is described in Section 3.2.2.

The use of Raman spectroscopy to identify polyPs is reviewed in Section 3.3. A description of the preliminary work undertaken to design, build, and test a lab-scale Raman spectrum is outlined in Section 3.3.3. This work involves a detailed optic components settings, which is presented in Section 3.3.3.2, and preliminary Raman spectrum collection in Section 3.3.3.3.

In this study, the main objective was the construction of a new Raman system, not to characterize an unknown sample at this stage due to practical restrictions. The objective was to build a Raman system with optic components in the laboratory. The design and construction were desired to be flexible to achieve the specific requirement based on the sample types. The P-O-P bond of polyP ($1145\text{--}1175\text{ cm}^{-1}$ (Omelon, Georgiou, Variola, & Dean, 2014)) in the waste activated sludge is the final desired sample for this setup. This chapter reviews the optical and Raman options for polyP identification, and presents the preliminary work performed to design and build a Raman spectroscopy system, starting with a few individual optical parts.

3.2. Polyphosphate Identification with Optical Microscopy

3.2.1. Introduction and Literature Review

High concentrations of polyP were identified within waste activated sludge bacteria by positive Neisser staining (Uhlmann, Röske, Hupfer, & Ohms, 1990) within volutin granules, which are known to include rich polyP (Widra, 1959). In fact, the metachromatic cationic stains of TBO, Neisser and Loeffler's Methylene Blue have a similar affinity for anionic volutin granule contents, including polyP, and are required to visualize the intracellular polyP contents with optical microscopy. These stains also interact with other anionic polymers, such as nucleic acids (Ohtomo, Sekiguchi, Kojima, & Saito, 2008) and are more efficient at labeling longer chain of polyP, with more than 10 PO_3^- mer-units of length (Hupfer et al., 2008).

TBO is an important and useful stain for identifying polyP. Although it is not specific for polyP, TBO is used as a fast and simple technique to identify polyP (Gu, Saunders, Neethling, & Stensel, 2008; He, Gu, & McMahon, 2008; Majed, Chernenko, Diem, & Gu, 2012a). This staining was used in this thesis to be expected to roughly but qualitatively conjecture the success of the polyP-extraction techniques examined in this work. This cationic TBO binds to the negatively charged polyP to specifically dye the polyP or polyP-liked granules instead of the background cell (Bartholomew, 1981). However, TBO staining with other non-polyP negatively charged compounds was suspected to be minimized by staining at acidic pH, which protonates many species (Ashford, Lee, & Chilvers, 1975).

Other stains than TBO, such as Neisser (Figure 3-1 (a),(b)) and Loeffler's Methylene Blue stains (Figure 3-1 (c)) were shown to identify the intracellular polyP storage pools found in WWTPs (Lindrea, Seviour, Seviour, Blackall, & Soddell, 1998). However, the interpretation of Loeffler's Methylene Blue as a polyP-specific stain was considered to be untrustworthy, as it also stains filamentous bacteria (Lindrea et al., 1998). Besides, it might not be an effective stain for shorter chain polyPs (Bartholomew, 1981). A review paper on the use of Methylene Blue to identify polyP by Rees et al. (Rees, Vasiliadis, May, & Bayly, 1992) is in agreement with a more recent review of this technique (Serafim et al., 2002).

Although Fluorescence In Situ Hybridization (FISH) and its associated techniques had not been undertaken in this project, their use may provide more desirable results than general

metachromatic cationic stains to identify polyPs within PAOs (Figure 3-1 (d) and (e)). The phylogenetic discovery of PAOs was undertaken with DAPI-FISH (DAPI: 4,6-Diamidino-2-phenylindole) staining for products from an enhanced biological phosphorus removal (EBPR) process (Kawaharasaki, Tanaka, Kanagawa, & Nakamura, 1999). A modified applications of the double-Fluorescence In Situ Hybridization (d-FISH) with DAPI for polyP staining have been reported (N. Lee et al., 1999; Liu et al., 2001).

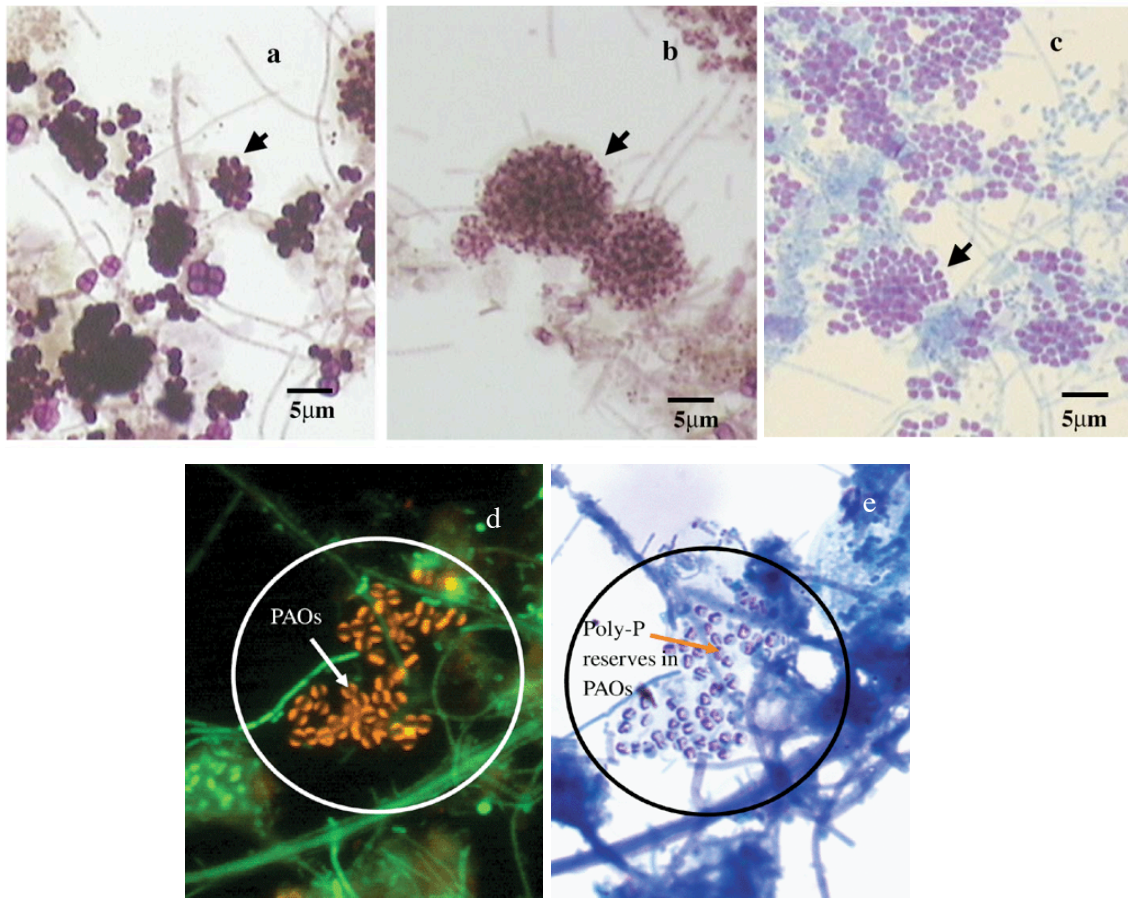


Figure 3-1 Microscopic images with certain strains of polyP and PAOs in the municipal sludge. (a,b) Neisser staining (x1000) at the end of aerobic and anaerobic digestion at EBPR, respectively. The black arrow indicates the PAOs cluster. The dark purple of cells displays the numerous polyP-rich granules. (c) Loeffler's methylene blue staining (x1000) at the end of aerobic digestion at EBPR. The black arrow indicated the violet-dyed polyP on the sky-blue colored of cells. (d,e) FISH staining shows that the presence of PAOs and polyP within the PAOs (Ginige, Lashkajani, & Cheng, 2013). (Images (a)-(c) adapted from (Serafim et al., 2002) and (e) and (f) from (Ginige et al., 2013).)

Moreover, the complicated task of quantifying microscopic measurements of waste activated sludge with optical staining or chemometric analytical techniques is summarized in the literature (Mesquita, Amaral, & Ferreira, 2013). A comprehensive summary table of the specificity of each

technique for polyP and PAOs identification is in the review work by Tarayre *et al.* (Tarayre *et al.*, 2016).

3.2.2. Polyphosphate Staining with TBO

3.2.2.1 *Material and Methodology*

The TBO staining solution preparation was based on the technique of Hirschman (Hirschman, 1967). 1% Toluidine blue (TBO) (Sigma® CI52040; Basic blue 17) was prepared as the stock solution. 0.01% TBO was created by diluting 100 times of the stock solution in 0.01 M acetate buffer to 100 mL at pH 4 with distilled and deionized water as the working solution.

TBO staining was applied to the waste activated sludge sample for 15 min. If the stained sample became blue instead of violet color during the waiting time of 15 min, a new sample with a higher dilution was prepared for staining. 10 μ L of the stained sample with TBO was transferred by pipette to a glass slide with a coverslip for optical microscopy (Olympus BX40). Samples of polyP-extracted waste activated sludge were also stained with TBO to appraise the efficiency of polyP extraction.

3.2.2.2. *Results and Conclusion*

Figure 3-2 shows the variable appearances of TBO staining in the untreated sludge. Many live microorganisms were also observed. Because PAOs is accounted as the majority organisms in the wastewater (Crocetti *et al.*, 2000), the presence of PAOs in the sludge sample was assumed to be confirmed. However, due to the limitation of objectives (x2, x20, x40, x60) in this experiment, the stained regions are not clear. Besides, it is difficult to ensure by TBO staining images if the stained granules are polyP or if polyP is otherwise within these organisms. For the purposes of this thesis, a positive purple stain was assumed to indicate polyP.

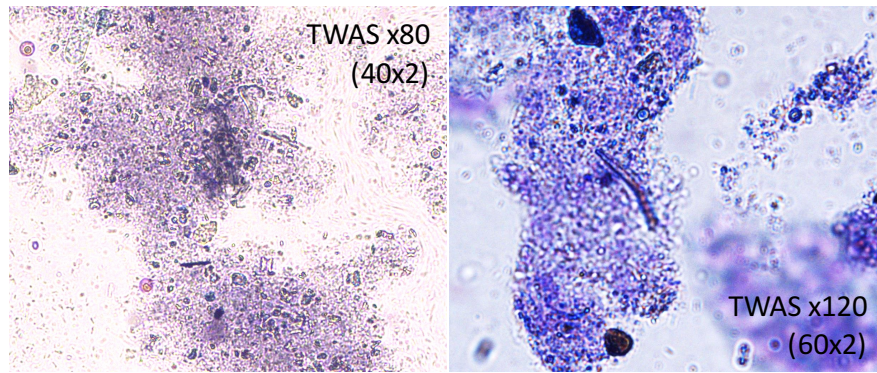


Figure 3-2 Microscopic images of diluted municipal wastewater thickened sludge from ROPEC with TBO staining.

In the Figure 3-3, a series of microscopic images display the variable patterns for the alkaline (1M NaOH) treated waste activated sludge samples after TBO stain. The comparison between the unstained samples and stained is present at the Annex C (Figure C-1). The identification of all of the PAO species is not the subject of this thesis. In Figure 3-3 (e.g., AE-6s.8), the stained granules are visible within the body of *Bacillus cereus* or the similar looking of bacteria. The variable area of blooming violet stain was observed in all of the images. These bloomings are suspected to be released polyP from the PAOs, or polyP that was extracted by the extraction process. Even if within the same sample, the pattern of violet staining was diverse (the sample of AE-6s in Figure 3-3). Therefore, it is almost impossible to judge the effectiveness of polyP extraction if the TBO-stained image is the only evaluation method. Other methods such as confocal microscopy may be used to the greater effect of polyP identification within cells (Sviben et al., 2017).

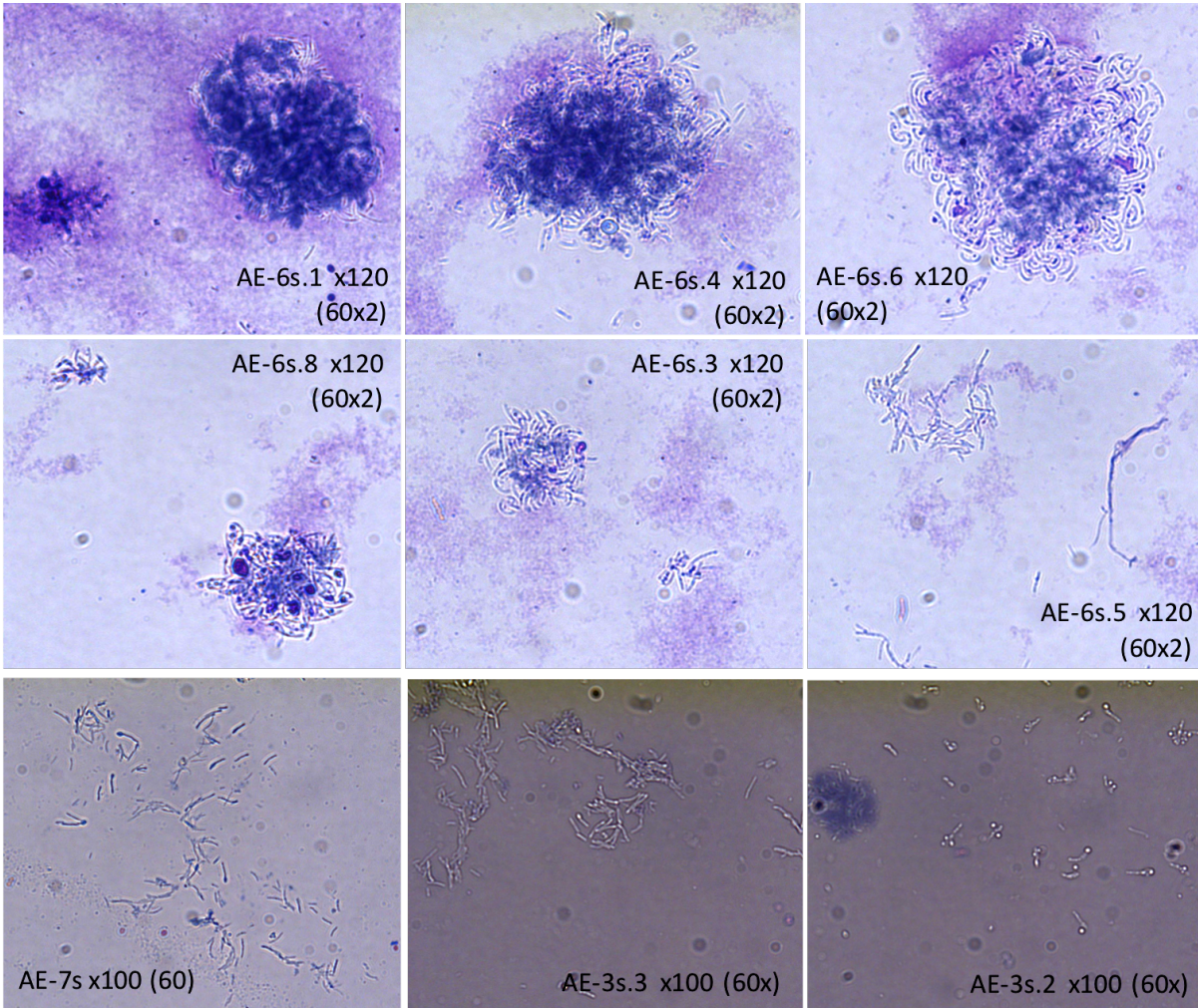


Figure 3-3 Microscopic images of TBO-stained polyP in the alkaline extracted sludge (objective x60).

3.3. Raman Spectrometer

3.3.1 Introduction

Raman spectroscopy utilizes the excited molecular vibrations of inelastic Raman scattering to identify the different composition and structure of the molecules with Raman-active chemical bonds (Schuster, Urlaub, & Gapes, 2000). As a portable, flexible and nondestructive analytical instrument, Raman spectroscopy is gradually taking the place of infrared spectroscopy (IR) (Dieing, Hollricher, & Toporski, 2011). However, the relationship between Raman and IR are complementary. A molecular bond signal is often strong for either Raman or IR analysis, but not both technique. Besides water, silicon (Si) is another material that provides strong signals with both two techniques. It has the similar emission frequencies at both IR and Raman spectrum, but with different intensities. Near infrared reflectance (NIR) Raman spectrometry, which uses a combination of IR and Raman techniques, was applied to organic phosphorus characterization in the cell (Malley, Lockhart, Wilkinson, & Hauser, 2000). However, the application of NIR-Raman is still limited in practice currently because of its complexity.

The significant advantages of material characterization by Raman spectroscopy, especially in the context of this study of P recovery from wastewater sludge, are that extraction or drying are not required. Furthermore, it is sensitive and spatially resolved, non-contact, and nondestructive (Dieing et al., 2011; Schrader, 1995). Therefore, Raman spectroscopy is a preferred technique for characterizing biological materials.

3.3.2. Literature Review

3.3.2.1. Polyphosphate Detection with Raman Spectroscopy

PolyP is a molecule of interest in many fields, including the evolution of life (Achbergerová & Nahálka, 2011). Raman spectroscopy was developed to allow for chemical species identification within a specific location that could be magnified by a microscope (Puppels et al., 1990). This technique to detect polyP in biological samples was first reported in 1957 (Steger & Simon, 1957). Until today, the techniques for Raman spectroscopic analysis of biological materials and live cells still continue to evolve (Smith, Wright, & Ashton, 2016). Raman spectroscopy can discriminate the ortho-Pi in amorphous calcium phosphate, apatite (Kazanci, Fratzl, Klaushofer, & Paschalis, 2006), and polyP, even within the PAOs. The strongest polyP Raman shift attribute to the out-of-chain P-O-P stretch is expected in the region of $1145\text{--}1175\text{ cm}^{-1}$ (Figure 3-4) (Omelon et al., 2014).

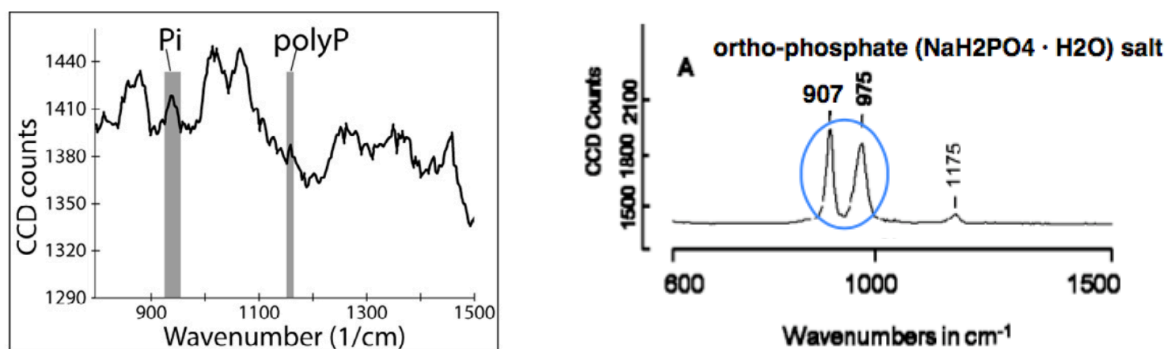


Figure 3-4 Two examples of Raman spectra. A) the spectral regions of polyP at ($1145\text{--}1175\text{ cm}^{-1}$) and Pi at ($930\text{--}980\text{ cm}^{-1}$) as the gray scanning lines, respectively (Omelon et al., 2014). B) Raman spectrum for the reference chemical of ortho-Pi salt ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) to indicate the P-O stretch by the peaks of higher intensity (Majed, Matthäus, Diem, & Gu, 2009).

In Figure 3-4, when polyP associated with a divalent cation, such as calcium ion (Ca^{2+}), this peak may be specifically at 1167 , 1169 and 1172 cm^{-1} (Majed & Gu, 2010). Or when associated with a monovalent cation, such as sodium ion (Na^+), this peak was detected at $\sim 1158\text{ cm}^{-1}$ (Bednářová et al., 2012; Majed et al., 2009). Besides, the interaction between polyP and Na^+ , cesium (Cs^+), Ca^{2+} and barium (Ba^{2+}) was discussed, including the effect of the different P-O bond types (Koda, Kawakami, & Nomura, 1994). The dissimilarity of the P-O bonds in the polyP, $\text{P}(\text{OH})_3$ and (ortho-)phosphoric acid were assigned in the kinetic investigation by the hydrolyzing rate from polyP to Pi in the water (de Jager & Heyns, 1998b). These results were also associated with the mathematical calculations (de Jager & Heyns, 1998a).

Yeast is considered to be a member of the PAO family (Melasniemi & Hernesmaa, 2000). Many studies of polyP with Raman spectroscopy techniques have been applied to yeast. The first Raman spectroscopic evidence of polyP within living cells was within the *Schizosaccharomyces pombe* yeast cells (Rao, Gomez-Garcia, & Kornberg, 2009). The accumulation of polyP within the vacuoles of *Candida albicans* yeast cell was investigated with confocal Raman spectroscopy (Bednářová et al., 2012).

Raman spectromicroscopy was applied to testify and monitor the waste activated sludge from a lab-scale EBPR process to provide the real-time of identification and quantification of polyP and other intracellular granules in a certain individual PAO cell (Majed et al., 2009). Majed and Gu investigated the feasibility of combining Raman and other techniques to identify polyP and the phylogenetic population in waste activated sludge (Majed & Gu, 2010). Both intracellular PAOs and glycogen accumulating organisms (GAOs)⁸ was discovered for their metabolism in the waste activated sludge from a lab-scale EBPR system. Researchers identified and quantified polyP within the granules by a modified Raman microscopy (Majed et al., 2012a).

Raman spectroscopy was identified carbonate apatite precipitates generated by the preliminary precipitation efforts from synthetic anaerobic digester solutions (Ross, 2017). Powder X-ray diffraction confirmed the apatite observation from Raman spectroscopy⁹ (Meouch, 2016; Meouch & Omelon, 2016; Ross, 2017). The difference between crystalline and amorphous solids by Raman spectroscopic analysis was compared by Tuschel (Tuschel, 2012; 2017).

3.3.2.2. Polyphosphate Detection with Raman Spectroscopy with mapping

Raman confocal microscopy with mapping has become a popular analytical tool for biological materials, especially in a single cell (Krafft & Popp, 2015). That is because researchers believed the studies of the location of polyP within a living cell (Diem, Romeo, Boydston-White, Miljkovic, & Matthéus, 2004) could break a current bottleneck, and support the further exploration for bio-evolution (Rao et al., 2009). This technique was applied to the detection of

⁸ GAOs is another group of bacteria in wastewater, which could compete for carbon sources with PAOs, resulting in the failure of EBPR process (Crocetti et al., 2000).

⁹ Mico-Raman was expected to be complementarily of micro-X-Ray diffraction (μ XRD) (Tuschel, 2012).

hydrocarbons within the *Botryococcus braunii* (Largeau, Casadevall, Berkaloff, & Dhamelincourt, 1980) and carotenoids within a single *Pyrocystis lunula* cell (Dupaix et al., 1982), soon after the first invention of the Raman microscope (Delhay & Dhamelincourt, 1975).

The study of polyP in an algal cell by Raman microscopy with mapping is expected to further understand their utilization and their capability to uptake phosphorus (Prochazkova, Branyikova, Zachleder, & Branyik, 2014) to remove P from waste streams (Solovchenko, Verschoor, Jablonowski, & Nedbal, 2016)

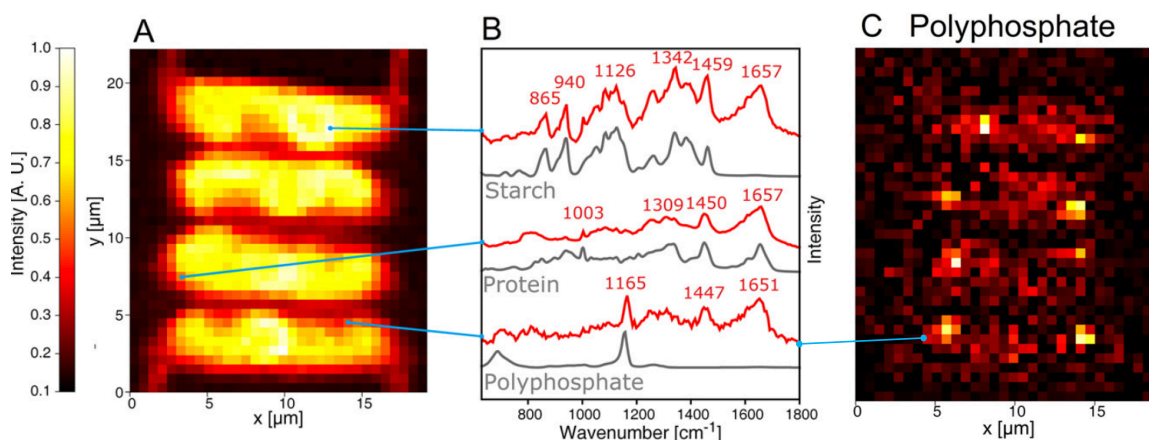


Figure 3-5 Raman maps and Raman spectra resulting from a single alga - *D. Quadricauda coenobium*. (A) and (C) The Raman maps of the distribution of entire intensity region (620–1800 cm^{-1}) and polyP only by many tiny granules in every cell, respectively. (B) The red and gray Raman spectrum represent the region of the algal cell and corresponding reference materials of starch, bovine serum albumin and sodium hexametaphosphate (from top to bottom as the order), respectively. The relationships between the maps of (A), (c) and the spectrum of (b) are shown by the blue lines crossing these panels. (Figures adapted from (Moudříková et al., 2016).)

In an algal cell of *Desmodesmus quadricauda* (Figure 3-5) or *Chlorella vulgaris*, the fundamental constituents of photosynthetic energy biomolecules, such as the reserves of polyP granules, pigments and lipids¹⁰, starch, have been detected, mapped and located by a confocal Raman microscopy (Moudříková et al., 2016).

¹⁰ The Raman application for pigments and lipids is available at the papers (Parab & Tomar, 2012; Wei et al., 2014). More detail of microbial pigments is available at the review paper (Jehlicka, Edwards, & Orenc, 2014).

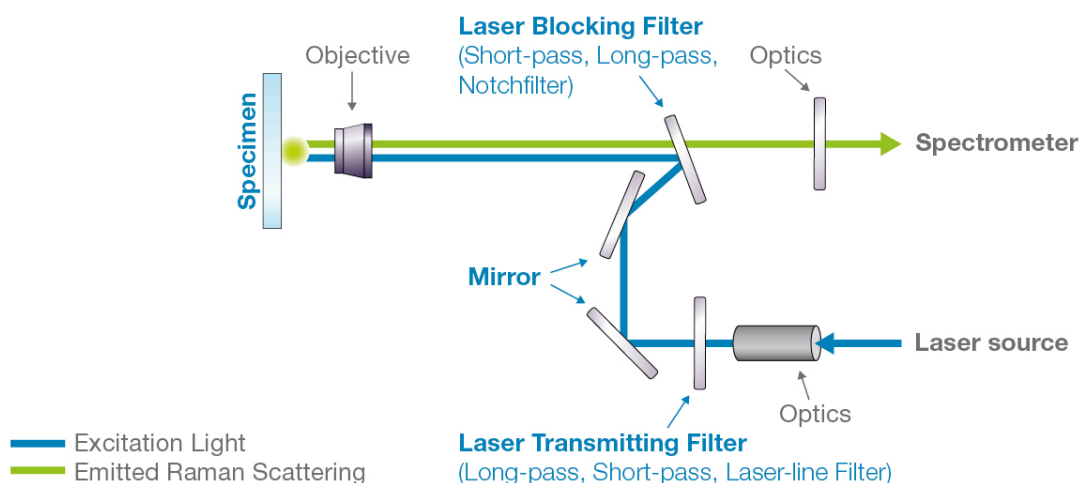
3.3.3. The Preliminary Design and Construction of a Dispersive Raman Spectrometer

3.3.3.1. Introduction

3.3.3.1.1. The Theory of Raman Spectrometer Construction

The main building blocks in the Raman Spectrometer (Figure 3-6) include a laser, objective(s), laser blocking filter, coupling optics to connect with a spectrometer and a spectrograph with a selection of gratings. A microscope, CCD detector(s), and computer control of the Raman instruments also could be part of the set-up. The laser transmitting filter and laser cleaning filter might be revised according to the laser itself. In the experiment setup (Figure 3-7), the wavelength of the laser could be controlled by the computer for any wavelengths. The presence of “laser transmitting filter” is a short-pass beamsplitter and the “laser blocking filter” in this figure is called the “Raman filter” in this thesis.

Because of Raman scattering, the narrow beam of the laser is important as a monochromatic source to excite the chemical bonds for their Raman shifts. The laser transmitting filter (beamsplitter) delivers the selected incident laser passing through the sample, and it also reflects the Raman-shifted beam towards the spectrograph. Then, the Raman signal from the sample was split passing through the Raman filter into spectral wavelengths inside of the spectrograph. With additional supplements, for example, a charge-coupled device (CCD), the spectral coverage and dispersion are managed with a grating device, and these shifted wavelengths and intensities could be detected by the CCD.



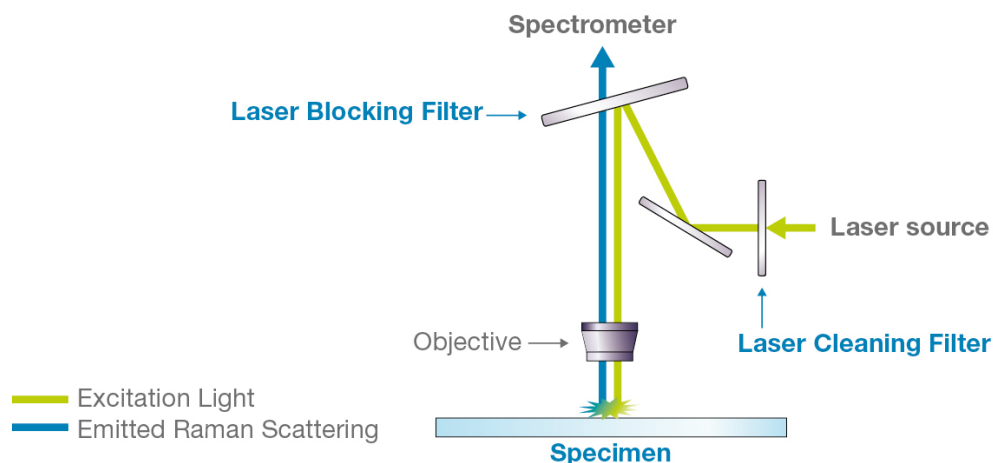


Figure 3-6 Two simplified schematics of the Raman spectroscopy set-up¹¹ for lab-scale without a complete microscope (top) and the arrangement with a possible complete microscope as shown as objective (bottom).

3.3.3.1.1. The Raman Spectrometer Setup

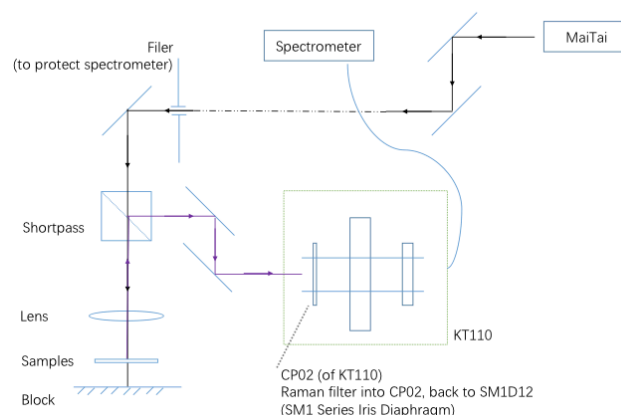
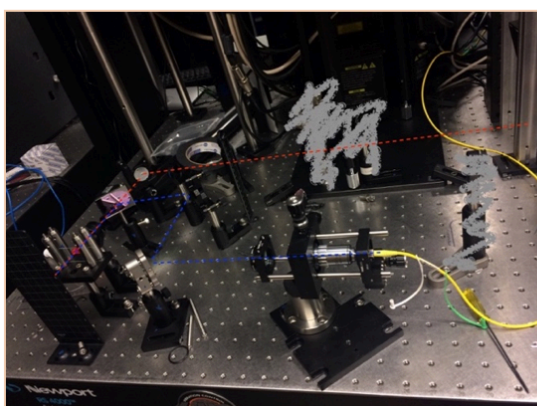


Figure 3-7 An image (A) and the schematic layout of version IV (B) for the preliminary Raman spectra devices. (B) The black line is the incident beam from MaiTai laser with wavelength ~ 785 nm, which corresponds to the red dotted line of (A). The violet line is the reflect Raman signal, which corresponds to the blue dotted line of (A). The light blue line is an optical fiber that is connected to the spectrometer.

A research group at the Max Planck Centre for Extreme and Quantum Photonics (University of Ottawa) was interested in building a Raman microscope in their facility. Before arranging the relevant Raman optics and optic-mechanics into the nonlinear microscope with other functions, a

¹¹ Available in <https://www.opticsbalzers.com/en/products/filters/raman-filters.html>

setup (Figure 3-7) was built to verify the feasibility for a basic Raman setup through a group collaboration, with their laser, and other infrastructures.

An excitation wavelength of ~785 nm is assumed to generate useful Raman spectrum for the biological samples detection. With this Raman spectrometer configuration, this NIR incident laser is achieved the cut-off frequency by a dichroic short-pass beamsplitter¹². As a result, this laser is excited through the beamsplitter to the sample. Then, the scattered light passes through the beamsplitter again from the sample towards spectrometer. This scattered light includes a Rayleigh scattered beam with an equal energy of the incident beam, the reflected laser incident beam, as well as the much weaker shifted light energies caused by Raman scattering. The fiber transfers the Raman signals into the spectrometer after passing through the Raman filter to achieve the material characterization.

3.3.3.2. *Optic-Components and Sample*

3.3.3.2.1. *Excitation: Laser and Laser Wavelength*

The laser is the best light source with a monochromatic output and high intensity to obtain the most distinct excitation Raman signals from samples. In principle, the range of the laser wavelengths is from ultraviolet (UV) to NIR based on the sample characteristics. Figure 3-8 is a table of commercially available laser wavelengths with maximum intensity and laser types. The laser wavelengths commonly used for Raman spectroscopy are 227, 244, 257, 325, 364, 413, 332, 457, 473, 488 (classic blue), 514, 532 (classic green), 633, 647, 660, 785 (classic NIR), 830 and 1064 nm¹³. The wavelengths commonly reported for Raman spectroscopy of biological samples are 488, 514.5, 532, 785nm (Wood et al., 2005), 830 nm (Nafie, 2011) and 1064 nm (Nikoobakht, Wang, & El-Sayed, 2002). While laser wavelength usually is reported in nanometers (nm) as the unit, Raman spectral shifts ($\Delta \nu$) are commonly reported in wavenumbers

¹² This filter is called a dichroic (short-pass or long-pass) filter (mirror) or beamsplitter (including both long-pass and short-pass). In Figure 3-7, it also is named a “laser transmission filter”.

¹³ More information is available Raman Q&A at HORIBA website: <http://www.horiba.com/scientific/products/raman-spectroscopy/raman-academy/raman-faqs/what-laser-wavelengths-are-used-for-raman-spectroscopy/>

(cm^{-1}) by chemists, and in wavelength (nm) by physicists. The reciprocal relationship between the wavelength (λ , nm) and wave numbers (ν , cm^{-1}) is defined in Equation 1:

$$\Delta\nu = \nu_{\text{Laser}} - \nu_{\text{Raman}} \quad \text{Eq. 1}$$

$$\text{where } \nu \text{ (nm)} = \frac{1}{\lambda \text{ (cm}^{-1}\text{)}} = \frac{1}{\lambda \text{ (10}^7 \cdot \text{nm}^{-1}\text{)}}$$

The software of contemporary Raman spectrometers allows for the Raman shift data to be presented at either wavelength (nm) scale or wavenumber (cm^{-1}) scale as the more common unit. Commercial Raman spectrometers also offer multiple lasers as variable excitation sources on a single Raman spectrometer system.

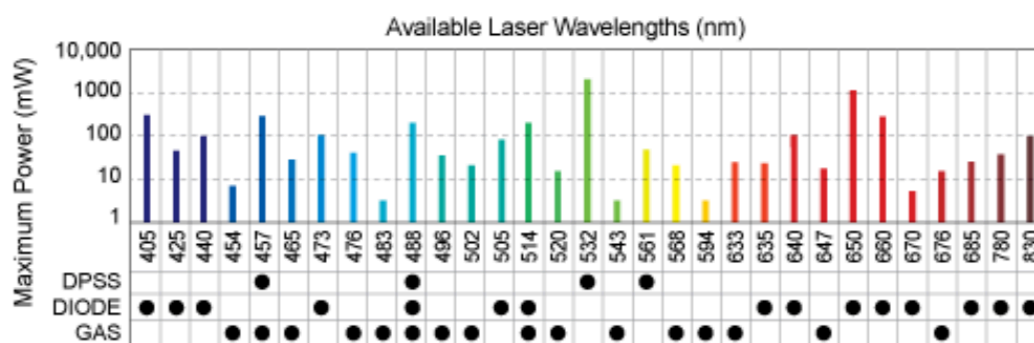


Figure 3-8 The guide for available laser selection by diode-pumped solid-state (DPSS) lasers, semiconductor diode lasers and gas laser (such as HeNe) (IDEX Optics Photonics Marketplace, 2014).

Fluorescence is the biggest enemy and current limitation to Raman spectroscopy. The autofluorescent background might easily overlay the Raman spectral region created by the laser (e.g., green laser of 532-nm wavelength) because of the stronger intensity (Figure 3-9). The trade-off for selecting a laser with a longer wavelength is a lower spatial resolution and lower Raman signal efficiency (Tuschel, 2016). More information about laser selection criteria was reported by Tuschel (Tuschel, 2016).

The unique molecular structures of molecules dictate their Raman shifts, and this shift is not affected by the excitation wavelength. However, shorter excitation laser wavelengths trigger sample fluorescence (Dieing et al., 2011), which makes it difficult or impossible to detect the weaker Raman shifts. Because of relatively less impact of fluorescence background (Figure 3-

9), 785 nm is a commonly used laser to generate Raman spectra from biological samples, and 1064 nm is also useful for measuring biological samples (Creasey, 2017) although it is not as available as the 785 nm laser. Given this information, and the goal of the thesis being the detection of polyP in waste activated sludge bacteria, ~785 nm was selected as the laser wavelength for this study.

To avoid auto-fluorescence, biological samples are often measured with the lowest laser excitation (785 nm). Therefore NIR (red) laser was selected as the excitation laser energy for this setup. However, a technique for adjusting wavelength could apply for easier alignment. A wavelength slightly higher than 785 nm to be close to the elimination of IR was used for the alignment for easy to visually determination with IR sensor card (pink card). Otherwise, it is possible that the laser beam would have been undetectable, even in the dark room.

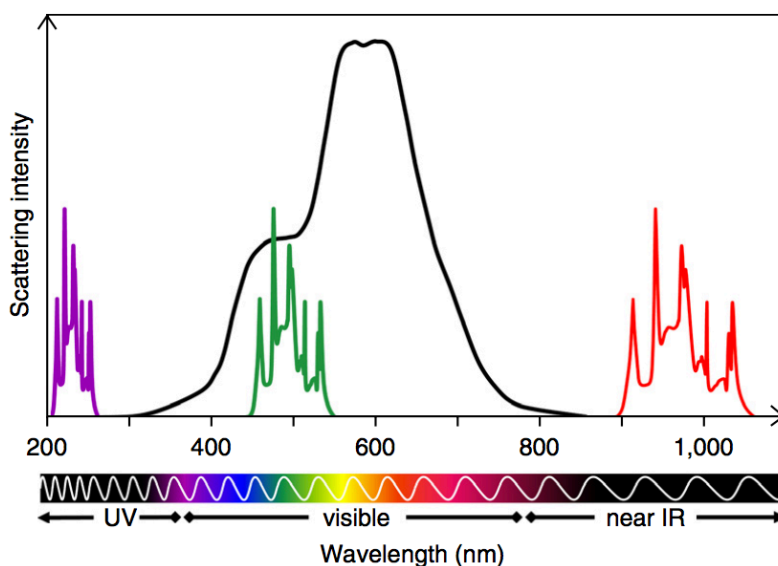


Figure 3-9 The impact of excited laser wavelengths (purple: high energy UV; green: 532-nm; red: low energy (near) IR) on the defined fluorescence background (back) (Butler et al., 2016).

MaiTai (Spectra-physics®) is a broad field laser available for the Raman spectrometer. Wavelength selection is often a function of the sample type. The laser should be warm 30 min to wait for the pump IR power value up. The humidity parameter should report the lab manager after 10%. A mounted reflective or variable neutral density filters (ND filter) might be applied as an optical barrier at the beginning of the setup to limit the intensity of the laser to protect the spectrometer.

The power meter and detector for this whole setup are Newport® 2936-C and 818 – SL (S/N: 21440), respectively. The “SL” in the detector refers to silicon detector. If the letters are “IG”, the detector is applied to the beams at IR level.

Moreover, the laser wavelength also affects the spot size of focus beam because the spot size of the laser is usually defined based on the laser wavelength and objective in principle. The minimum of laser spot size limits diffraction and this size is essential for the spatial resolution of Raman microscopy. However, in this setup, the laser and the laser spot size were fixed so the objective was inversely chosen in Section 3.3.3.2.6.2. - Objective Lens.

3.3.3.2.3. Beamsplitter

The term “dichroic” refers to the properties of both reflection and transmission as a filter. Before the entrance to the lens objective, a dichroic beamsplitter transmits the excitation laser to the sample. Then, it also emits the laser into the objective lens through the Raman filter after the signals reflect from the sample. But this time, it only can emit the longer wavelengths of the Raman scattering.

Both the notch and edge filter can be conducted as the beamsplitter. The notch filter filters the laser wavelength specifically, allowing the Stokes and anti-Stokes scattering signals to pass through the lens. Both of Raman Stokes and anti-Stokes shifts are generated by the excited laser, though the Raman Stokes have more energy and anti-Stokes have less energy than the excitation. With an edge filter, it only allows the Raman Stokes shifted energies to pass through the filter, and the laser and anti-Stokes energies are blocked out. Edge filters are more physically robust, are lower cost, and provide better long-term stability due to the material property (Bleisteiner, 2010). The loss of the anti-Stokes scattering information usually is less important than the other benefits of the edge filter. However, the notch filter is the only option if the anti-Stoke signal is to be detected.

Based on the wavelength-dependent property, the short-pass dichroic beamsplitter, Semrock® FF791-SDi01-35.9x50 (unmounted 25.2mm X 35.6mm X 1.1mm, Figure 3-10) was chosen as an edge dichroic beamsplitter for the Stokes scattering only. The mount of this short-pass is model

number F30FOPT (Olympus®). It is an alternative model for mounting on the inside of the future non-linear microscope (Olympus® FluoView FV1000). This edge filter guides the laser to the samples through the objective. It is able to limit transmit the incident shifted wavelengths (697-787 nm). After the Raman scattering is generated from samples, the scattered Raman signals that pass through the edge filter with shifted wavelengths of 795-940 nm are directed to the association by “Free space launch KT110” (3.3.3.2.6), which directs the Raman signals to the spectrometer.



Figure 3-10 The transmission capability with an edge wavelength of 791 nm BrightLine® single-edge short-pass dichroic beamsplitter (Source: product information at Semrock®) ¹⁴ as the exciter filter or called as laser transmission filter.

3.3.3.2.4. Lens

The applied lens was a LA1131-A - N-BK7 Plano-Convex Lens, Ø1", $f = 50.0$ mm (THORLABS®). As one of its core functions, this lens can focus the incident laser beam onto the sample surface. The choice of the lens was not considered in this study due to the early stage of this Raman study.

¹⁴ Available at <https://www.semrock.com/FilterDetails>

3.3.3.2.5. Samples

The incident laser could damage samples if the laser power is too high or the sample absorbs the laser energy. Therefore, the minimization of laser absorption is desired to protect sample. The laser power usually is restricted with a ND filter, which reduces the intensity of all of the incident wavelengths. However, reducing the incident laser power might require an increase in the measured time for the better spectrum. Otherwise, it has significant background noise with an inferior signal-to-noise ratio (SNR). In fact, the spectrum of a complex material may present as a lower and wider peak. Therefore, the sample preparation is also critical to achieving a good signal to noise ratio in order to identify the Raman-shift.

Crystal violet has a stronger Raman signal than other Raman-active molecules, so it offers easy initial detection of its Raman shifts (Angeloni, Smulevich, & Marzocchi, 1979). Therefore, it was selected as the test material for this setup. A 1%(w/v) (0.0245 M) concentrated crystal violet (certified biological stain, C0775 SIGMA) solution was prepared as the stock substance to test the Raman spectrometer system configuration (Figure 3-11).

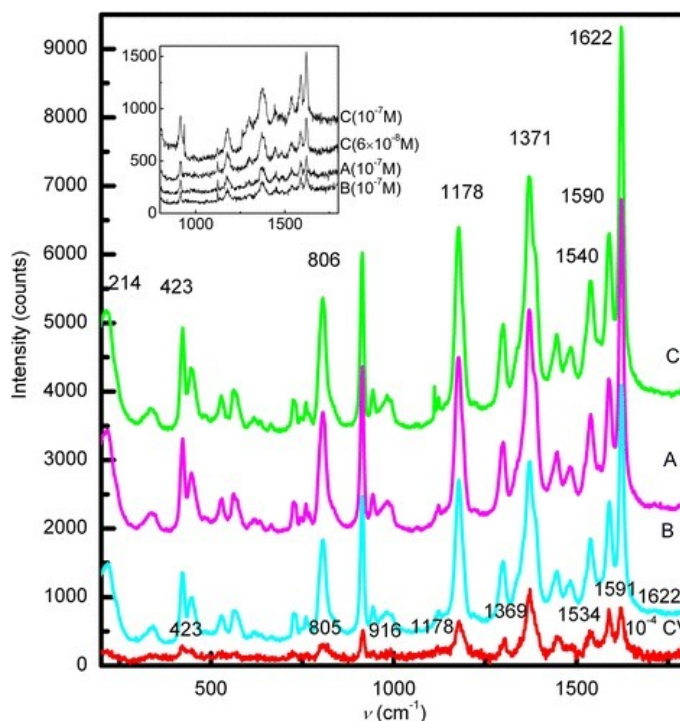


Figure 3-11 The surface-enhanced Raman scattering (SERS) (green, pink, blue) and Raman spectrum (red) of crystal violet (Meng, Hu, Jiang, & Lu, 2015).

3.3.3.2.6. Free Space Launch

The Free space launch (Figure 3-12) was model KT110 (THORLABS®)¹⁵. This collects the energy from the reflected Raman signal through the objective and directs the reflected sign into the spectrometer through fibers and couplings.

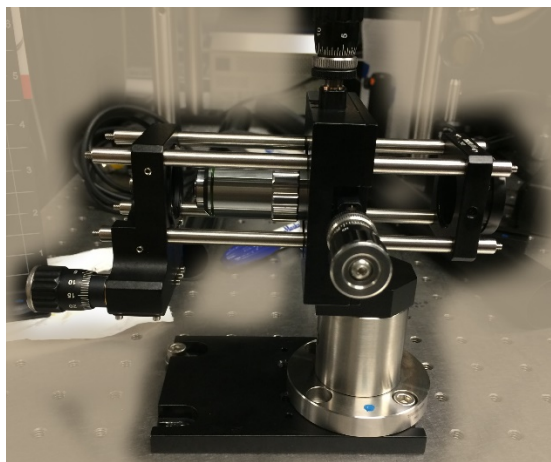
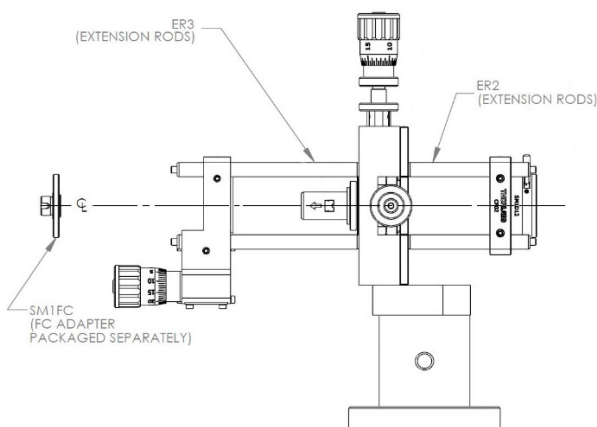


Figure 3-12 (left) The revised layout of KT110 free space launch (partial redraw based on the AutoCAD drawing at official product information website). The original drawing is in Annex B, Figure B-6 (right) The photo of KT110. The main differences are the exchanged position of extension rods ER3 and ER2 to assemble the longer objective and the selection of SM1FC FC adapter to connect with fiber.

3.3.3.2.6.1. Raman Filter

The Raman filter, as the laser block filter, can be enabled to direct the light into the objective. The objective focuses the light and collects the Raman shifted light into the spectrometer by coupling fiber and optics. It is a single-band bandpass, FF01 855/2-10-25 (Semrock®, Figure 3-13), with a center wavelength of 855/210 nm (25mm X 3.5mm). It can transmit above 90% at 750-960 nm to restrict the longer wavelengths of the Raman signal. In this version of the setup, the main function of this filter is to concentrate the beam into the objective lens, instead of restricting transmission.

¹⁵ Available at <https://www.thorlabs.com/thorproduct.cfm?partnumber=KT110>

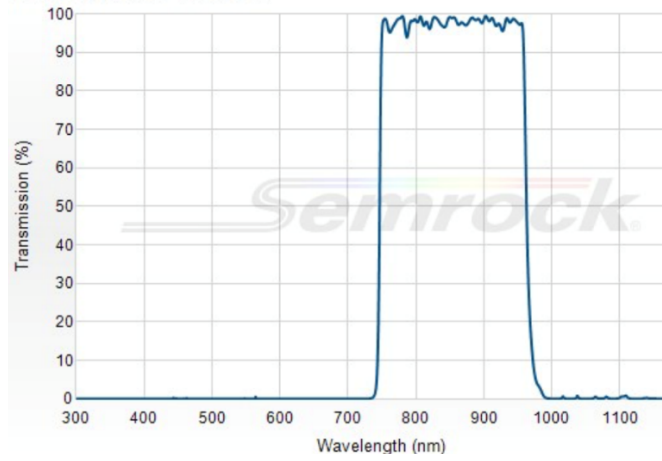


Figure 3-13 The transmission capability with a wavelength of the customized 855/210nm BrightLine® single-band bandpass filter (Source: Semrock®) ¹⁶ as the emitter filter or called it laser block filter.

3.3.3.2.6.2. Objective Lens

The microscope for the Raman systems is infinity focused, which means that the beam entering and leaving the back aperture of the objective is collimated. Because of the absence of a microscope at this preliminary stage, while the Raman optical system was set up, the objective was installed into the Free space launch. The distance between the objective and the sample could not be adjusted with this setup. The alignment of the setup also requires that this single objective cannot be changed.

The performance of a coupling beam into an optical fiber is defined by Equation 2,

$$f = D \frac{\pi \omega}{4\lambda} \quad \text{Eq. 2}$$

where,

f is the focal length of required coupling lens,

D is the collimated beam diameter of the light source entering the coupling lens($\frac{1}{e^2}$),

¹⁶ The similar product is available at <https://www.semrock.com/FilterDetails.aspx?id=FF01-445/40-25>. This Raman filter was customized based on the physical size of the channel of FV30-RXD in the microscopy (Olympus FluoView FV1000) (Figure 3-17).

ω is the single mode fiber mode field diameter,

λ is the wavelength of light source.

The beam diameter (D) of Mai Tai laser (Spectra-physics®) is $D < 1.2\text{mm}$ ¹⁷.

The single-mode fiber mode field diameter of the fiber (THORLABS ®), @850nm: $\omega = 5 \pm 0.5\mu\text{m}$ ¹⁸. The f_{ave} for the experimental configuration is calculated with Equation 3:

$$f_{\text{ave}} = D \frac{\pi\omega}{4\lambda} = (1.2\text{mm}) \frac{\pi(5\mu\text{m})}{4(785\text{nm})} = 6.003\text{mm}$$

The overlap extent of the Gaussian mode of the incident beam and the nearly Gaussian fundamental mode of the fiber determines the coupling efficiency. It was assumed that the difference between the focal length and working distance was negligible. The working distance of the 20x objective (MDE173 Elliot®, N.A.=0.4) ¹⁹ is $\sim 1.3\text{ mm}$, and the 10x objective (MDE172 Elliot®) ²⁰ was $\sim 6.5\text{ mm}$, as the closest fit for this application. Therefore, the focal length is larger by a factor of 1.08.

3.3.3.2.7. Fiber

The selection of fiber was based on the wavelength. For this setup, the operating wavelength of the fiber should be 780-970 nm. Therefore, the chosen fiber for this work was 2 x (1 m), (THORLABS®) P5-780A-PCAPC-1, T0412037 to meet the wavelength requirement. The loss of fiber coupling cannot be measured precisely because there was no proper connected optics for

¹⁷The beam diameter (D) of MaiTai laser <http://www.spectra-physics.com/products/ultrafast-lasers/mai-tai - 3>

¹⁸ Single mode fiber mode field diameter of the fiber, @850nm <https://www.thorlabs.com/thorproduct.cfm?partnumber=P5-780A-PCAPC-1>

¹⁹MDE173 - Microscope Achromatic Objective x20 <http://www.elliotscientific.com/Elliot-Martock-MDE173-Microscope-Achromatic-Objective-x20>

²⁰MDE172 - Microscope Achromatic Objective x10 <http://www.elliotscientific.com/Elliot-Martock-MDE172-Microscope-Achromatic-Objective-x10>

the fiber and the detector of the power meter. Usually, 1 – 2% coupling efficiency²¹ was the maximum for the rough detection for this setup.

3.3.3.2.8. Spectrometer

Acton[®] SpectroPro SP-2500 is a sensitive spectrometer, so mounted round ND filters and a power meter may be required accessories for this setup. Unfortunately, the calibration of the spectrometer was not completed, but a plan was made based on a published technique note (Princeton Instruments, 2014). The calibration of the spectrometer was not included in the objectives for this setup. But this calibration should be conducted for accurate sample characterization in the future.

3.3.3.3. Results and Discussion

3.3.3.3.1. Results and Discussions at the First Stage

By removing the extra ND filters, only one ND filter was left in place to restrict the beam intensity, and to protect the spectrometer detector. Dried, concentrated crystal violet samples on a glass slide with coverslip were used as a test sample. The setting wavelength of the laser was 715 nm. As the actual wavelength was known, the maximum wavelength measured by the software was revised to 708 nm (Figure 3-14).

Without spectrometer calibration, priority was given to solving some of the other problems associated with this result, such as peak shift, peak breadth, and peak interference. The physical interference-diffraction phenomenon may be a consequence of the glass slide thickness. In Figure 3-14, interference patterns for each Raman shift seem to be built up by multiple peaks. As known, the laser was the only light source. However, this result shows that there were a few beams (waves) that passed through the sample. The interference comes from the interactions between two slits - the slide and the coverslip. They were close to each other, as is the requirement for slits. The diffraction patterns could arise from interactions with the slits.

²¹ Coupling efficiency refers to the percentage of beam power transferring from the beginning of the free space launch KT110 to the end of fiber.

However, the glass slide for mounting the samples was thick. It could also cause many diffraction patterns, which could contribute to the interference phenomenon.

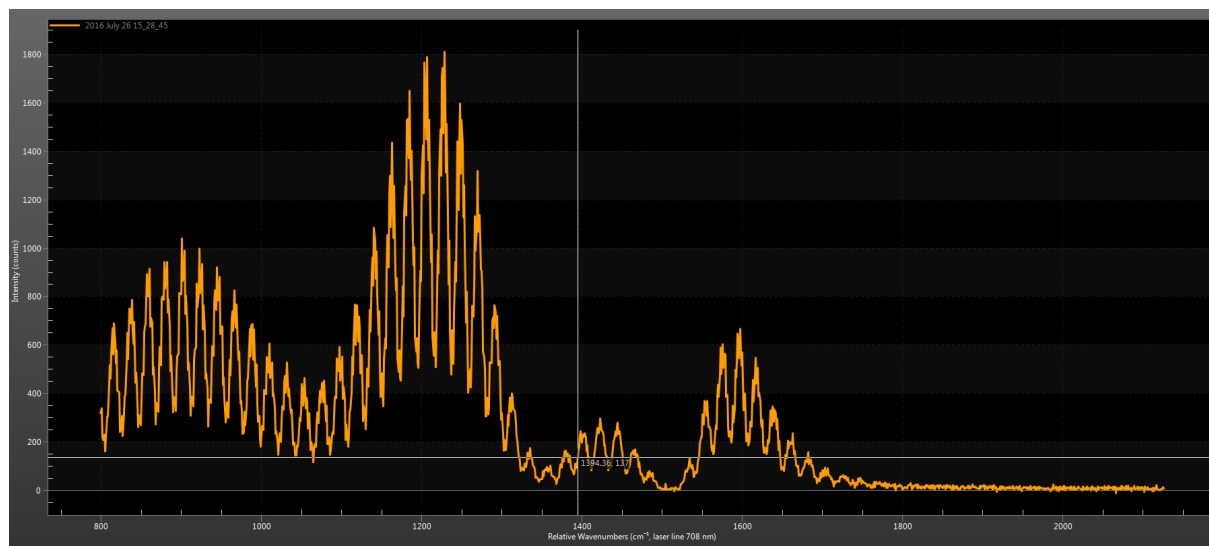


Figure 3-14 Raman signal of dried crystal violet samples without calibrating, generated by 715 nm laser (708 nm indicated with the software) with the version IV setup.

It is evident in the Figure 3-14 that the peaks are broad, which may be caused by the physics of the wide spectrum generated by the Mai Tai laser. Because of the strong interference, the glass slide of the sample may also be responsible for the Rayleigh peak shift. Regarding the result in the Figure 3-11, the Raman shifts from crystal violet were expected at 1369, 1591, 1178 and 916 cm^{-1} from the peaks with highest to lower intensity. The estimated measured peak maxima in Figure 3-14 were near 1200, 900, 1600, and 1425 cm^{-1} . These Raman shifts of dried crystal violet generally correspond with the published results, which implies that the preliminary setup was built and it has the potential capability to measure unknown samples. However, the uncommon broad peaks and interference require future work to be understood and mitigated.

3.3.3.3.2. Results and Discussions at the Second Stage

Considering the possible adverse effects of the glass sample holder in Figure 3-15, an appropriate slide mount was proposed to replace the glass slide in the last trial.

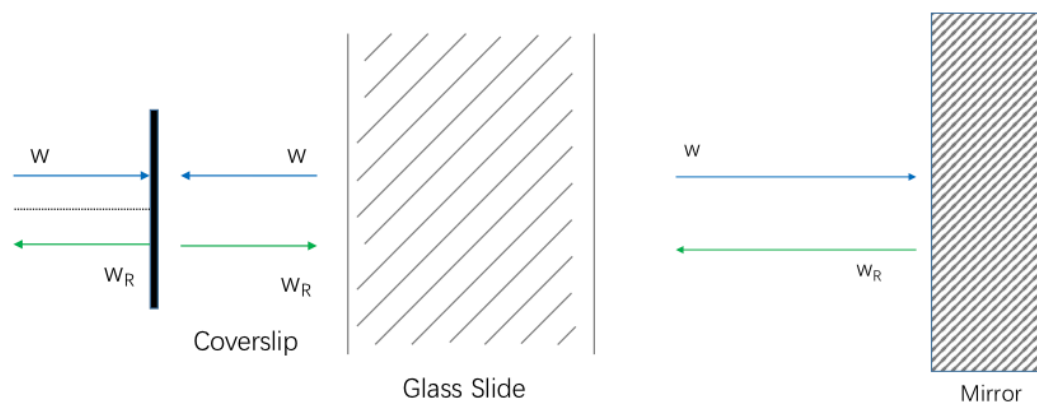


Figure 3-15 The schematic of glass slides with a coverslip, and the schematic for the proposal of a mirror as the sample holder, respectively.

Because of the signal generation by the NIR lasers on the glass slide, quartz slides are a better substitute for glass slides. Four options were also considered to eliminate the effect of the glass slide thickness without extra cost: (1) replacement the glass slide by one or two coverslips; (2) replacement by a micro-slide to reduce the thickness, (3) replacement by a mirror as the sample holder (Figure 3-15 right), (4) measurement of a clean glass slide with coverslip may be measured as the background, and subtracted from the sample signal in the software.

To limit the interference that might have been caused by the powdered sample, the sample was changed from solid powder to a solution of 1% crystal violet that was air-dried on a single coverslip. The results with the variable laser powers by ND filter adjusted are shown in Figure 3-16. Changing the laser power changed the Raman shifts. The lower laser power of 5 mW should be considered for the future trials. However, the discrepancy of the measured spectrum and the standard material (Figure 3-11) suggest that the calibration of spectrometer will be required in the future.



Figure 3-16 The Raman spectrums through the setup measured by the variable power of the laser with 10s of exposure time at 785 nm of both of wavelength and central wavelength.

3.3.3.4. *Conclusions and Future Work*

In conclusion, this preliminary setup proved the possibility to build a Raman spectrometer with a fiber connection. The Raman shifts for crystal violet were detected, but they were not close to the standard material shifts. The calibration of the spectrometer and the loss of coupling fiber are required future work. More work is required to build the Raman system further.

There are three suggestions for future work.

Alternative laser and two interchangeably lasers Regarding the broad peaks, a single wavelength laser may replace the present Mai Tai laser, and reduce the Raman shift peak width. The present Mai Tai laser will be used to generate an optical-wavelength beam to observe and focus the sample when it is mounted in the nonlinear microscope. A second, single wavelength laser, such as a narrow-line 780 nm laser, could be installed and switched to excite the samples to generate the Raman scattering. Besides, it is important to reduce the intensity of the incident laser in the reflected light beams including the Raman shifts, so the CCD will be protected from the high-energy incident laser.

Laser Wavelength A different laser wavelength may be beneficial for detecting the laser beam while the system is constructed. Because of the current short-pass filter restriction upstream of the Raman setup, neither the transmitted nor reflected beam could be detected by an IR sensor card (VRC2, THORLABS®). This made it more difficult to build and align the system efficiency.

Build the Microscope Setup After calibrating the spectrometer with the crystal violet solutions, the bandpass filters, and the short-pass beamsplitter will be transferred into the nonlinear microscope (Figure 3-15). The upper and low stand should prevent undesired light from entering the eyepiece and proceeding through the short-pass beamsplitter. If this occurred, the incoming light would be of higher intensity than the relatively weak Raman signal, and the Raman signals would not be measurable with the spectrophotometer.

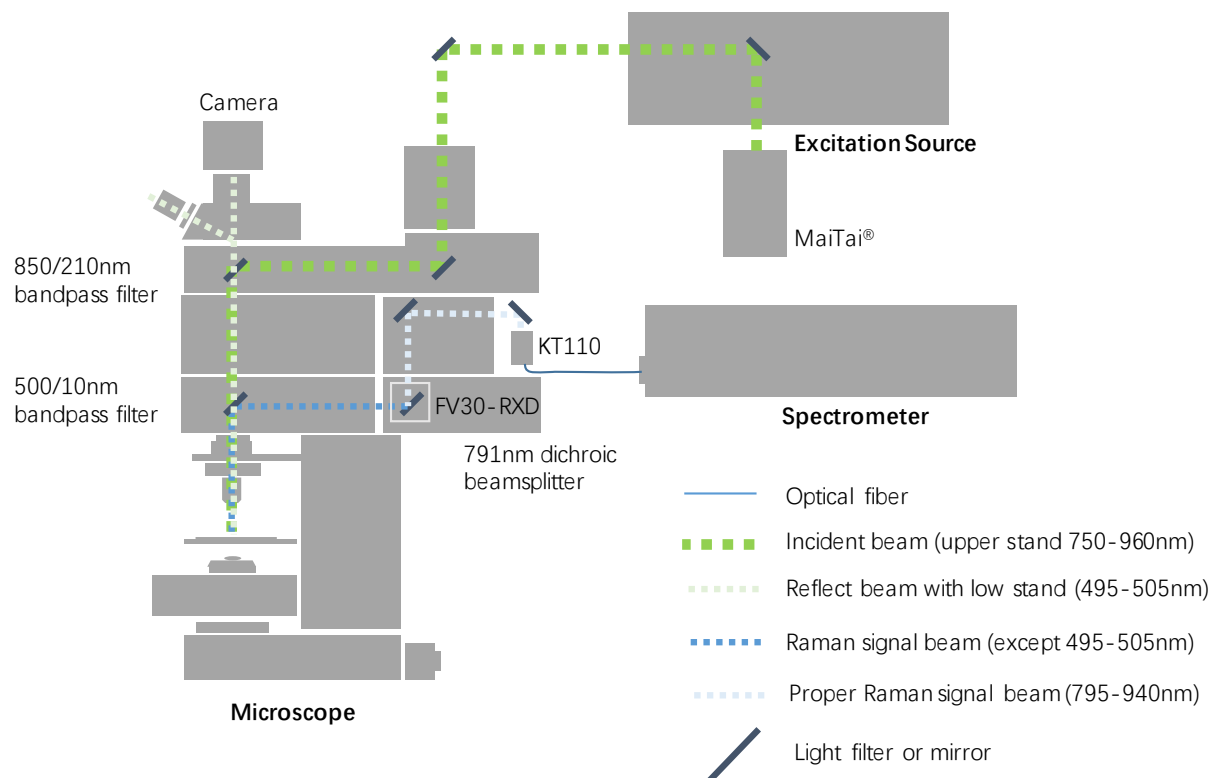


Figure 3-17 Raman spectroscopy experiment schematic layout. The bold green dotted line corresponds to input beam with assuming wavelength $\sim 785\text{nm}$. The light blue line is an optical fiber to connect microscope (Olympus FluoView FV1000) and spectrometer. The low stand filter could consider by 500/10nm filter 500/10 nm BrightLine® single-band bandpass filter (Part number: FF01-500/10-25, 25 mm X 3.5 mm, Semrock®). The upper stand filter is the Raman filter in this setup. The dichroic beamsplitter and the free space launch will be placed at proper locations.

3.4. References

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4. Extraction and Hydrolysis of Polyphosphate from Thickened Waste Activated Sludge

4.1. Introduction

4.1.1. Background Chapter Summary

This chapter includes background information on the TWAS samples (Section 4.1.2), a literature review of the topics relevant to these extraction studies (Section 4.2), methods, results and discussion for each study (Section 4.3), and an overall conclusion (Section 4.4). Among them, the study of each extraction method is conducted into three subsections – introduction, material and methodology, results and discussion. The same organization is also applied in Chapter 5.

At the end of P cycle, phosphorus in human and commercial waste is directed through sewage pipes where it is concentrated into municipal wastewater treatment plants, where it takes many different forms. One of the forms is polyphosphate (polyP), which is a bioavailable P-sink that also is found naturally in the lake and river sediments (Hupfer, Gloess, & Grossart, 2007), and in benthic marine bacteria (H. N. Schulz & Schulz, 2005). It is produced by and contained within PAOs in the aerobic waste activated sludge (WAS) as the secondary sludge generated by municipal wastewater treatment plants (WWTPs) (Kenney, Schelske, & Chapman, 2001). However, within the anaerobic conditions of anaerobic digestion plants that follow the aerobic treatment process in larger WWTPs, intracellular polyP contents are hydrolyzed into soluble inorganic phosphate (Pi). Smaller WWTPs do not include an anaerobic digestion step (personal communication, Dr. Christian Kabbe), so P recovery from aerobic WAS with the goal of creating a carbonate apatite (cAp) apatite mineral, should involve a polyP extraction and /or hydrolysis step. To study the extraction of polyP from the dewatered aerobic WAS, thickened waste activated sludge (TWAS) from the Robert O. Pickard Environmental Centre (ROPEC, Ottawa, Figure 4-1) that includes iron salts added at ROPEC for chemical precipitation of Pi was used to perform this polyP extraction study.

The experimental section in this chapter aims to extract and measure the polyP and then convert it to Pi. Using different polyP extraction methods, polyP was quantified and compared with total phosphorus in the TWAS to estimate the fraction of polyP to total P in the TWAS and therefore estimate the efficiency of the extraction methods. The literature reports that most P-extraction

techniques for wastewater sludge are to increase or decrease the pH to accelerate P-hydrolysis and solubilize it as Pi (Table 4-1). So the effects of pH and the metal-ion chelating agent ethylene diamine tetraacetic acid (EDTA) on Pi release from TWAS and TWAS pellets was evaluated.

In Section 4.3.1, the TWAS supernatant Pi concentration was measured, and the possibility of extracting P from TWAS by using a 1:1 vol/vol 1 M HCl:TWAS mixture at a temperature of 118°C, as performed by Meouch (Meouch 2016). This experiment was expanded to determine the 0.5 M HCl acid-extractable polyP content in TWAS solids by measuring the extractable P in this heated 0.5 M HCl digestion from the centrifuged TWAS pellet that was resuspended in distilled and deionized water (ddH₂O).

Following the confirmation of feasible P-extraction, total P was estimated by acid digestion with persulfate acid or concentrated HCl or extraction by EDTA followed by acid hydrolysis (Section 4.3.2.). Revised and modified published polyP extraction techniques with hot water (Section 4.3.3) and a sequenced biochemical extraction (Section 4.3.4.3.4) were also tested.

The results of this experiment suggested that there was a significant metal-phosphate content in TWAS. It is known that acid digestion hydrolyzes P-compounds indiscriminately, but alkaline extraction may target polyP extraction (Pick, Bental, Chitlaru, & Weiss, 1990), as well as dissolve Pi from precipitated metal phosphates (Sano et al., 2012). Sano et al. extracted phosphate from the iron phosphate content in wastewater sludge under alkali conditions of varying concentrations (0.5 – 5 % NaOH) and for various time points (10 - 120 min) at room temperature. So its effectiveness for polyP extraction and hydrolysis, as well as its potency for dissociating phosphate from iron phosphate precipitates in ROPEC was tested. TWAS was investigated with alkaline extraction, followed by acid hydrolysis. Acid hydrolysis of alkaline-dissolved Pi from metal phosphates would not increase the extracted Pi concentration, because it is a simple iron salt dissolution process, but acid hydrolysis of alkaline-extracted polyP would increase the Pi concentration, as it polyP degradation generates new Pi molecules (Section 4.3.5.).

4.1.2. Waste Activated Sludge Samples and Their Origin

In this chapter, all municipal wastewater sludge samples are TWAS, and were collected at various times from ROPEC. At ROPEC, chemical precipitation is performed to precipitate P_i from wastewater by adding soluble $FeCl_3$ at the point where the waste activated sludge (WAS) is recycled to the activated sludge aeration tanks (Figure 4.1). It should be noted that iron chloride is added before thickened waste activated sludge (TWAS) is produced by centrifugation of WAS. It was also learned that aluminum hydroxide from the Lemieux Island potable water treatment plant is sent to ROPEC, which represents a significant iron and aluminum content for phosphate precipitation (personal communication, ROPEC). After the secondary clarifier, the WAS is dewatered by centrifugation to become TWAS.

The fresh TWAS samples were distributed into volumes of approximately 40 mL in ten, 50 mL closed centrifuge tubes and stored at 4 °C (Model 146, low-temperature incubator, Fisher) until the next collection of fresh TWAS. The initial P_i concentration of the supernatant was measured with colorimetry (Section 4.3.1). Seasons, optimal storage conditions (de Haas & Dubery, 1989) or other unknown factors might have impacts on P hydrolysis in the sludge. The differences in each sample's initial P_i concentration is likely due to inherent errors that are not the subject of this thesis. The impact of ddH₂O water is assumed to be negligible for extraction though water might have the ability to hydrolyze P. Variabilities in sludge samples should be expected, due to variations in sampling times during the year. This variability was not controlled in this work because of the different utilization date, the amount of pellet in the TWAS or other unknowns factors from wastewater.

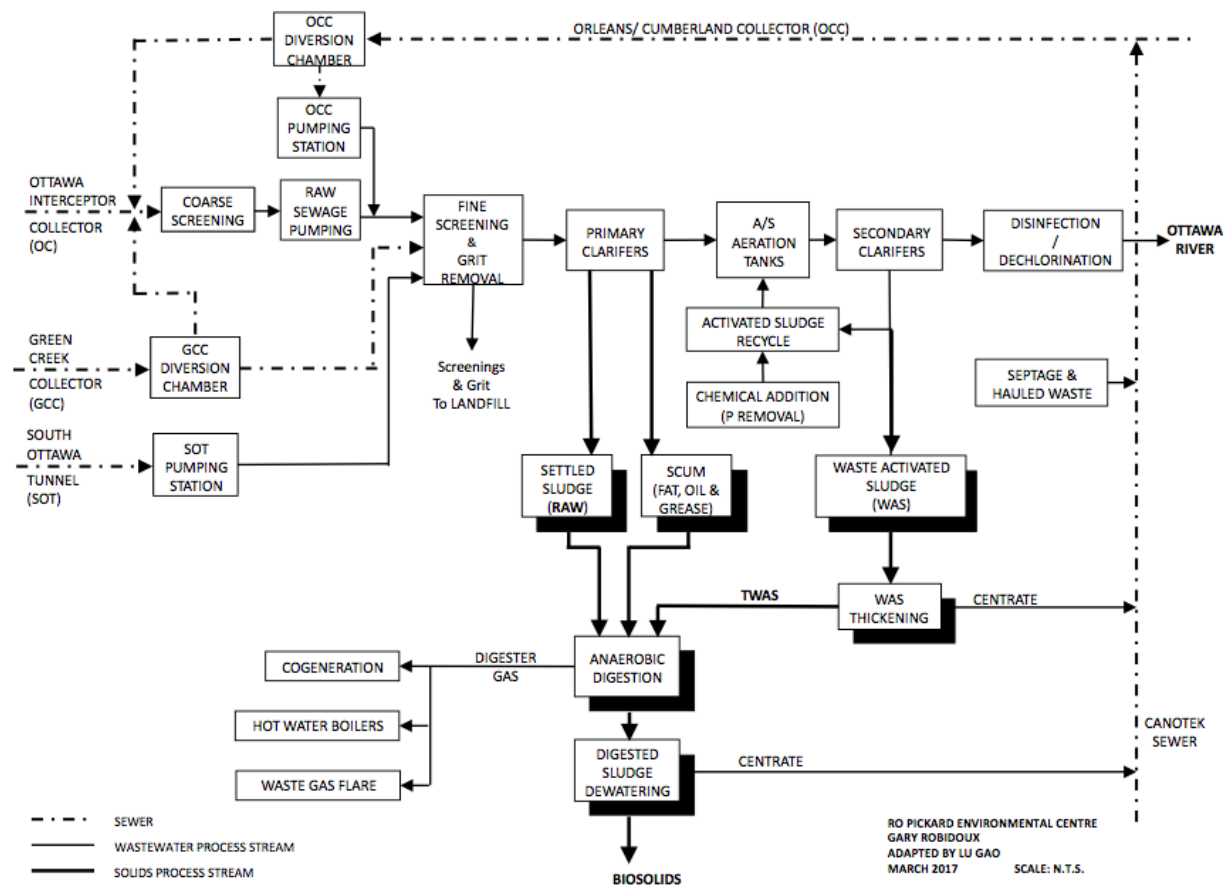


Figure 4-1 The process flow diagram for the Robert O. Pickard Environmental Centre (ROPEC), March 2017.

4.2. Literature Review

PolyP is used extensively in industry for many applications such as inhibition of scale in water pipes, and strong metal chelation properties (Kulakovskaya, Vagabov, & Kulaev, 2012). In recent years, attention from academic and industrial researchers is directed to P-recovery through microbial polyP production in municipal wastewater sludge (Kuroda, Takiguchi, & Gotanda, 2002). The developments of P-recovery through polyP-production from these biological wastes remains largely in the research and developmental stages. In contrast, the P-extraction techniques and measurement of various P-relative fractions for other research areas, such as studies of P in lakes, soils or cells, have been developed over several years. Unfortunately, there are still no standard protocols for P-extraction, or agreements about the results of P compositions by extractions from any of the P-pools, such as polyP or other organic-P compounds. With no consensus on a “gold standard” technique for polyP extraction, a range of published methods was applied to TWAS.

PolyP is difficult to extract and quantify from biological samples because it can be bound in different forms within one biological sample. It can have variable chain lengths, can be bound to multivalent metal ions or monovalent cations, or reside within cellular modules. All of these factors increase the difficulty of the extraction and identification of polyP in biological samples. Therefore, the older polyP extraction methods required a series of extraction techniques including pre-treatments of samples that included purification steps (Majed, Li, & Gu, 2012). The methods of extraction and purification of polyP in the biological materials such as cells are summarized by Kulaev et al., along with different analytical methods for polyP identification and quantification (Kulaev, Vagabov, & Kulakovskaya, 2005).

PolyP extraction remains a difficult process, largely because the mechanisms nor dynamics have not been completely understood for polyP extraction. The polyP extraction processes also were observed to increase the polyP hydrolysis rate into P_i . Dissolved polyP is even suspected to degrade into P_i at neutral pH (Hupfer & Gächter, 1995). The thermal effect on P hydrolysis was reviewed, with a view to improve on waste activated sludge dewatering and digestion (Neyens & Baeyens, 2003).

According to the activated sludge literature, polyP is considered to be a major P-component when compared to other intracellular and extracellular P components (Röske, 1994; Serafim et al., 2002; Uhlmann, Röske, Hupfer, & Ohms, 1990). In principle, this polyP content should be hydrolyzed into Pi in the anaerobic digesters. However, the Pi concentration after polyP hydrolysis might be still low, because of the excess of metal ions in solution that precipitates with the hydrolyzed Pi. In the review paper by de Haas et al. (de Haas, Wentzel, & Ekama, 2000a), the chemical precipitation with soluble metal salts was also used to improve the biological P removal (BNR) process in practice. This review also summarized polyP extraction techniques for characterization of P fraction in the WAS with two categories: if the method included cold perchloric acid or not. The cold perchloric acid was concluded to discriminate the P from Pi from chemical precipitation, and the P from biological P removal of polyP by its dissolution from an insoluble metal-polyP complex (de Haas & Dubery, 1989; de Haas, Wentzel, & Ekama, 2000b; Mino, Kawakami, & Matsuo, 1985). The idea of cold perchloric acid might be to decrease the rate of acidic polyP hydrolysis. Detailed methods for intracellular P extraction and fractionation are described, for components such as nucleic acids and phospholipids (Mino et al., 1985).

In Table 4-1, selected polyP extraction techniques from WAS are summarized. The impact of stirring, temperature, soil concentrations and the natural property of P-release on P-extraction was first discovered in 1967 (Shapiro, Levin, & G, 1967). Research on the effect of acid and alkaline conditions on phosphatase (Pi-cleaving) activity in anaerobic digesters, resulted in an empirical result that could provide a rapid assessment of abnormal operating conditions (Ashley & Hurst, 1981). Enzymatic degradation of polyP to Pi could also increase the dissolved Pi concentration (Hill, Benefield, & Jing, 1989). ³¹P NMR was used to conclude that polyP is the major fraction in hot water extraction of WAS from biological P removal processes (Uhlmann et al., 1990). Simultaneous chemical addition in biological P-removal processes could lead to significant improvement of the P-removal of biological P removal processes, although this impact was reported to be negligible for metal concentrations less than 3 mg/L Fe or Al (Röske, 1994). Sequential extraction protocols for P-species were developed for soil, but also applied to sediments (Pettersson, Boström, & Jacobsen, 1988). Sequential P- extraction with EDTA (Reina, Serrano, & Golterman, 2011) was first developed for algal cultures, modified for sediments with

Goltermans'²² procedure for polyP quantification. It should be noted that the complex mixture of P-compounds in lake sediment makes it impossible to dissolve all P-compounds, or to extract one type of P-molecule without altering the others (Reina et al., 2011).

The sequence extraction (Psenner, Pucsko, & Sage, 1984) is considered to be one of the classic techniques for P-extraction from sewage sludge (Röske, 1994; Uhlmann et al., 1990), though it was originally designed for analysis of lake sediments. This procedure is used to estimate the P fractions of WAS produced by simultaneous chemical precipitation and biological P removal (de Haas, Wentzel, & Ekama, 2000b). The first step is a cold water wash to remove dissolved Pi in order to eliminate the impact of soluble Pi on further P-extraction. The second step is to use bicarbonate-dithionite (BD) to dissolve P from iron phosphate salts and to desorb Pi from Fe(OH)₃-P complexes, which are produced by excess Fe ions from the process of chemical precipitation. The third, alkaline step is to hydrolyze the P content in polyP, organic P, as well as P from Al-P, and to desorb P from Al(OH)₃-P. Soluble aluminum ions can be added to the chemical P removal process to precipitate Al-P, or they may report to the WWTP as a byproduct of potable water treatment. The fourth extraction step is to dissolve P from Ca-P, CaCO₃-P, and Mg(CO₃)-P.

Generally, acid digestion or alkaline extraction is the first step towards polyP extraction and polyP hydrolysis. However, polyP hydrolysis during the extraction step would underestimate the extracted polyP content measured after extraction, if it is converted to Pi during polyP extraction (Ahlgren et al., 2007; Bonting, Kortstee, & Zehnder, 1991; Clark, Beegen, & Wood, 1986; Hupfer, Glöss, Schmieder, & Grossart, 2008; Lockwood, Lindrea, & Seviour, 1990; Mino et al., 1985). It should be noted that it is likely that strong acid polyP extraction processes with long retention times might also digest other P-fractions. In this case, polyP could be quantitatively overestimated with the higher final Pi concentration. Gel electrophoresis is a technique to separate the variable chain lengths of polyPs (Clark & Wood, 1987). However, the complexities of sample preparation and determination of the loading density resulted in this powerful

²² Personally speaking, H L Golterman is legend, whom studied P extraction in the lake sediments for decades. About himself and his researcher, see at Gulati, Ramesh D. 1995. "Dr Han L. Golterman, a Limnologist: a Tribute on the Occasion of His 65th Birthday and Retirement." *Hydrobiologia* 315 (1). Kluwer Academic Publishers: 1–6. doi:10.1007/BF00028624.

technique not being undertaken in this thesis, although the ability to measure polyP chain length with this technique might be helpful to better understand the polyP extraction processes.

As mentioned earlier (Chapter 2), the rise of chemical precipitation (mainly with iron chloride) in the wastewater treated process avoids the common and serious problem of eutrophication, as happened the Great Lakes in North America. Both of Fe(II) and Fe(III) bind with soluble Pi to precipitate with completed hydroxide (OH^-) (Wiechers, 1986). However, the ferrous (Fe^{2+}) ions are assumed to oxidize to ferric (Fe^{3+}) ions in aerobic conditions (Yeoman, Stephenson, Lester, & Perry, 1988). Rasmussen and Nielsen (1996) also reported that Fe^{2+} could not be detected in the supernatant of fresh combined sludge from biological nutrient removal with additional ferrous sulfate system (Rasmussen & Nielsen, 1996). Moreover, Fe^{2+} was the minor, but Fe^{3+} was a detectable major component of 70-90% total iron in the fresh sludge (Rasmussen & Nielsen, 1996). $\text{Fe}(\text{OH})_3$ also slowly exchanges OH^- with Pi (Rabinowitz, van Rooyen Marais, University of Cape Town, 1980) to support the P removal process. Soluble Fe is a significant chemical precipitation reagent for the ROPEC water treatment process. Its presence as iron phosphate and/or iron hydroxide in TWAS may affect polyP extraction results. Figure 4-2, Figure 4-3 and Figure 4-4 indicate the speciation of iron and aluminum as a function of pH in water. It provides the evidence that insoluble iron and aluminum salts precipitate at neutral pH, and they dissolve in strong alkaline conditions as the $\text{Fe}(\text{OH})_3$ and $\text{Al}(\text{OH})_3$, respectively. It predicts that the ortho-Pi in the Fe- or Al-P precipitates is dissolved and might be extractable during alkaline extraction. It also reveals that pH value is an essential parameter to decide the speciation of Fe and Al.

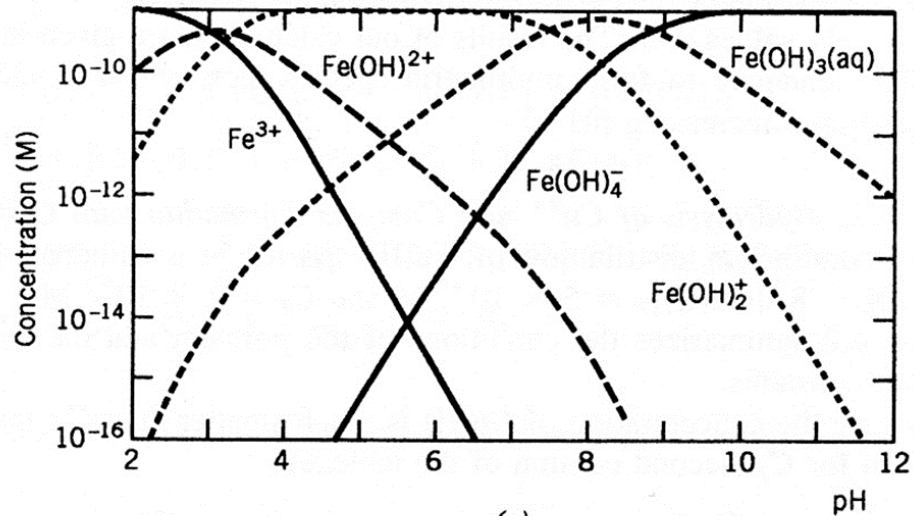


Figure 4-2 Iron speciation diagram as a function of pH in water. From Demopoulos, G.P. Hydrometallurgical Engineering course notes, McGill University, 1998

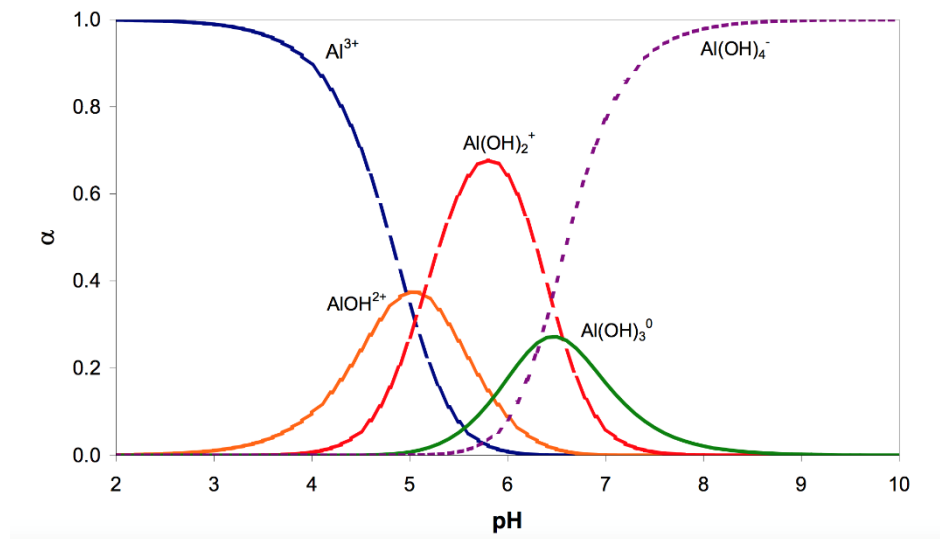


Figure 4-3 The distribution of Al-H₂O considering only mononuclear species as a function of pH (Holt, 2006).

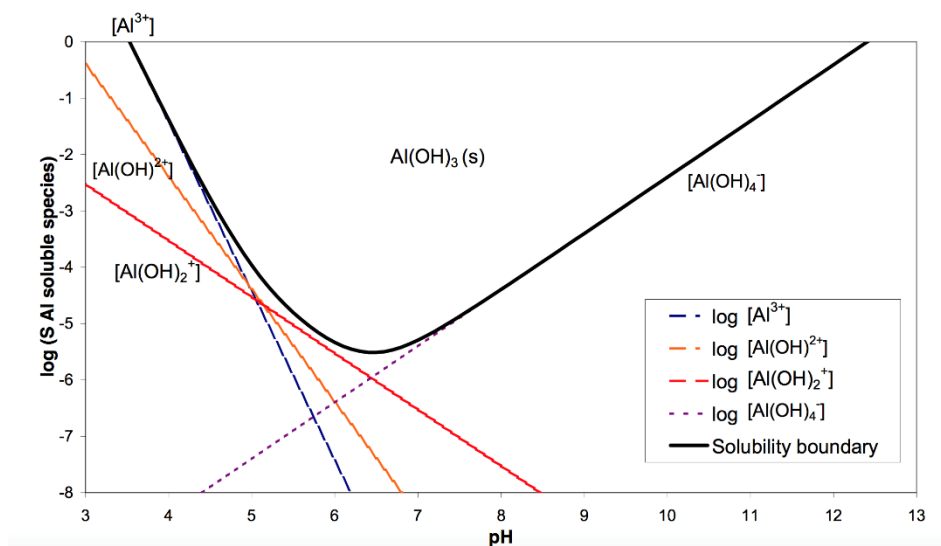


Figure 4-4 The solubility of aluminum hydroxide (Al(OH)_3) as a function of pH. The boundary of solubility represents the thermodynamic equilibrium. The 0.03 mg Al/L of minimum solubility occurs at 6.3 of pH value and it increases as the solution becomes more alkaline or acidic (Holt, 2006).

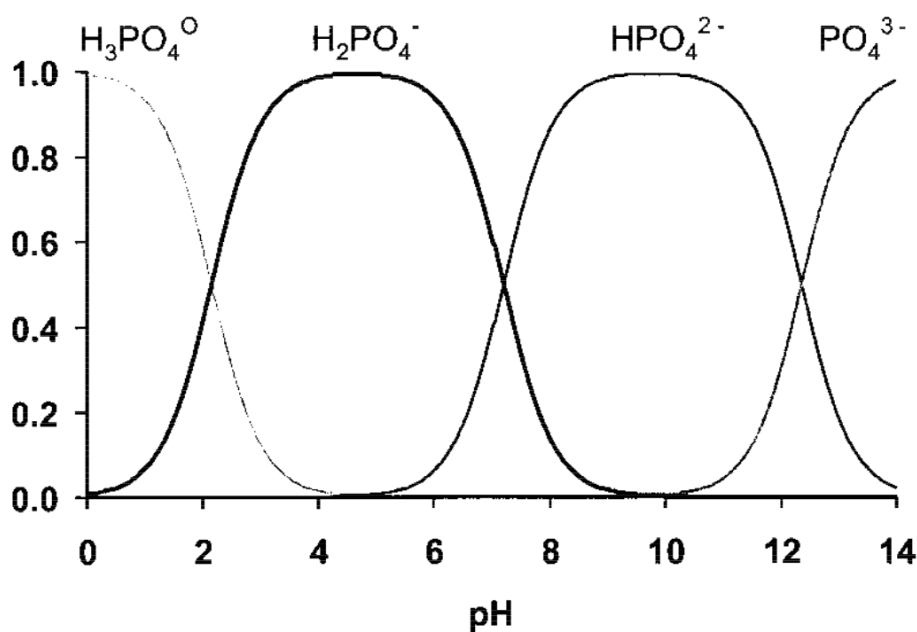


Figure 4-5 The speciation of Pi as the mole fraction of total phosphorus as the function of pH in the solution (Hinsinger, 2001).

The literature reports that the most common P-extraction technique in the sludge is to add NaOH (e.g., M. Kim, Han, & Kim, 2015; Sano et al., 2012) or to add strong acids (e.g., Latif, Mehta, & Batstone, 2015; Ntuli, Falayi, Mhlungu, & Thifhelimbilu, 2014; Ottosen, Kirkelund, & Jensen, 2013; Shanableh & Ginige, 1999; Shiba & Ntuli, 2016; Vaneeckhaute, Zeleke, Tack, & Meers,

2016) to control the pH to accelerate P-hydrolysis and solubilize it as Pi (e.g., Ali & Kim, 2016; Liao, Mavinic, & Koch, 2003; Stark, 2005b; 2005a; Stark, Plaza, & Hultman, 2006; Y. Xu et al., 2015; X. H. Zhao & Zhao, 2009). Therefore, the acid and base are the classical reagents to extract P from sludge. Besides, the effects of pH and the metal-ion chelating agent ethylene diamine tetraacetic acid (EDTA) on Pi release from TWAS and TWAS pellets was evaluated.

Table 4-1 Summary of P-extraction methods for waste activated sludge

Reference	Type of Extraction	Objectives	Chemicals &/ Fraction	Sample	Supplements
(Shapiro et al., 1967)	Physical impacts of P released in anoxic conditions	Discovery of the phenomenon of higher Pi concentration from secondary clarifier after aeration	Stirring and elevation of temperature may stimulate P release, but external added P may not.	Aerobic sludge from Baltimore WWTP, MD, USA	The background: (Feng, 1962), (Sekikawa et al, 1966), (Levin and Shapiro, 1965), (Srinath et al, 1959) and (Vacker, 1967)
(Ashley & Hurst, 1981)	pH controlled of extraction	Detection of phosphatase activity extraction to rapidly test uncommon operating conditions of anaerobic digesters	pH adjusted of 4.2, 6.6, 9.4 and 11.4 with 0.2 M NaOH	Anaerobic sludge from Naburn, York and Wakefield, Yorkshire, England	Total P: {CHEN:1956jl}. Acid and alkaline phosphatase assays: {Bessey:1946vy}
(Van Groenestijn, Bentvelsen, Deinema, & Zehnder, 1989)	Pre-treatment of PAOs cells by EDTA	Discovery the polyP-degrading enzymes in WAS	Based on (F. M. Harold & Harold, 1965)	Anaerobic sludges from 5 WWTPs, Netherlands	Sample preparation: {Harold:1965to}. Pi and total P (persulfate): {Association:2013ub}.
(Hill et al., 1989)	Sonication, EDTA and hypochlorite treatment	³¹ P-NMR: to investigate the characteristics of polyP in EBPR processes.	NMR: variable ways to wash and centrifuge samples with 1 mM KNO ₃ and 0.1 M EDTA	Activated sludge (EBPR) in bench-scale (SBRs), City of Auburn WWTP	Cell treated with EDTA: Cell Disruptor by Branson Sonifier
(Continued)					

(Uhlmann et al., 1990)	Sequence extraction	Discovery of the P fractions in WAS	(1) Cold water; (2) bicarbonate-dithionite; (3) NaOH; (4) HCl	WAS (bio-removal with added FeSO ₄), Munchehofe, Liebenwerda	Based on Psenner et al. (1984, 1988)
(Röske, 1994)	Sequence extraction	Explore the relationships between PAOs and bio-chemical combined sludge and precipitation	(1) Cold distilled water (CW); (2) Bicarbonate-dithionite (BD); (3) 1 M NaOH; (4) 0.5 M HCl	Combined and bio-sludge at the full-scale Mfinchhofe plant and Marienfelde plant	Based on Psenner et al. (1984) and modified (Uhlmann et al., 1990)
(Kuroda et al., 2002)	Heat	An easy way to extract polyP and to reuse P from WWTPs	Heat with 60 °C for 1 hour as the optimal condition	All-and-draw type reactor of the bench-scale EBPR process.	
(Reina et al., 2011)	Sequence extraction	PolyP extraction and quantification	(1) Ca-EDTA with 1% dithionite; (2) Na ₂ -EDTA; (3) H ₂ SO ₄ ; (4) Trichloroacetic acid (TCA) (4 °C); (5) NaOH (90 °C)	WAS from WWTP (E.D.A.R. Tablada, Sevilla)	The EDTA method: (Golterman 1996) with modified for poly-P as proposed by Golterman (2006).

Table 4-2 The summary table for the classical P-extraction methods and comments (Pettersson et al., 1988).

	Extraction	Proposed fraction	Shortcomings
1. Chang and Jackson (1957)	a. NH_4Cl 1 mol/l b. NH_4F 0.5 mol/l pH 8.2 c. NaOH 0.1 mol/l d. HCl 0.5 mol/l e. CDB f. NaOH	labile P Al-bound P Fe-bound P Ca-bound P reductant-soluble P refractory P	extraction of Fe-P by NH_4F resorption by CaF_2 precipitation of phosphate with iron
2. Williams <i>et al.</i> (1971)	a. NaOH/NaCl 0.1/1.0 mol/l b. CDB 85 °C c. HCl 0.5 mol/l	non-occluded Fe-, Al-P reductant-soluble P apatite P	resorption by carbonates in calcareous sediments Ca-P released by CDB
3. Williams <i>et al.</i> (1976)	a. CDB 0.22/1.0 g/0.1 M b. NaOH 1 mol/l c. HCl 0.5 mol/l	non-apatite P apatite P	resorption by carbonates in calcareous sediments Ca-P and org-P released by CDB
4. Hielijes and Lijklema (1980)	a. NH_4Cl 1 mol/l pH 7 b. NaOH 0.1 mol/l c. HCl 0.5 mol/l	labile P Fe- and Al-bound P Ca-bound P	dissolution of small amounts of Fe-P and Al-P by NH_4Cl
5. Psenner <i>et al.</i> (1985)	a. H_2O b. DB 0.11 mol/l 40 °C c. NaOH 1 mol/l d. HCl 0.5 mol/l e. NaOH 1 mol/l 85 °C	water-soluble P reductant-soluble P Fe- and Al-bound P Ca-bound P refractory P	resorption by carbonates in calcareous sediments

4.3. Materials, Methodology, and Results

4.3.1. The feasibility of 0.5 M HCl extraction from TWAS and TWAS solids

4.3.1.1. Introduction

The protocol used for measurement of dissolved inorganic phosphate (Pi) is the vanadomolybdate assay (Robinson, Roughan, & Wagstaff, 1971) modified by Tomson (Tomson, Barone, & Nancollas, 1977) (details in Appendix E). This protocol was used to measure dissolved Pi in TWAS supernatant, as well as in extracted and hydrolyzed samples.

Protocols for acid digestion, also known as acid hydrolysis, have been developed to break down the bacteria cell, extract and solubilize the intracellular polyP or organic phosphate fractions, and then to convert these P-compounds to soluble inorganic phosphate to be measured (Table 4-1). The mechanisms for polyP extraction and hydrolysis from TWAS have not been clarified, so as a starting point, TWAS samples were acid digested with a 1:1 volume mixture of 1 M HCl and TWAS and heated at 118°C for 1 h to compare the results with those of Meouch (Meouch, 2016) and to measure the variability between TWAS samples. PolyP concentration was measured by the change in soluble Pi concentration caused by a 1.7 M HCl (final concentration when HCl is mixed with the sample) acid hydrolysis at 100 °C for 7 min.

1.3.1.2. Material and Methodology

To measure the initial Pi concentration in the supernatant of the untreated TWAS, 1 mL of TWAS was centrifuged in a 1.5 mL microcentrifuge tube at 6,000 rcf, for 5 min. It should be noted that the Pi test of the supernatant of the TWAS sample required solubilization of the organic components with Triton X-100 to suppress the precipitation of organics caused by the acidic Pi indicator solution. In order to eliminate the effect of the additional volume on the colorimetric assay, regardless of the dilution factor of the sample, the volume of 10% Triton X-100 always was 25 µL for all samples on the 96-well plate. Therefore, the sample has to be diluted 2 times at least on the plate because the total volume of the sample and diluted solution is 50 µL for the vanadomolybdate assay as the until of mM Pi. The detail of dilution volume and the colorimetric agents are recorded in the Appendix E.1 with the microplate spectrophotometer (Epoch, BioTek®).

The 0.5 M HCl hydrolysis of TWAS and the solid fraction of TWAS was conducted to determine if the same content of the 0.5 M hydrolyzed P was from the TWAS solids. The 50 mL TWAS sample tube was shaken before TWAS was pipetted into a microcentrifuge tube. After centrifugation of 1 mL TWAS in a 2 mL microtube at 6,000 rcf for 5 min, the pellet volume was estimated to be more than 0.4 mL and less than 0.5 mL. It was noted that with longer storage times, the centrifuged pellet volume decreased. New TWAS was collected from ROPEC if the pellet was less than 0.3 mL per 1 mL TWAS.

The acid digestion solution was made by mixing 500 μ L of 1 M HCl with either 500 μ L of TWAS or a centrifuged TWAS pellet, with the supernatant decanted, and replaced with an equivalent volume of ddH₂O to create a 500 μ L sample of TWAS pellet + ddH₂O. These acidified samples were pipetted into a screw neck Pyrex tube with a Teflon-lined cap, and placed in an oven at 118°C (Precision® Gravity convection oven, economy model) for 1 h (Meouch, 2016). The acidified sludge samples were cooled, transferred to a 1.7 mL or 2 mL microcentrifuge tube, and centrifuged at 6,000 rcf for 5 min to sediment the residual undissolved solids. The Pi assay without adding Triton was conducted following the protocol.

4.3.1.3. Result and Discussion

In the group of TWAS hydrolysis, the initial Pi concentration of TWAS supernatant was 0.79 ± 0.86 mM (n=12). Then, the Pi concentration after 0.5 M HCl acid digestion of similar samples of TWAS pellet suspended in ddH₂O water was 43.68 ± 16.30 mM (n=12) (Figure 4-6). This digested result is not statistically significantly different from previous results from the laboratory (Student's t-test, $P=0.25>0.05$, Table G-1 in Appendix G.1). The analysis of the initial supernatant Pi concentration for the experiments that analyzed the acid-hydrolyzable P-content in the TWAS pellets was 1.22 ± 1.02 mM (mg P/L) (n=8) (Figure 4-6). The Pi concentration after acid hydrolysis of the TWAS was 55.7 ± 13.0 mM (n=12).

However, this difference in acid-digested Pi concentration is an unexpected result ($p>0.05$, Table G-2 at Appendix G.1). The only distinction between these two groups was assumed to be the initial soluble Pi from wastewater. However, the initial supernatant P values for the TWAS and TWAS pellet samples are not statistically different ($p=0.21 >0.05$, Table G-2 at Appendix G).

Besides, the difference of acid-digested Pi concentration is about 12 mM, which is much more than the initial soluble Pi. There are two ways to explain this result. One is that the centrifugation force was not large enough to separate the soluble and insoluble P-rich components. Some P-rich small particles might remain in the supernatant, and contribute to the total P value for the supernatant. Another is that beside soluble Pi in the supernatant, the other P-compounds are also soluble (e.g. organic P). These P-compounds cannot be detected by the colorimetric Pi-assay. During the acid digestion of whole (S non-removed) TWAS samples, these soluble P could be degraded to Pi, which could be measured.

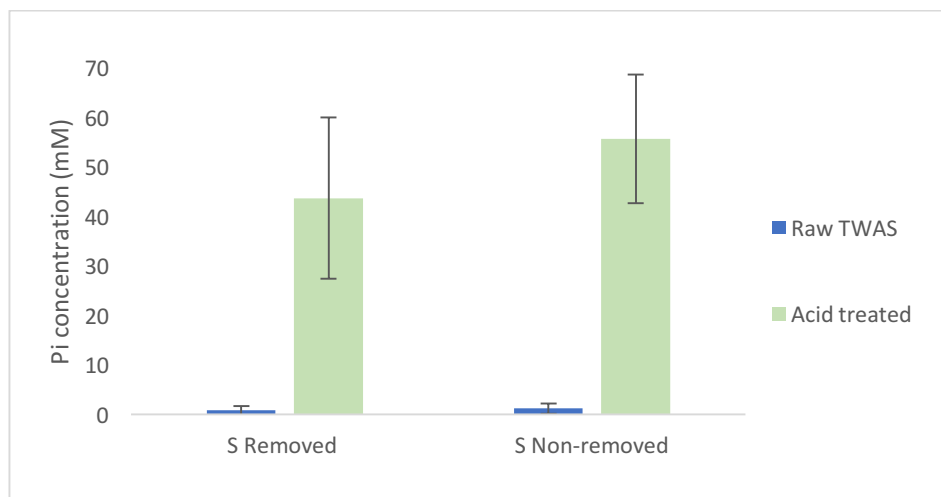


Figure 4-6 Feasibility assay of extracting P from TWAS by acid digestion in 0.5 M HCl between 110-120° C for 1 hour. “S” refers to the TWAS supernatant.

4.3.2. Total Phosphorus

4.3.2.1. Total Phosphorus - Persulfate Digestion

4.3.2.1.1. Introduction

In the last section, acid digestion as a promising method of extracting P from TWAS was demonstrated. In this section, the goal was to measure the total P extractable in the TWAS samples, including organic P that requires oxidative conditions (Müssig-Zufika, Kornmüller, Merkelbach, & Jekel, 1994). The U.S. Environmental Protection Agency (EPA) publishes many standard protocols to measure different components related to water quality. This experiment used a modified version of the standard protocol “SOP Phosphorus, Total, Persulfate Digestion / EPA/600/R-93/100, Method 365.1” for total phosphorus digestion for a liquid sample, including

sludge and wastes (EPA, 2016). However, there was an incubation condition of 15 - 20 psig (pounds per square inch) pressure in this protocol, which was not available in the laboratory. Therefore, a modification was undertaken to extend the digestion retention time to determine if this would compensate for the atmospheric pressure conditions for this digestion.

4.3.2.1.2. Material and Methodology

The material and method followed EPA (SOP Phosphorus, Total, Persulfate Digestion / EPA/600/R-93/100, Method 365.1) (EPA, 2016), except for its execution at ambient pressure and extended reaction times. The working solutions were prepared daily. The reagents were prepared in small volumes to perform the preliminary tests. The stock sulphuric acid was 25 mL of 5.6 M acid, produced by diluting 7.61 mL of 98% (18.4 M) sulphuric acid, filling to the final volume with dd H₂O, in the fume hood. 25 mL of working acid was composed of 8 mL 5.6 M stock sulphuric acid and 3.2 g ammonium persulfate ((NH₄)₂S₂O₈, Fisher), diluted with dd H₂O. Following the EPA protocol, 1 mL untreated TWAS sample was placed in a screw neck tube with a Teflon-lined screw cap with 0.685 mL acid solution. This was placed on a lab bench dry incubator with temperatures that varied between 110-120 °C. Each of two samples was heated for 0, 20, 40, 60, 80 or 100 min. Samples were cooled down with a cold-water bath immediately after their time point of heating. Samples were transferred to microtubes and centrifuged (6000 rcf, 5 min) to sediment residual solids before the Pi concentration in the supernatant was measured with the vanadomolybdate method (calculation exemplifying at Appendix E, Section E-2).

4.3.2.1.3. Result and Discussion

Figure 4-7 plots the total Pi concentration versus the acidic, oxidative, hydrolysis time. The results were back-calculated in TWAS samples. The average maximum Pi concentration from persulfate digestion measured was 25 mM, which is lower than the digested Pi concentration of 43 mM in the feasibility test (Figure 4-6).

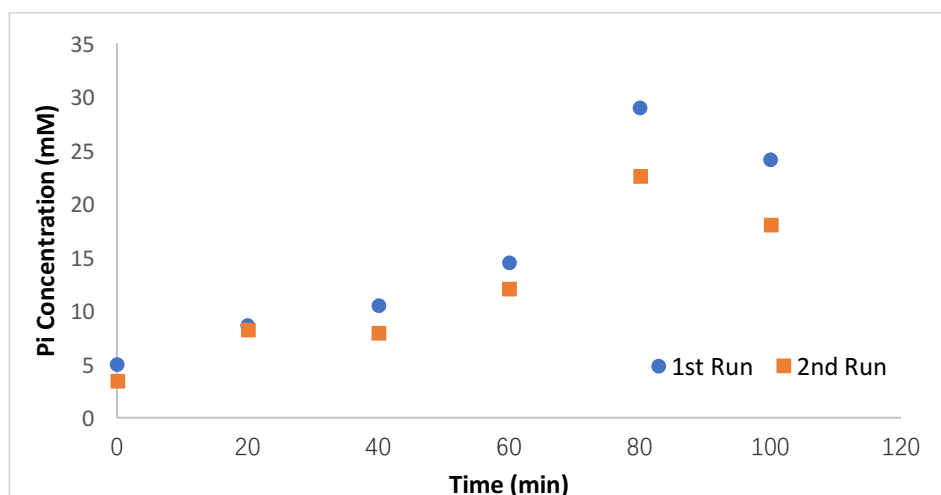


Figure 4-7 Total phosphorus was measured by persulfate digestion from a simplified method from EPA, n=2.

An interesting result is a decrease in soluble Pi between 80 min and 100 min hydrolysis times. This is a difficult result to explain, as it is assumed that there would be an increase in Pi hydrolysis with time, approaching a steady-state value. The reduction in Pi between the 80 min and 100 min time points could be caused by a Pi-precipitation event, the use of an ambient pressure instead of a recommended pressurized hydrolysis, or another unidentified error source. After 2 repeats to confirm this reduction in Pi at 100 min, this method of Pi hydrolysis was abandoned. According to the following results in the next sections, it is concluded that the solubilized Pi generated by 80 min likely precipitated, possibly due to the iron and aluminum ions in the TWAS.

4.3.2.2. Acid Digestion – Effect of Varying HCl Concentration on TWAS and TWAS Pellet P-Extraction

4.3.2.2.1. Introduction

For acid-hydrolyzable P measurement of waste activated sludge samples, Uhlmann *et al.* (1990) compared their modified method (Table 4-1) with the method of (Psenner *et al.*, 1984). The protocol of Psenner *et al.* uses a NaOH-extraction, while the Uhlmann *et al.* protocol uses HCl-extraction. The use of HCl to extract polyP from waste activated sludge is supported by the HCl-P-extraction of Harold for other microorganisms (F. M. Harold, 1960).

The feasibility experiments (Section 4.3.1) gave the interesting result of HCl-hydrolyzable P in the TWAS. The TWAS from ROPEC was collected end of the stage of the secondary

clarification, before which iron salts are added for P-removal. Aluminum ions are also a component of TWAS due to the disposal of aluminum hydroxide from the Lemieux potable water treatment plant into the Ottawa sewer system (personal communication, ROPEC). During an acid extraction process of TWAS, the solubility of iron and aluminum phosphates likely increases as iron and aluminum phosphates dissolved, which would increase the extracted soluble Pi result (de Haas, Wentzel, & Ekama, 2000b). It is also possible that P-adsorbed to metal hydroxides and other organic polymers in TWAS is released by this acid hydrolysis (de Haas, Wentzel, & Ekama, 2000b).

Meouch measured the acid hydrolyzed Pi concentrations when the acid hydrolysis was undertaken with 2 M or 4 M of HCl or H₂SO₄, mixed in a 1:1 volume ratio with TWAS, and heated (Meouch 2016). Based on her data, the acid type and 2 or 4 M concentration did not significantly change the acid-hydrolyzed P value of 55 mM (Meouch, 2016). It is possible that high concentrations of these two strong acids generated the maximum acid-digestible Pi. The goal of this section is to measure the Pi extracted with acid hydrolysis conditions of less than 0.5 M HCl produced by mixing 1:1 volume ratios of TWAS with 1 M HCl or less concentrated HCl at room temperature, in order to determine the minimum HCl concentration required for maximum Pi extraction. A cold perchloric acid extraction process was reported to release chemically-bound Pi as well as polyP (de Haas, Wentzel, & Ekama, 2000b), so it was thought that heat was not required. A cold extraction process was used to reduce polyP hydrolysis during P-extraction in the acidic conditions (de Haas, Wentzel, & Ekama, 2000b).

The hypotheses for this section are (1) the extracted Pi concentration increases with increasing HCl concentration to a stable value, (2) decanting the TWAS supernatant after centrifugation, will remove soluble iron that could precipitate with Pi released by acid hydrolysis, (3) At the same time, the potential effects of centrifugation may also adversely affect (Peterson, Sharma, van der Mei, & Busscher, 2011) the gram-negative bacteria at least²³, causing them to release their intracellular polyP.

²³ Because the gram-negative bacteria have thinner wall than gram-positive bacteria.

4.3.2.2.2. Materials and Methodology

500 μL of TWAS samples were placed in 1.7 mL or 2 mL microcentrifuge tubes. The samples were centrifuged at 1,200 rcf for 5 min. For one group of samples, the supernatant was decanted, and replaced with deionized and distilled, H_2O up to 1 mL in total, while the other pellet was re-suspended in its supernatant. The acid digestion step was conducted by mixing the TWAS or TWAS pellet mixed in dd H_2O with 500 μL of 0.01, 0.05, 0.1, 0.5, or 1 M HCl for 3 hours at room temperature. The acidified samples were centrifuged again at the same speed and time length to isolate the supernatant and sediment the particles before the Pi assay.

4.3.2.2.3. Result and Discussion

Figure 4-8 demonstrates a trend of increasing Pi concentration with increasing HCl concentration. The hypothesis is increasing HCl concentration increased acid hydrolyzed Pi concentration was supported for the TWAS sample and for the TWAS sample with the supernatant removed. A two-way ANOVA was used to determine the effects of supernatant removal ($p=0.040 < 0.05$) and the concentration of HCl ($p=0 < 0.05$) on the Pi released by acid hydrolysis. Therefore, the impact of removing supernatant and varying the concentration of HCl cannot be neglected. The reason for this result is not understood, as it was assumed that removal of dissolved components in the supernatant could remove the potential for Pi-precipitates to form during hydrolysis.

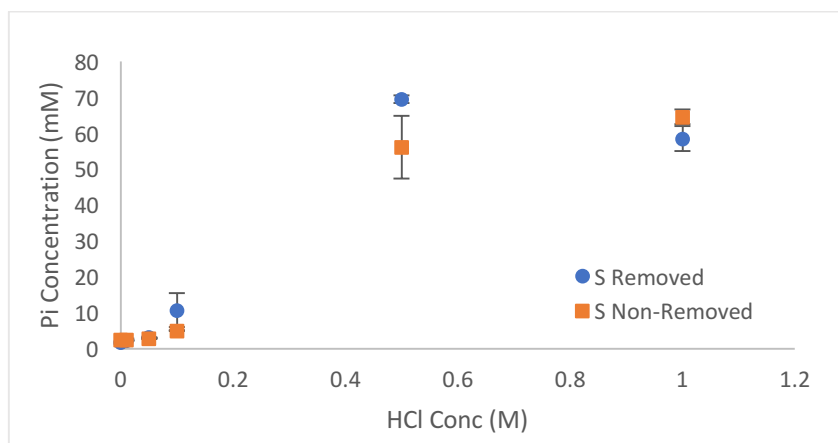


Figure 4-8 Acid hydrolyzable Pi by different HCl concentrations. Except for the control group of dd H_2O ($n=1$), other trials were repeated ($n=4$). “S Non-removed” refers to the control group, for which acid digested the entire TWAS sample. “S removed” refers to the experiment group, for which acid digested was of the pellet suspended in dd H_2O .

However, although the differences are visible in Figure 4-8, the hypothesis that S-removal (and therefore dissolved metal ion removal) would increase the dissolved Pi concentration in acidic digestion was too complex to prove without a two-way ANOVA analysis to show their significant difference (Appendix G-2, Table G-9). For example, in the Figure 4-8, the S-removed has a higher Pi concentration than the S non-removed group with 0.01 M HCl digestions ($p=0.28 > 0.05$, Table G-8) and 0.5 M HCl ($p=0.091 > 0.05$, Table G-10), but a Student's t-test did not prove the significant difference between them. Furthermore, although the extracted Pi seems lower for the S-removed sample digested with 1M HCl, there is no statistical difference between these results ($p=0.84 > 0.05$). The acid-hydrolyzed TWAS pellet (S removal group) had no statistical difference in the Pi concentration between 1 M HCl acid and 0.5 M HCl acid ($p=0.39 > 0.05$). In fact, in the Bonferroni test of means comparisons, it proves that regardless of the categories of two groups, there was no statistical difference between 0.05 M and 0.01 M HCl, between 0.01 M and 0.1M HCl, and between 0.5 M and 1 M HCl digestion. These results reveal that more study of acid digestion of TWAS with HCl should be conducted with the additional HCl concentrations ranging from 0.1 M to 0.5 M. The t-test and ANOVA reports are presented in Appendix G.2 - Table G-8, G-9, and G-10, G-22.

4.3.2.3. PolyP Extraction – Combination of EDTA and Acid Hydrolysis

4.3.2.3.1. Introduction

The presence of iron and aluminum in TWAS complicates P-extraction experiments, as Pi has an affinity for iron and aluminum cations, as well as their hydroxyl forms (de Haas, Wentzel, & Ekama, 2000b). To reduce the effect of these multivalent metal cations on P-extraction, EDTA was used as a P-extractant to solubilize metal phosphates (Reina et al., 2011; Ting Zhang, Jian He, Xingzhang Luo, 2017). For the larger purpose of P-extraction from municipal waste activated sludge for P-mineral precipitation, EDTA extraction would not be cost-effective in industrial application. However, it is convenient to work with at laboratory scale because of its ability to bind multivalent metal ions into metal-EDTA complexes, effectively reducing the metal ion free concentration, and allowing Pi and/or other P-species to dissolve. In this section, concentrated EDTA was compared with HCl as a room-temperature Pi extractant from TWAS. The extracted P-forms were examined for polyP content with a 7 min, 100 °C, 1.7 M HCl (total)

acid hydrolysis (Weimberg & Orton, 1965). The difference in Pi concentration before and after the acid hydrolysis represent the Pi released by polyP hydrolysis. EDTA chelates metal ions from many sources, and since WAS is complex material, the interpretation of these data will be limited.

4.3.2.3.2. Material and Methodology

(1) EDTA compared with HCl for Pi extraction

In this experiment, three types of Pi-extraction reagents were tested: ddH₂O, EDTA (Na-EDTA, pH 8) and HCl. 500 µL TWAS was mixed with 500 µL of either ddH₂O, 0.25 or 0.5 M EDTA, 1 or 3.5 M HCl in 2 mL microcentrifuge tubes, with four repeats. The samples were placed on a shaker for 5 days. Samples were centrifuged at 6,000 rcf for 5 min, then the Pi concentration in the supernatant was measured with the vanadomolybdate colorimetry method.

(2) The effect of EDTA concentrations on Pi extraction

Another TWAS sample was used to test the Pi extraction potential of different EDTA (Na-EDTA, pH 8) concentrations. 500 µL of TWAS was mixed with 500 µL of either ddH₂O or 40, 60, 80, 120, 160, 180, or 200 mM EDTA in the microcentrifuge tubes, with four repeats. samples were shaken for 5 days, and after centrifugation, the Pi concentration assay was performed.

(3) Test for PolyP extraction with EDTA

The same EDTA-extraction procedure was undertaken with a third TWAS sample, with EDTA concentrations ranging from 0, 100, 120, 140, 150, 160, 200 mM EDTA. After 5 days of shaking, the samples were centrifuged, and 500 µL supernatant decanted into a new microcentrifuge tube, leaving 500 µL with the pellet. The supernatant and pellet samples were mixed with 500 µL 3.5 M HCl, and placed in a boiling water bath that hydrolyzes poly P (Weimberg & Orton, 1965). The samples were cooled down with an ice-bath after 7 min at 100 °C. All samples were centrifuged, and the Pi concentrations measured with the vanadomolybdate method. The polyP “concentration” is calculated from the difference between the acid-hydrolyzed Pi concentration and the Pi concentration before acid hydrolysis, as this increase in Pi concentration arises from polyP hydrolysis under these conditions. For the ddH₂O

group, n=7, for the 100 mM EDTA group, n=8, and for all other EDTA concentration groups, n=6.

4.3.2.3.3. Result and Discussion

(1) EDTA compared with HCl for Pi extraction

Figure 4-9 presents a comparison of Pi extraction at room temperature with ddH₂O, EDTA, and HCl. The Pi value of TWAS exposed to ddH₂O for 5 days was 3.26 ± 0.52 mM Pi. EDTA successfully extracted Pi from TWAS. A concentration effect was observed. 0.25 M EDTA extracted (54.9 ± 12.9) mM Pi, and 0.5 M EDTA extracted (84.8 ± 4.80) mM Pi. They are statistically significantly different because the p value for the t-test is 0.012 (<0.05). The comparable HCl digestion results were (72.0 ± 9.37) mM Pi for 1 M HCl acid and (87.0 ± 8.25) mM Pi for 3.5 M HCl acid. The Student's t-test p-value for this two group is 0.054, which means they are not statistically significantly different. Besides, the p-value for the comparison of the 0.5 M EDTA and 3.5 M HCl extractions with a Student's t-test was 0.67 (>0.05), which indicates that Pi extracted was not different with these extractants at these concentrations. The detailed t-test reports are in the Appendix G.2 – Table G-11, G-12, and G-13.

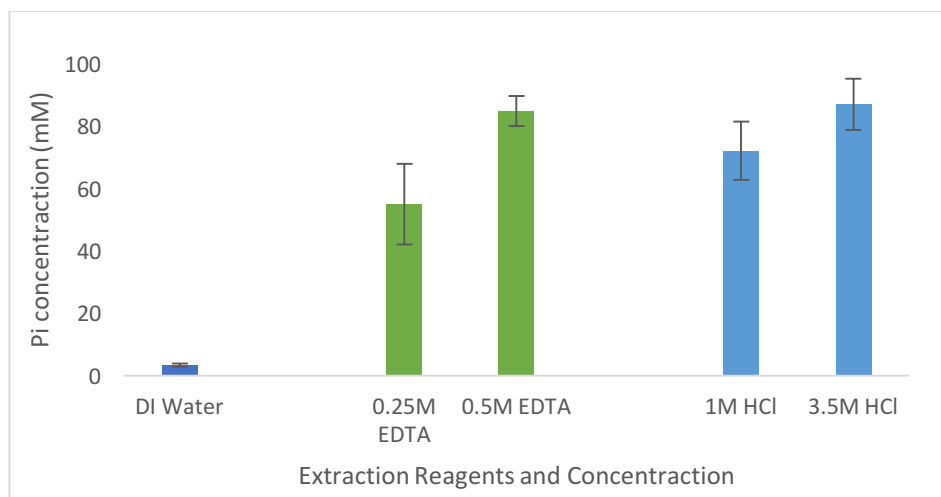


Figure 4-9 Extraction results for EDTA, HCl and dd water (n=4).

(2) The effect of EDTA concentration on Pi extraction

Figure 4-10 plots the Pi concentrations extracted from varying EDTA concentrations. The Pi concentration extracted by ddH₂O as a control was 3.58 ± 0.32 mM/L Pi (n=8). Increasing EDTA concentration correlates with an increasing extracted Pi concentration. At and above 160 mM EDTA, the change in extracted Pi concentration was not statistically significant ($p < 0.05$, in Appendix G, Figure G-3, others in Figure G-4). Another interesting result is that the extracted Pi concentration with 0.2 M EDTA was 70.8 ± 2.74 mM/L. However, in the previous section (Figure 4-9), the extracted Pi concentration with 0.25 M EDTA was 54.9 ± 12.9 mM/L. The P value in the Student's t-test is 0.13 (> 0.05), which reveals P-extracted with the 0.2 M EDTA in this experiment (Figure 4-10) is statistically higher than in the last experiment (Figure 4-9) with 0.25 M EDTA. This difference might be caused by a few factors. The same TWAS sample is not ensured in this complex mixture, when the samples are taken at different times, or from different TWAS batches. However, the Pi extractions with ddH₂O for the two groups were the same ($p = 0.53 > 0.05$) in two experiments. Besides, this experiment was conducted 19 days earlier than the previous experiment, so the TWAS sample could have changed with time. The Student's t-test reports are in Appendix G.2 – Tables G-14 and G-15.

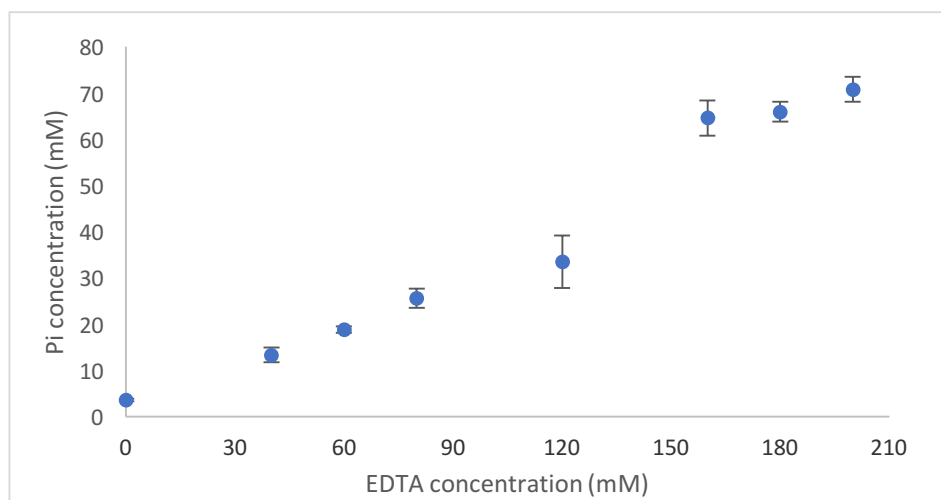


Figure 4-10 Pi extraction as a function of EDTA concentration (n=4, except with dd H₂O and 200 mM EDTA trials n=8).

(3) Test for polyP extraction with EDTA

This section addresses the question if polyP is extracted from TWAS by EDTA, by measuring the difference between the Pi concentration before and after EDTA extracted supernatant or supernatant and pellet together are exposed to a polyP-specific acid hydrolysis process. The concentrations of Pi after EDTA extraction, in the supernatant and in the small amount of supernatant and pellet after polyP-specific acid hydrolysis are presented in Figure 4-11. It was assumed that EDTA would chelate iron, aluminum, and other multivalent cations, allowing for the extraction and detection of Pi and other P-species (Gong, Liu, & Jiang, 2009). The 7-minute acid hydrolysis test was meant to determine if polyP was one of the extracted P-compounds. According to the trends in Figures 4-10 and 4-11, it is clear that an increased concentration of EDTA resulted in more Pi extracted by EDTA. There is an increasing trend of released Pi with an increase in EDTA concentration, even in the range between 160 and 200 mM EDTA. It implies that more concentrated EDTA might have able to extract more P from TWAS, such as 84.8 ± 4.80 mM Pi mM released Pi by 500 mM EDTA in Figure 4-9. However, the polyP released Pi by the 7 minute hydrolysis method in both supernatant and pellet for 0 and 100 mM EDTA. This results confirmed that polyP is considered to be a P-component in TWAS (Röske, 1994; Serafim et al., 2002; Uhlmann et al., 1990). With iron and aluminum ions in the TWAS. However, it is difficult to distinguish the contribution of Pi from polyP after the acid hydrolysis from the contribution of Pi from Fe- and Al-OH-Pi species in TWAS.

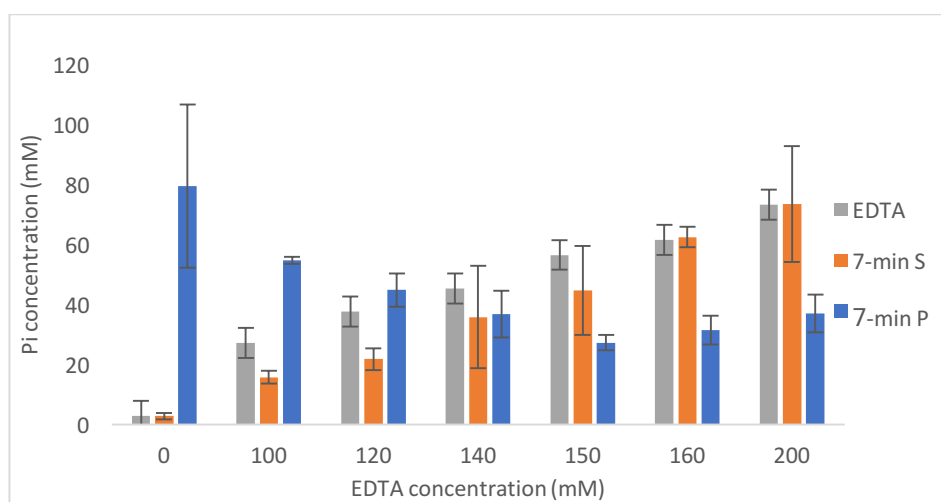


Figure 4-11 Pi extraction with EDTA, followed by a 7-minute acid hydrolysis to measure the polyP extracted (n=6). “7-min P” refers to the results from 7-min hydrolysis on the pellet after EDTA extraction. “7-min S” refers to the results from 7-min hydrolysis on the supernatant after EDTA extraction.

The same extraction results are also presented in Table 4-3. The Pi extracted by ddH₂O was 2.89 ± 0.29 mM/L (n=6). The average Pi extracted by EDTA increased with increasing EDTA concentration (gray bars), up to an average value of 73.3 ± 8.39 mM/L Pi, which confirms the extraction results in the previous section. This value was not statistically significantly different than the 200 mM EDTA Pi extraction value for the next test (Figure 4.10) (Student's t-test, p= 0.22, Appendix G, Table G-16).

One interesting result is that the Pi concentration in the hydrolyzed supernatant was not significantly different for some of the EDTA concentrations (ANOVA report in the Appendix G, Table G-21). However, only the decreased tendency of acid-hydrolyzed Pi concentration on EDTA-treated residual supernatant with complete pellet decreased following with the increasing EDTA concentration. This suggests that the pellet did contribute significantly to the polyP hydrolysis result. Theoretically, the TWAS pellet was expected to contain more (intracellular) polyP. However, after 5 days in 2x dilution with the extractant, it is also possible that osmotic pressure ruptured the cells, and released the Pi and polyP-contents into solution.

Table 4-3 Extraction results with EDTA, followed by a 7-minute acid hydrolysis (n=6).

<i>Extractant</i> <i>EDTA (mM)</i>	[Pi] after extraction (mM)	[Pi] in supernatant after polyP hydrolysis (mM)	[Pi] in pellet after polyP hydrolysis (mM)
<i>ddH₂O</i>	2.90 ± 0.29	2.72 ± 1.14	79.5 ± 27.2
<i>100</i>	27.1 ± 2.06	15.7 ± 2.08	54.8 ± 1.19
<i>120</i>	37.6 ± 8.15	21.8 ± 3.64	44.8 ± 5.56
<i>140</i>	45.4 ± 15.5	35.7 ± 17.11	36.7 ± 7.72
<i>150</i>	56.5 ± 12.2	44.7 ± 14.8	27.2 ± 2.60
<i>160</i>	61.6 ± 13.2	62.5 ± 4.43	31.4 ± 4.86
<i>200</i>	73.3 ± 8.39	75.5 ± 19.4	36.9 ± 6.27

The interpretation of these results may require another level of complexity. This value could represent both the Pi present in a colloidal form (associated with colloidal iron and/or aluminum hydroxides that did not sediment upon centrifugation) that dissolved in the acidic hydrolysis conditions, as well as the Pi from acid-hydrolyzed polyP. The opposed pellet and supernatant Pi

hydrolysis trends suggest that it is possible that colloidal P-rich compounds and/or polyP compounds that were sedimented by the centrifugation process, and could have been intracellular before osmotic rupture, are an important P-component of TWAS. These P-components were dissociated at an acidic pH, and 7 min residence time at 100 °C. These compounds could be Pi associated with iron and aluminum hydroxides and soluble polyP complexes.

Moreover, the acid-hydrolyzed Pi concentration in the supernatant was similar to the EDTA-extracted Pi concentration without significant difference (Table G-21) and then gradually generated a similar Pi concentration with increasing EDTA concentration. The interesting result of a decreased Pi concentration from the acid hydrolysis of the pellet with increasing EDTA concentration may be due to the loss in EDTA-chelating power at the acidic pH of the polyP hydrolysis step. The H⁺ competes for metal ion chelation with EDTA (Harris, 2012), so the EDTA effect would be reduced by the polyP hydrolysis conditions. Metal ions would be freed at this pH that protonates EDTA, allowing the metal ions to precipitate with the extracted Pi.

4.3.3. Thermal Heating/Hot Water Pi Extraction at 70 °C

4.3.3.1. *Introduction*

A hot-water extractable P-fraction was identified in polyP-accumulating cells in 1966 (Fitzgerald & Nelson, 1966). Hot water was also reported to extract intracellular polyP and nearly all cellular Pi (Weimberg, 1970; Weimberg & Orton, 1965), this extractable fraction was named “surplus phosphate” (Uhlmann et al., 1990). It was not clear if other intracellular P-compounds, such as RNA and DNA are also extracted with this technique, especially when it is applied to wastewater activated sludge.

Kuroda et al. (2002) suggested that heating the polyP-rich waste activated sludge from a laboratory-scale bench EBPR process disrupted the cell of PAOs that released polyP from the cytoplasm. The most simple polyP-extraction from cells accomplished by heating found in the literature is heating the sample at 70 °C for 1 h to extract intracellular polyP, which was equal to 87% of total P measured by Kuroda et al. (Kuroda et al., 2002). This group performed high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE) to

identify the presence and the chain length of extracted polyP, respectively. Kuroda *et al.* also demonstrated that treatment of EBPR-sludge in 70 °C water extracted 115 mg Pi/L (3.68 mM Pi). Extracted polyP was detected by its 7-minute acidic hydrolysis after either 70, 80 or 90 °C water, and its extracted concentration was approximately 90 mg P/L (2.88 mM Pi). However, the polyP-releasing mechanism of PAOs was not visually verified after a 90°C heating process for 120 min, even though the PAO's were imaged before and after extraction by scanning electron microscopy (SEM) (Kuroda et al., 2002).

It is interesting that the extracted Pi values from this laboratory EBPR sludge sample were an order of magnitude lower than those from ROPEC sludge. However, there was no iron addition or aluminum in their sludge. Therefore, this preliminary test was conducted to determine if the hot water extraction process could hydrolyze polyP from ROPEC TWAS with its iron and aluminum contents.

4.3.3.2. Material and Methodology

1000 µL of TWAS sample was placed in glass vials with screw necks and Teflon-lined caps. Seven vials for each group were placed on a hot plate, and the heating setting controlled by hand with a thermometer on the hot plate in order to maintain 70 °C. Samples were heated for 0, 20, 40, 60, 80, 100, or 120 min, and the experiment was repeated twice. After heating, tubes were placed in a cold-water bath, and the contents transferred to microcentrifuge tubes to centrifuge at 6,000 rcf for 5 min to sediment the solids. The supernatant was measured for Pi concentration with the vanadomolybdate assay.

4.3.3.3. Result and Discussion

The average initial Pi concentration in the TWAS was 0.73 mM Pi. Figure 4-12 illustrates how the released Pi increased to an average value of 2.58 mM Pi with heating time up to 60 min and then dropped to an average value of 0.60 mM Pi at 120 min. The maximum value of 2.58 mM Pi at 60 min is similar to the 3.68 mM value reported by Kuroda et al. (Kuroda et al., 2002) although their samples were WAS from EBPR, which did not contain soluble metal salts.

The increased then decreased extracted Pi concentration with increased heating time could be attributed to the heating set-up; instead of using a water bath, the heating was direct from the dry bath heater to the sample tubes, and the thermometer was outside of the sample tubes. Therefore an indirect temperature was taken. It is possible that these thermal fluctuations could have overheated or underheated the sample, with unknown consequences for Pi and polyP extraction. The effects of iron and aluminum hydroxides in the heated samples is also unknown.

However, phosphorus fixed by chemical precipitation with iron cannot be dissolved with hot water (Uhlmann et al., 1990). Based on the experimental results from extracting P from activated sludge from a biological P-removal process, Uhlmann *et al.* (1990) reported that hot water extraction might be similar to NaOH extraction, which was regarded as the extractant for polyP. However Uhlmann et al. also indicated that the concentration of hot-water-extractable phosphate decreased in the presence of additional iron (Uhlmann et al., 1990).

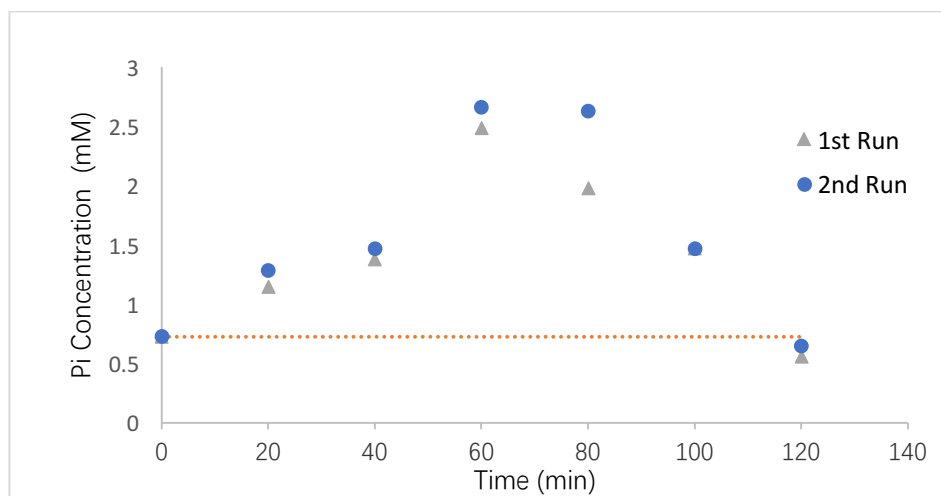


Figure 4-12 Thermal heating on the TWAS at 70 °C for 0-120 min when the initial Pi of raw TWAS is 0.73 mM.

In this study, hot water extracted Pi, and possibly polyP from TWAS, as reported in the WAS literature. Previous P extraction from TWAS in this thesis attained 85 mM Pi with EDTA and acid extraction (Figure 4-9), in conditions that chelated the metal ions. However, in the presence of metal ions, it is not possible to determine if the extracted Pi concentrations were affected by excess free iron, as it could precipitate with the extracted Pi. The maximum measured Pi concentration is 3.04 % of the maximum Pi extracted by EDTA, which could represent the polyP extracted by this process, although it is difficult to support this conclusion due to the

complexities of this experiment. Due to thermal instabilities and the presence of metal ions in the TWAS, it was determined that this method, although simple, does not provide a useful quantification of extractable polyP from ROPEC TWAS.

4.3.4. Sequenced PolyP Extraction: Modified from Weimberg

4.3.4.1. Introduction

The sequence extraction techniques are designed to separate the different P fractions with specific stepwise extractions (Bezak-Mazur & Mazur, 2009). The first P-extraction sequence was published by Chang and Jackson (Chang & Jackson, 1957). The applications of the stepwise techniques applied to wastewater activated sludge were listed and described in Section (4.2), Table 4-2 in particular.

In this experiment, the modified procedure from Weimberg (Weimberg, 1970; Weimberg & Orton, 1965) was tested, as it was designed for polyP extraction and quantification from cells. Given the previous experience with metal ions, and the complex composition of TWAS, the same chemical reagents from this protocol were used, but the extractions were extended for up to 20 hours to determine the possible optical extraction time.

4.3.4.2. Material and Methodology

(1) Modified extraction sequences

Figure 4-13, it is the schematic layout for the stepwise extraction process (Weimberg, 1970; Weimberg & Orton, 1965). Six different iterations of this stepwise extraction process were tested; they are listed in Table 4-4, and the details of each step are described after the details of each step are described in this table.

Table 4-4 Table of the experimental process for modified sequence extraction from (Weimberg, 1970; Weimberg & Orton, 1965).

<i>Extraction Process</i>	Step 1	Step 2	Step 3	Step 4
<i>A</i>	ddH ₂ O wash	Alkaline extraction (AE)	100 °C water bath (n=4)	Acid hydrolysis (AH) (n=2)
<i>B</i>	ddH ₂ O wash	100 °C water bath	Alkaline extraction (AE) (n=6)	Acid hydrolysis (AH) (n=2)
<i>C</i>	ddH ₂ O wash	Alkaline extraction (AE) (n=4)	Acid hydrolysis (AH) (n=2)	
<i>D</i>	ddH ₂ O wash	100 °C water bath (n=6)	Acid hydrolysis (AH) (n=2)	
<i>E</i>	Alkaline extraction (AE) (n=2)	Acid hydrolysis (AH) (n=2)		
<i>F</i>	100 °C water bath (n=2)	Acid hydrolysis (AH) (n=2)		

(2) 20-hour Pi extraction

Three separated extraction methods were tested for 20 hours in duplicate without sample washing and polyP hydrolysis. For the first extraction method, 500 µL of TWAS was mixed with 500 µL ddH₂O, and placed in a boiling water bath. The second extraction was of the 500 µL TWAS pellet in 500 µL of 0.2 M NaOH at room temperature. The third extraction process was of the 500 µL TWAS pellet with 500 µL of 3.5 M HCl. The individual samples were centrifuged and an aliquot taken at the time points for Pi measurement, then the samples were not returned to their extraction processes. After centrifugation and supernatant decanting, soluble Pi was measured.

4.3.4.3. Result and Discussion

(1) Modified sequenced polyP extraction

The original protocol was designed for extraction of different polyP fractions from cells. The modified series of sequence polyP extractions performed in this experiment (Figure 4-14). Process B was wash, 100 °C (boiling-water) bath, AE (alkaline extraction by 0.2 M NaOH) and AH (7-min hydrolysis) procedure in the original publication. However, it did not produce the produce maximum Pi extraction.

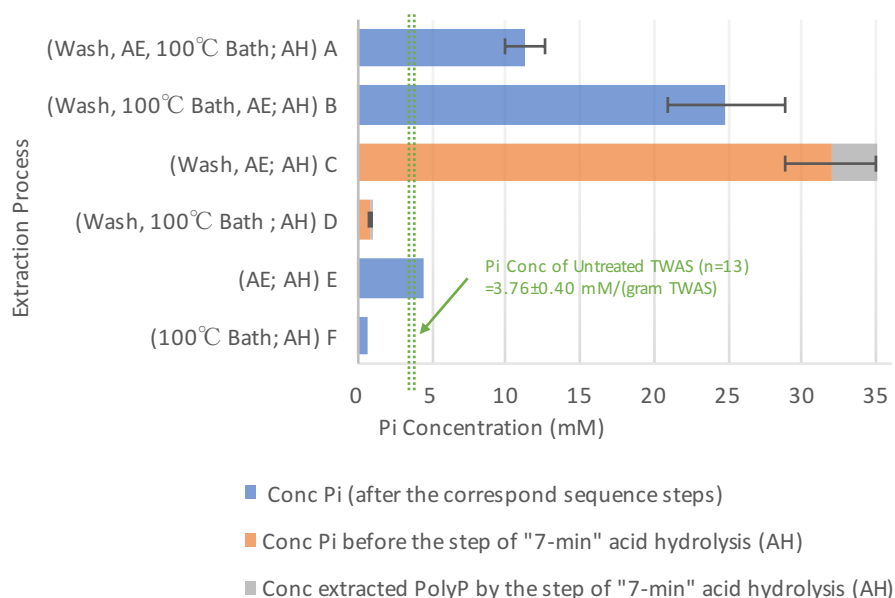


Figure 4-14 Sequence extraction results based on the method from (Weimberg, 1970; Weimberg & Orton, 1965). See Table 4-4 for the acronym key.

Regardless of boiling water extraction, process C extracted the largest amount of Pi. After 7-minute hydrolysis, there was about 35 mM Pi in total more than about 32 mM from alkaline extraction. It implies that there was about 3 mM of Pi from 7-min hydrolysis at least. It reveals at least 3.53% P from total P is polyP under the previous results of 85 mM total P in the TWAS. In the trial E, the released Pi was only 4.39 mM. It implies that ddH₂O washing could help to release Pi from sludge. The possible reason is that ddH₂O washing contributed to removing not only the soluble Pi in the original sludge sample, but also the soluble free iron ions in the supernatant, which is induced from chemical precipitation in the plants. Another possible explanation is during the washing process; the samples were also applied several times of centrifugation, which might kill the PAOs, that might then release polyP. However, the worst

yield generated by process D confirmed that cold washing does not have a significant impact on the extraction. But it also verified that the 7-minute hydrolysis in this sequence extraction could hydrolyze extracted P to soluble Pi.

(2) Extraction for 20 hours

The effect of time on three Pi-extraction conditions for up to 20 hours is presented in Figure 4-15. It is interesting that at the maximum Pi extraction appears to have been observed between 3 and 5 hours for the acid and base extractions. The average highest and last extracted Pi concentrations for 3.5 M HCl were 93.7 mM at 5 hours and 65.2 mM at 0.25 hour, respectively. The average peak and lowest extracted Pi concentrations for 0.2 M NaOH were 45.7 mM at 2 hours and 22.6 mM at 0.5 hours, respectively. Besides, the average maximum extracted Pi concentrations by hot water were 5.60 mM at 16 hours, which is higher than released 2.58 mM Pi at 1 hour by 70 °C water extraction in the Section 4.3.3.

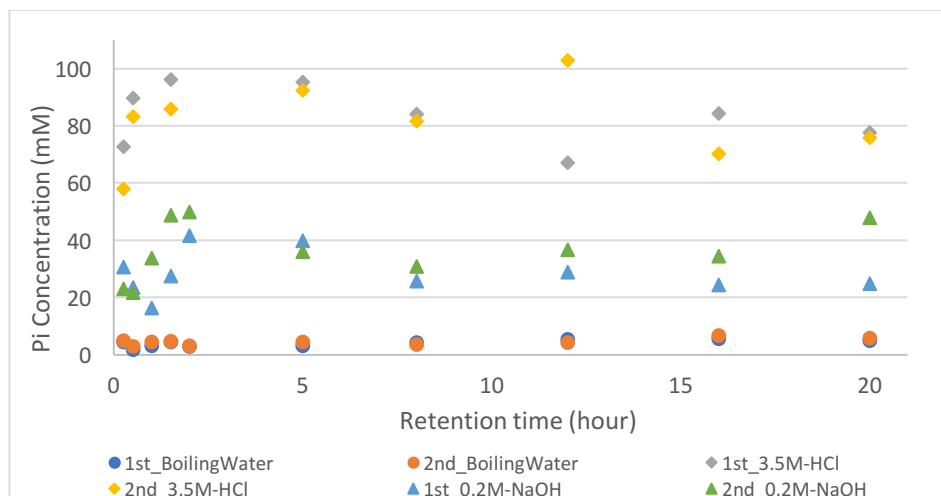


Figure 4-15 20-hour extraction in a boiling-water bath, 0.2 M NaOH or 3.5 M HCl (n=2).

The HCl extraction conditions are known to extract more than just polyP species. The maximum Pi released by 3.5 M HCl was an average of 92.3 mM, which is within the first standard deviation values of 86.98 ± 8.25 mM of acid extracted P previously from another TWAS sample

(Section 4.3.2.3, Figure 4-9). The alkaline extraction at 0.2 M NaOH extracted approximately 26% of the Pi extracted with the HCl acid extraction experiment in the supernatant.

4.3.5. Expanded Alkaline Extraction Investigation

4.3.5.1. Two-step extraction: Alkaline exaction and/or acid digestion

4.3.5.1.1. Introduction

PolyPs are extracted, and might remain intact with alkaline extraction conditions²⁵ (Uhlmann et al., 1990). It is possible that PAOs cannot survive under the strong base condition. However, the conclusion for the P-extractions of the NaOH extraction were difficult to determine based on the previous experiments. According to the previous experiments, unlike acid, alkaline could release a significant amount of P from certain P forms within sludge. Due to ion exchange against OH⁻, NaOH-extractable phosphate was expected to adsorb the metal hydroxides, like Al(OH)₃ (Uhlmann et al., 1990). Since Al-waste from the Lemieux water treatment plant is sent to ROPEC, it is possible in this experiment that NaOH could release Pi from Al(OH)₃-P. Regarding the study on the lake sediment instead of wastewater sludge, Pettersson et al. (1988) reported that the major NaOH-extractable P-contents is “organic phosphate” (Pettersson et al., 1988). However, polyP was later suggested to be the major “organic P”. Uhlmann *et al.* (1990) hypothesized that more polyP would be extracted from aerobic waste activated sludge than anaerobic waste activated sludge, and their experimental NaOH-extractable phosphate data confirmed this guess.

Therefore, in this study, alkaline extraction was expected to extract on polyP specifically. However, the acid may help to convert released polyP into soluble phosphate after alkaline extraction. Therefore, the desirable situation is that alkaline only extracts intracellular PAO polyPs, and then, acid digests these extracted, intact polyPs into soluble inorganic phosphate.

²⁵ This conclusion is also proved by which alkaline extraction as a pretreatment before the characterization of polyphosphate by NMR.

4.3.5.1.2. Material and Methodology

These six groups of two-step P-extractions were tested in this experiment. The details of these P-extractions from TWAS are presented in Table 4-5. The Pi concentration was measured after Step 1 and after Step 2. The Pi concentration was back-calculated to the initial TWAS volume.

Table 4-5 Table of extraction process detail for the two-step alkaline and/or acid hydrolysis extraction test.

<i>Extraction Series</i>	<i>Name</i>	Step 1 – 2 hours, room temperature	Step 2 – 2 hours, room temperature	n Step 1, 2
1	BLK	250 µl TWAS + 500 µl ddH ₂ O	+ 500 µl ddH ₂ O	2, 2
2	H ₂ O + AD	250 µl TWAS + 500 µl ddH ₂ O	+ 500 µl 0.5 M HCl	2, 2
3	H ₂ O + 2AD	250 µl TWAS + 500 µl ddH ₂ O	+ 500 µl 1 M HCl	2, 5
4	AE + H ₂ O	250 µl TWAS + 500 µl 0.5 M NaOH	+ 500 µl ddH ₂ O	2, 2
5	AE + NEU	250 µl TWAS + 500 µl 0.5 M NaOH	+ 500 µl 0.5 M HCl	2, 5
6	AE + AD	250 µl TWAS + 500 µl 0.5 M NaOH	+ 500 µl 1 M HCl	2, 5

4.3.5.1.3. Result and Discussion

Figure 4-16 presents the extracted Pi concentrations from each of the 2-step P-extractions. The average Pi concentrations measured in the blank experiment of ddH₂O were 2.26 mM Pi (Step 1) and 2.88 mM Pi (Step 2). These values are within the first standard deviation of 3.18 ± 0.38 in the Section 4.3.2. The average first step of alkaline extraction values were 57.68, 59.33, and 59.34 mM Pi. These results are within the first standard deviation of 0.96 (average 58.79). The different step 2 processes resulted in different Pi extraction values. Dilution of the alkaline solution with water slightly increased the average extracted Pi concentration (64.4 mM Pi (step 2), over 57.7 mM Pi (step 1)).

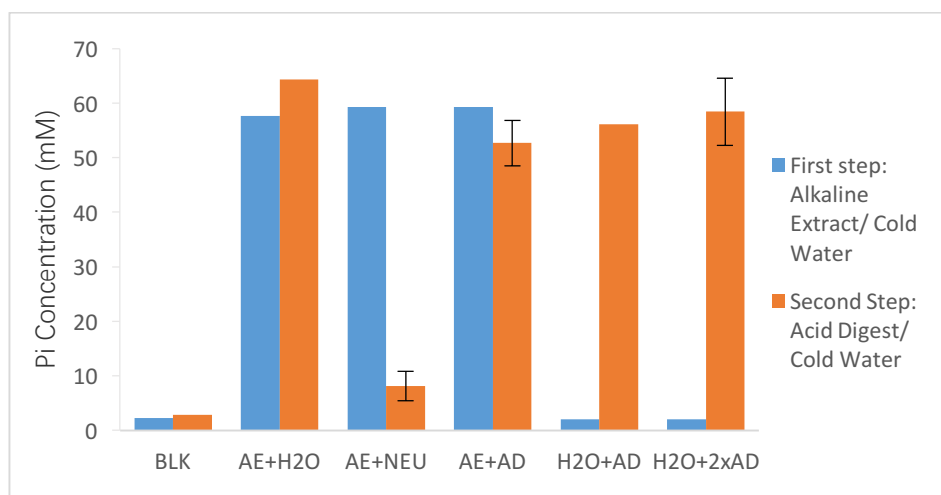


Figure 4-16 The two-step extraction, using water or alkaline extraction for the first extraction, and water or acid for the second extraction (n=2 except for data with error bars (n=5)).

However, neutralization of the NaOH solution with HCl resulted in a marked decrease in Pi concentration (8.15 ± 2.68 mM Pi). Acidification of the solution in step 2 reduced the average Pi concentration (52.7 ± 4.17 mM Pi) below the step 1 average Pi concentration (59.3 mM Pi). It is assumed that the pH affects the metal ion-phosphate relationship through the metal ion speciation change with pH. The pH values were above 12 when 250 μ L TWAS sample was mixed with 250 μ L 0.5M NaOH. In alkaline conditions, the metal hydroxide complexes could loosely bind to extracted Pi (Chen, Lu, Zhao, Wang, & Huang, 2011), the Pi could be measured with the vanadomolybdate assay. However, in neutral pH conditions, dissolved metal ions could precipitate with the extracted Pi, reducing the dissolved Pi concentration. In acidic conditions, where the pH values were less than 1 when 250 μ L of TWAS was mixed with 250 μ L 0.5 M HCl, it is assumed that the Pi speciation is mostly H_3PO_4^0 , which is not favourable for metal-phosphate precipitation as metal- PO_4 salts, and the metal ions are not in metal-hydroxide forms that could adsorb Pi.

Uhlmann *et al.* observed that polyP in the wastewater sludge performed as the non-reactive phosphate with 1M NaOH extraction and it cannot be detected until hydrolyzed to ortho-Pi (Uhlmann *et al.*, 1990). However, this undetectable property of polyP did not occur in this experiment. Besides, it is barely possible to judge the re-precipitation of metal phosphate particles by visually observing the pellet volume, as the packing density might be different, and

metal-P particles may pack more closely than TWAS. It is possible NaOH is able to dissolved Pi from Fe-P in other sediments (Chen et al., 2011).

As previous mentioned, NaOH solutions may extract polyP from TWAS instead of generally extracting other P-species. But NaOH may also degrade polyP to Pi by base catalysis of polyP. PolyP chains may also incompletely hydrolyze into ring shapes (Ivey & Shaver, 1977). Alkaline polyphosphate enzymes in WAS may also cleave Pi from polyP in the basic condition (Xie, Lu, Huang, Wang, & Xu, 2010). This possibility could be tested by gel electrophoresis separation of the extracted solutions. Another possible reason for the high Pi concentrations in the NaOH-extracted solutions could be the catalysis of the polyP hydrolysis reaction by Na-catalysis (McCullough, Van Wazer, & Griffith, 1956). The high concentration of free Na^+ ions from alkaline NaOH may trigger this catalytic event.

4.3.5.2. AE – Effect of cation type

4.3.5.2.1. Introduction

Based on the previous experiments (result in Figure 4-14), it is possible that polyP extraction by NaOH solutions may also induce polyP hydrolysis with the catalytic effect of Na^+ on polyP hydrolysis (McCullough et al., 1956). In this section, another alkaline agent - potassium hydroxide (KOH) was compared with NaOH to determine if there is a cation effect on polyP hydrolysis during alkaline polyP extraction.

4.3.5.2.2. Materials and Methodology

(1) Different concentrations of KOH or NaOH for alkaline P-extractions from TWAS

500 μL of TWAS was mixed with 500 μL of either NaOH or KOH at concentrations of 0 (ddH₂O), 0.2, 0.5, 0.8 and 1 M in the microcentrifuge tubes. The samples were mixed on a benchtop shaker for 5 days²⁶ at room temperature to ensure a complete extraction. The samples

²⁶ The retention time was designed and assumed to react completely in 5 days. The ideal reaction time is expected to be verified in the future as required.

were centrifuged, and the supernatant tested for Pi concentration assay. Each alkaline concentration and type were repeated 4 times.

(2) Alkaline P-extraction from TWAS by KOH or NaOH, followed by a 7-minute acid hydrolysis

Five P-extraction groups were tested: a control group with ddH₂O water and experiment groups with 0.5 M NaOH, 0.5 M KOH, 1 M NaOH or 1 M KOH. 8 repeats were performed for each base type and concentration, except for 0.5 M KOH, for which n=7. 500 µL of TWAS was mixed with 500 µL of the different alkaline extractants. The samples were placed on a benchtop shaker for 3 hours at room temperature. The samples were centrifuged to separate the supernatant from the pellet. The supernatant Pi concentration was measured. The supernatant and the pellet were mixed with 500 µL of 3.5 M HCl, and placed in a 100 °C water bath for 7 min to hydrolyze any extracted polyP. The hydrolysis experiments were repeated 4 times. Soluble Pi in the acidic supernatant was measured after the samples were cooled and centrifuged at 6,000 rcf for 5 min.

4.3.5.2.3. Result and Discussion

(1) Different concentrations of KOH or NaOH for alkaline P-extractions from TWAS

The extracted Pi concentrations produced by the alkaline extractions at different concentration and with KOH or NaOH are presented in Figure 4-17. The Pi extractions increased with increasing NaOH or KOH until 0.8 M NaOH/KOH. P-extraction with NaOH or KOH was not significantly different for all concentrations (Appendix G-2, Table G-23). The reason for this difference ($p=0.024 < 0.05$) between 0.6 M NaOH and 0.6 M KOH is not known. It is possible that the chaotropic nature of potassium, which means that it disrupts the hydrogen bonding network in water, may catalyze the polyP hydrolysis reaction of water and polyP. It is not known if there is a significant extracted polyP content at the lower OH⁻ concentrations.

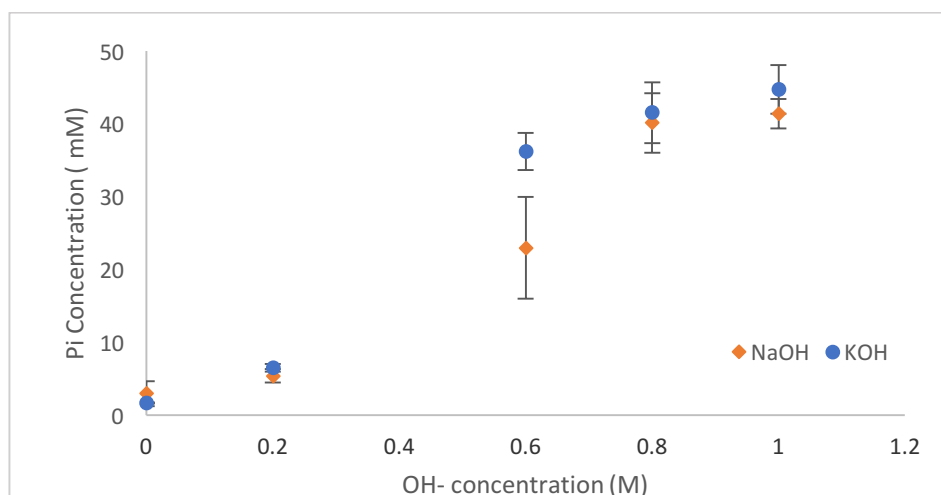


Figure 4-17 Pi extraction results from different concentrations of KOH and NaOH extractions (n=4).

(2) Alkaline P-extraction from TWAS by KOH or NaOH, followed by a 7-minute acid hydrolysis

Figure 4-18 shows the Pi measured by colorimetry after alkaline extraction and after acid hydrolysis of the supernatant and pellet. These data are summarized in Table 4-6. The control Pi concentration, which was Pi extraction in ddH₂O water, was 1.76 ± 1.24 mM. Pi extracted by KOH or NaOH showed a trend of increasing Pi extraction with increasing alkaline agent concentration. A 2-way ANOVA analysis of the effect of alkaline concentration and type showed that the reagents of KOH and NaOH affect the extraction significantly but the interaction between the two alkaline types and a variable concentration of alkaline is not significant (Appendix G-2, Table G-24). Acid hydrolysis that targets polyP hydrolysis in the pellet and supernatant samples showed a distinct difference in the polyP content in the pellet and supernatant, with a higher polyP content in the pellet than the supernatant for both KOH and NaOH alkaline extractions.

Table 4-6 Extracted alkaline Pi concentrations, before and after polyP hydrolysis (mM Pi).

<i>Extractant</i>	[Pi] after extraction (mM) (n=8)	[Pi] in supernatant after polyP hydrolysis (mM) (n=4)	[Pi] in pellet after polyP hydrolysis (mM) (n=4)
<i>ddH₂O</i>	1.76 ± 1.24	0.53 ± 0.21	-
<i>0.5 M NaOH</i>	11.7 ± 4.45	6.69 ± 3.57	88.3 ± 4.57
<i>0.5 M KOH</i>	13.4 ± 6.50 (n=7)	8.25 ± 5.51	80.5 (n=2)
<i>1 M NaOH</i>	21.7 ± 10.2	17.6 ± 13.2 (n=3)	65.9 ± 5.06
<i>1 M KOH</i>	29.7 ± 9.0	22.3 ± 12.7	57.8 ± 12.5

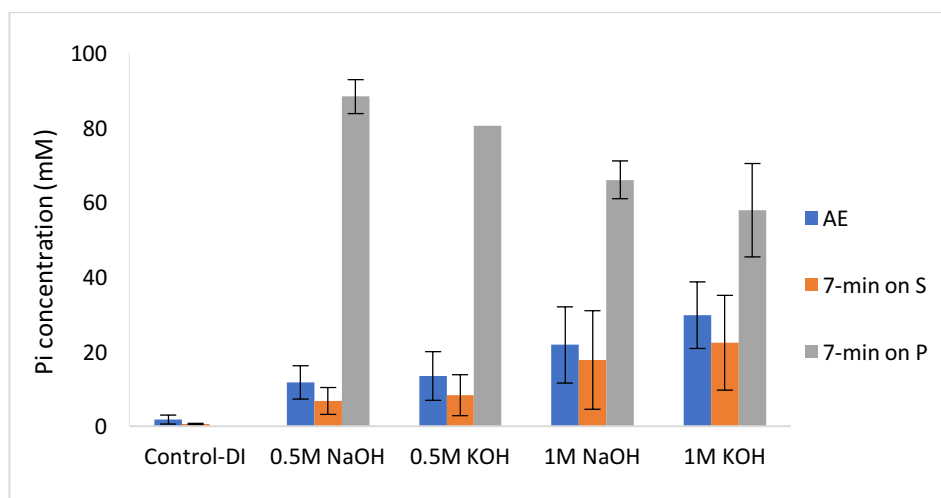


Figure 4-18 The Pi-extraction results from alkaline extraction by KOH and NaOH, and extraction followed by a 7-minute acid hydrolysis at 100 °C.

The errors are large, but they may be due by the over one month time interval between two runs, although the initial TWAS samples were the same. There is a trend of increasing Pi concentration after hydrolysis in the supernatant with increasing alkaline concentration in the extractant. This suggests an increase in extracted polyP in the supernatant with increasing extractant alkalinity. However, given the dissolved metal ions in the supernatant, these metal ions may have precipitated with some of the hydrolyzed Pi, and reduced the measured Pi

concentration. This effect is not known or quantifiable, as the techniques to measure dissolved iron or aluminum are not available in the laboratory.

PolyP hydrolysis of the pellets after alkaline extraction also offers interesting results. The 0.5 M NaOH extract group had the highest polyP-hydrolyzed Pi concentrations. A statistical comparison of this result with the 0.5 M KOH acid-hydrolyzed Pi from the pellet was not possible because of $n=2$ for this group. However, both values are within the first standard deviation of the maximum EDTA-extracted Pi values (84.8 ± 4.80 mM Pi, Section 4.3.2.3.(2).).

The groups with 1 M alkalinity in the extractant showed a decreased average acid-hydrolyzed Pi concentration than the 0.5 M alkalinity group. This diminished value could be caused by two effects. It is possible that the increased alkalinity decreased the effective acidity that is required for polyP hydrolysis, therefore less Pi was hydrolyzed. It is also possible that the final pH of the acidified solution for the 1 M alkaline extractants would be higher than the final pH of the 0.5 M extractant groups. This higher pH might result in Pi loss to residual dissolved metal ions that were released from the pellet. Without the capability to measure metal ions concentration, is not possible to distinguish between these two possible effects.

It can, however, be concluded that a polyP fraction was extracted from TWAS in these conditions, but that there was a significant polyP fraction that remained in the pellet. Pi from polyP may precipitate with metal ions in solution, which may depend on solution pH, and is not possible to quantify at this time. Assuming the acid extraction of TWAS extracts all but the organic phosphate compounds, and the average extracted 21.7 mM Pi by 1M NaOH or 39.7 mM Pi by 1 M KOH is 25.6% or 34.9% P, which is possibly polyP, in 85 mM Pi of total P from TWAS.

4.4. Conclusions and Recommendations

Waste activated sludge is complex. A summary table presents as Figure 4-7. Due to the chemical phosphorus removal process at ROPEC, major P components of TWAS are in the pellet as insoluble P salts. The supernatant P_i concentration varied between 0.5 - 4 mM (section 4.3.1). PolyP a major P-component in the sludge based on the literature. In the TWAS sample from ROPEC, it is concluded that the majority of phosphorus is acid and EDTA-extractable, and the P-form is difficult to identify. Different extraction methods suggest that polyP is approximately 30% (8.2 mg P/ 100 mL) by alkaline extraction of the total P ($\sim 85 \text{ mM} = 27.2 \text{ mg P/100 mL}$) in TWAS.

Acid induces a general reaction to dissolve many types P-compounds, generating soluble P_i . Therefore, it is an effective technique for non-oxidative, acid-extractable measurement. Concentrated EDTA solutions can chelate metal ions, and release almost all of precipitated P to soluble P_i in the TWAS. Alkaline extraction may have the potential to extract polyP exclusively in the TWAS. This ability has already been reported for WAS produced by biological P removal process. There is a subtle difference between NaOH and KOH extraction of P from TWAS which is attributed to a cation effect.

The potential impacts of changing polyP extraction variables are not commonly reported in the literature. Municipal waste activated sludge is a complex material, so it is difficult to assess the differences in polyP extraction techniques if there are larger differences in TWAS composition. It can be concluded that polyP extraction from TWAS has not been optimized, and could be the subject of future study for its extraction, hydrolysis, and reuse. It is recommended to compare the cold perchloric acid extraction method (de Haas, Wentzel, & Ekama, 2000b) with alkaline extraction to determine its suitability for polyP extraction from ROPEC TWAS. It is also recommended to develop methods for measuring dissolved iron and aluminum ions in these samples.

Confirmation of the presence of polyP in TWAS is still a challenge. If successfully extracted, polyP separation by gel electrophoresis is suggested in the future to confirm extraction as well as polyP chain length. Raman spectroscopy is a powerful tool for polyP detection, which should be pursued as a future analytical technique.

Table 4-7 A summary of applied extraction methods in this chapter.

Technique	Method	P Species	Comment	Usage section
Hydrochloric acid digestion	0.5M HCl with heating (118°C)	Total P	A common technique with a strong acid was applied to test the feasibility of extraction P from sludge sample.	4.3.1.
Persulfate digestion	Persulfate acid with heating, modified from (EPA, 2016)	Total P	Might work well. Hard to judge in this thesis because of replacement of the required incubation condition of high pressure by heating.	4.3.2.1.
Hydrochloric acid digestion	With variable concentrated HCl extractants	Total P	A series of supplied experiment after certain concentration of HCl digestion on TWAS. Mixing concentrated strong acid might be an easy method to measure total P for sludge samples.	4.3.2.2., 4.3.2.3. (1)
PolyP hydrolysis (7-min)	1.5M HCl in the boiling water batch for 7-min (F. M. Harold, 1960).	PolyP (?)	A wide applied method to hydrolyze polyP after certain extraction process. The precise extraction of polyP could follow up polyP purification process in other types of samples (e.g., cells). However, considering the complicated of sludge samples, the technique is expected to be verified with Raman results in the future.	4.3.2.3. (3)., 4.3.5.2.(2))
EDTA extraction	Variable (Na-) EDTA concentrations solutions as the extractants.	Metal-P (?)	EDTA was considered to reduce the effect of multivalent cations from original sludge samples and to release Pi from insoluble mental-P. However, it was not clear if EDTA is able to extract P. Personally, Raman Spectroscopy is expected to verify the ability of EDTA during this process in the future, especially for polyP.	4.3.2.3. (1)(2).,

(Continued)

EDTA extraction with 7-min polyP hydrolysis	Following polyP hydrolysis after the extraction of EDTA.	Metal-P (?), polyP(?)	Both of two techniques are expected to be verified their function and effeteness. Ideally, it might be an easy way to test the amount of polyP in the metal-containing sludge samples.	4.3.2.3. (3).
Thermal heating	Hot Water Pi Extraction at 70 °C (Kuroda et al., 2002).	PolyP for bio-sludge	Kuroda et al. (2002) suggested that heating EBPR-sludge to release polyP. Personally, I suppose the effect was attributed to aggravate polyP hydrolysis by heating from solid-steam with liquid-stream of sludge samples. Chemical additional sludges are not suggested to consider.	4.3.3.
Sequenced PolyP Extraction	Modified from Weimberg (Weimberg, 1970; Weimberg & Orton, 1965)	?	Without the technique of P-species characterization for the extracted samples, the sequenced extraction is not suggested to conduct.	4.3.4. (1)
20-hour Pi extraction	Conditions were inspired as HCl, NaOH and boiling water-bath	Total P, polyP (?), metal-P (?)	It seemed like that the strong acid (HCl) and base (NaOH) conducted extraction within 5-hour.	4.3.4. (2)
Two-step extraction	Alkaline extraction following with acid hydrolysis.	?	Acid might react generally with high efficiency. Alkaline was expected to extract polyP.	4.3.5.1.
Alkaline extractions (NaOH, KOH)	Extraction with variable concentrations of NaOH or KOH.	Metal-P (?), polyP (?)	Alkaline might dissolve metal-P to release Pi as well as extract polyP.	4.3.5.2. (1)(2)
Alkaline extractions with 7-min polyP hydrolysis	Following polyP hydrolysis after OH- extractions	Metal-P (?), polyP (?)	More tests and characterizations are expected in the future.	4.3.5.2. (2)

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5. Formation of Carbonate-Containing Apatite from Phosphorus-Rich WWTP Streams

5.1. Introduction

The objective of Chapter 4 was to extract Pi from intracellular polyP from the PAO community in TWAS, but it was determined that iron and aluminum complicate this extraction and its assessment. The objective of this chapter is to precipitate Pi that was released by PAOs in the anaerobic digester, and precipitate calcium phosphate with a calcium carbonate solution. The calcium-carbonate rich solution required for this precipitation will be produced from limestone mixed with carbon dioxide gas (CO₂). The main component of limestone rock is calcite mineral, which is composed of calcium and carbonate; the suitability of synthetic calcium carbonate was assessed (Ross, 2017), but the use of limestone was not thoroughly tested. Both the calcium and carbonate components are useful for precipitating a calcium-carbonate-phosphate solid phase known as “carbonate apatite”.

Carbonate apatite (cAp) contains carbonate substitutions for phosphate (1-5 wt %), which enhances its solubility and therefore its utility for phosphorous fertilizer production (Easterwood, Sartain, & Street, 1989). Besides, limestone is inexpensive and plentiful in the Ottawa area. The literature review by Ross indicates that there is no other publically-declared technology that strives to produce calcium carbonate phosphate from the P-content of municipal waste (Ross, 2017). This precipitated calcium phosphate product is envisioned to be an option for redirecting the Pi that is concentrated at ROPEC to an alternative P-resource to produce P-fertilizers.

The P-extraction work in Chapter 4 was conducted to determine the polyP content, and the acid and EDTA-extractable P content in TWAS. The acid and EDTA-extractable P content were estimated to be 87.0 ± 8.2 and 84.8 ± 4.8 mM Pi, respectively, and the polyP content was estimated to be 25.6% or 34.9 % of NaOH- or KOH-extractable P, respectively. As a demonstration of the feasibility of

precipitating cAp from Pi extracted from TWAS. The acid-extractable P content from TWAS (Section 4.3.1) was tested as a Pi source for the precipitation of cAp (Section 5.3.1), the feasibility of precipitating cAp was verified by neutralizing the acid-extracted TWAS sample with synthetic sodium bicarbonate and calcium carbonate powders to avoid diluting the phosphate concentration. The phosphate-rich precipitates were characterized with Raman spectroscopy and FT-IR.

With the goal of creating a continuous precipitation process, a calcium-carbonate-rich solution was desired to precipitate the solubilized phosphate ions. However, because of the low solubility of calcium and carbonate ions in water, it was not possible to precipitate cAp with a synthetic, 27 mM P-stream – an average value found in the literature for wastewater treatment (Phillips, Kobylinski, Barnard, & Wallis-Lage, 2006) and a saturated calcium carbonate solution (Ross, 2017). The conclusion was that the solubility of calcium carbonate solids was not high enough to achieve supersaturation condition when mixed with the phosphate solution for cAp nucleation or growth.

The possibility of precipitating cAp from P that was acid-extracted from TWAS with the addition of solid, synthetic CaCO_3 was tested to prove the concept in Section 5.3.1. With this successful result, the next goal was to increase the solubility of calcium carbonate from synthetic CaCO_3 and limestone by reducing the pH with carbon dioxide gas of different concentrations in Section 5.3.2. The gas produced by anaerobic digestion has a large fraction of methane (60-65 %), and a large fraction of carbon dioxide gas (30-40 %) (Kuo, 2015). When this methane-rich biogas is used to produce electric power, the carbon dioxide fraction in this combustion flue gas can range from 10-15 % CO_2 (Kuo, 2015). Therefore CO_2 gas is available on-site, from the anaerobic digester off-gas, or from the power plant off-gas. The CO_2 gas reacts with water to form carbonic acid; this increases the solubility of calcium carbonate. It is the objective of Section 5.3.2 to produce concentrated calcium-streams with carbon dioxide gas, synthetic calcium carbonate, and limestone, in potable water.

The complications of Pi-extraction from TWAS were discovered in Chapter 4, and the large neutralization required to precipitate cAp from acid-extracted P, caused a re-evaluation of the project, and a change in Pi source from another waste-stream from ROPEC. The slurry from the anaerobic digesters at ROPEC is also rich in P. The slurry from all anaerobic digesters are collected in anaerobic digester 2 (Figure 4-1), which feeds the centrifuge. This slurry stream is called “DIG-2” at ROPEC and in this thesis. During the process of anaerobic digestion, PAOs are known to hydrolyze the polyP to soluble Pi in anaerobic environments (Jenkins & Tandoi, 1991). Therefore, the major P composition in the DIG-2 is Pi, though it was possible, given the effect of metal ions on P-extraction in Chapter 4, that not all of the Pi may exist as soluble Pi.

There were two reasons to choose DIG-2 as the P-stream for this chapter (Section 5.3.3. and 5.3.4). It could be used for cAp precipitation without targeted extraction for polyP because the polyP was most likely completely hydrolyzed by the PAO's in the anaerobic environment. In this way, the stages of polyP extraction and precipitation from TWAS were completed in the anaerobic digesters, which reduced the risks and complexity of this process in the laboratory. The ROPEC stream analysis data showed that the soluble Pi concentration in DIG-2 is relatively low (0.9 – 1.8 mM Pi (27 – 56 mg Pi/L)) because most of P in this stream is stabilized in its corresponding solid phase, called biosolids. The slurry total P ranges from 20 – 25 mM Pi (620 – 780 mg P/L), so there is a significant P in the biosolids, possibly in the form of extractable Pi-solids. This chapter aims to utilize the Pi from DIG-2, and test if it is possible to precipitate cAp with this P-stream and an enriched calcium-carbonate stream made from limestone, potable water, and carbon dioxide gas.

5.2. Literature Review

This literature review focuses on the previous strategies to reuse P from municipal wastewater sludge. So the calcium phosphate-containing fertilizer in this section is generated by the recovery process. Among the P-pools, the recovery of P from municipal wastewater sludge has become a sustainability strategy (Coker & Carlton-Smith, 2016) for both commercial and government agencies. For example, the concepts of P reclamation and recycling as a sustainable process has begun to change the sludge management strategies for WWTPs in the EU. At the beginning of the 21st century, Fielding (2000) reviewed how Western Europe targeted a 25% P-reuse as P-recovery from waste in the last 10 years (Stark, 2005). Sweden also proposed a national goal to recycle 75 % of P from municipal wastewater treatment plants before 2010 (Bengtsson & Tillman, 2004). In 2004, P-recovered fertilizers generated from P recovered from municipal wastewaters were formally recognized in the Dutch fertilizer law²⁷. German P recycling goals were announced to Europe in 2016²⁸.

Researchers proposed the sustainable use and recovery of P from municipal wastewater treatment plants even in the 19th-century (Scott, 1879). The P-rich waste in the municipal WWTPs directs abundant P into the solid sludge phase because of the removal of soluble Pi from the wastewater by precipitation. Therefore, waste activated sludge from aerobic digestion contains approximately 80% P (Bird, 2015), metals, microorganisms and other components (Appels, Baeyens, Degre, & Dewil, 2008). Although this sludge is a potential P source to recycle it, there are many factors to consider for its re-use.

Landfill, incineration, and land application are the primary disposal methods for sludge generated from municipal wastewater plants (Epstein, 2002). From a sustainability perspective, disposing WWTP sludge to landfill is the least desirable route in recycling inverted pyramid (DessauGermany, 2015; Vats & Rissanen, 2016). Municipal waste activated sludge that has a significant metal content can be incinerated as sludge ash to generate a P-rich solid material, although the P must be extracted from this ash before it can be recycled (Schipper et al., 2001).

²⁷ <http://www.biorefine.eu/cluster/news/'recovered-phosphates'-included-dutch-fertilizer-law> Accessed December 2016

²⁸ <http://www.bmub.bund.de/themen/wasser-abfall-boden/abfallwirtschaft/wasser-abfallwirtschaft-download/artikel/abfklav-klaerschlamverordnung> Accessed December 2016

Residual, higher concentrations of Cu and Zn in the ash might restrict its use as a fertilizer component (Schipper et al., 2001). Incineration is an expensive process. Besides the study of P recovery, this technique is usually considered for recovery of valuable nutrients and metals simultaneously.

Besides, sewage sludges are also applied directly to land with the possibility of utilizing its nutrients (Egle, Rechberger, Krampe, & Zessner, 2016; Morse, Brett, Guy, & Lester, 1998; Shiba & Ntuli, 2016; Vaneeckhaute, 2015). The results of the land application of P-recovered solids from WWTPs are variable. Iron phosphates have low solubilities (Kobayashi & Nishizawa, 2012), so the P-uptake from these compounds by plants is unavailable. Aluminum at high concentrations can be toxic (Matsumoto, 2000). Different soils also have different properties such as pH, so the soil composition also affects the P-release from WWTP sludge. Low P-availability in the long-term might result in increased WWTP biosolids application, and the production of P-surpluses in the soils, with the risk of P-runoff from the soil to the watershed.

Based on an assessment of the economic feasibility of recovering P-rich products from municipal WWTPs, a P-removal process by producing struvite as a P-recovered fertilizer was proposed to reduce 8-31% of the sludge weight, and improve sludge dewatering characteristics (Matynia, Koralewska, Wierzbowska, & Piotrowski, 2006; Woods, Sock, & Daigger, 1999). Struvite (an ammonium magnesium phosphate mineral) as a slow-release P-fertilizer (Gaterell, Gay, Wilson, Gochin, & Lester, 2000; Jeanmaire & Evans, 2001), and is a successful example of P-recovery from WWTPs. Previous to engineering designs for its production, unintentional struvite precipitation occurred in anaerobic digesters as the ammonia, Mg^{2+} , and P_i concentrations exceed the supersaturation state for struvite (Marti, Bouzas, Seco, & Ferrer, 2008). Uncontrolled struvite precipitation can occur on the insides of the reactor, pipes, and pumps, requiring extra cleaning maintenance. Therefore, the benefits of purposeful struvite crystallization at dedicated reactors in the plants avoid the spontaneous struvite crystallization on reactor walls and pipes; this reduces maintenance costs, and the final sludge volume (Hao, Wang, van Loosdrecht, & Hu, 2013; Münch & Barr, 2001; Stratful, Scrimshaw, & Lester, 2001). With both N and P as nutrient components, struvite is considered to be a potentially viable commercial option for recycling P from the effluent to a useful P-rich product (Doyle, Oldring, Churchley, Price, & Parsons, 2003).

A limitation to struvite precipitation is that the municipal wastewater sludge anaerobic process must be a BNR or EBPR process (Xiu-Fen, Barnes, & Jian, 2011).

Figure 5-1 presents P-recovery technologies for different types of P-removal processes for WWTPs. The combined chemical-biological P removal process is grouped with the chemical precipitation processes. The main constraint on P-recovery from chemically treated sludge is due to the presence of the metal salts. Metal phosphate salts have a low solubility product, which results in difficult Pi-extraction. The current P-recovery techniques focus on the P-streams from biological P-removal processes, although most WWTPs apply chemical precipitation for more effective P- removal.

With the rise in the market demand for a P-fertilizer, Ca-P solids and struvite have both been considered as P-fertilizers (Gaterell et al., 2000). Kuroda et al. (2002) observed that nearly 75% of total P precipitated as calcium polyP after addition of a CaCl_2 solution to heat-treated waste activated sludge from EBPR systems. Ca-P has a lower Ca/P ratio than natural phosphate rock, which supports its identification (Kuroda, Takiguchi, & Gotanda, 2002). P-fertilizer in the form of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ was precipitated with calcium hydroxide ($\text{Ca}(\text{OH})_2$) and phosphoric acid produced from acid-leached sludge ash with sulphuric acid (Gorazda, Kowalski, & Wzorek, 2012). Dissolved CaCl_2 precipitated whitlockite ($\text{Ca}_9\text{PO}_3\text{OH}$, an unusual calcium phosphate mineral) with anaerobic digestion unit effluent (Huang et al., 2015). Based on an economic-feasibility analysis, it was summarized by Huang *et al.* (2015) that the economic benefits of P recovery as P-minerals from WWPTs were higher than the cost of extracting P from PR. However, this low bioavailability of extracted P-precipitates (Naidu et al., 2008) will require further processing, as is required of PR, to produce effective P-fertilizers.

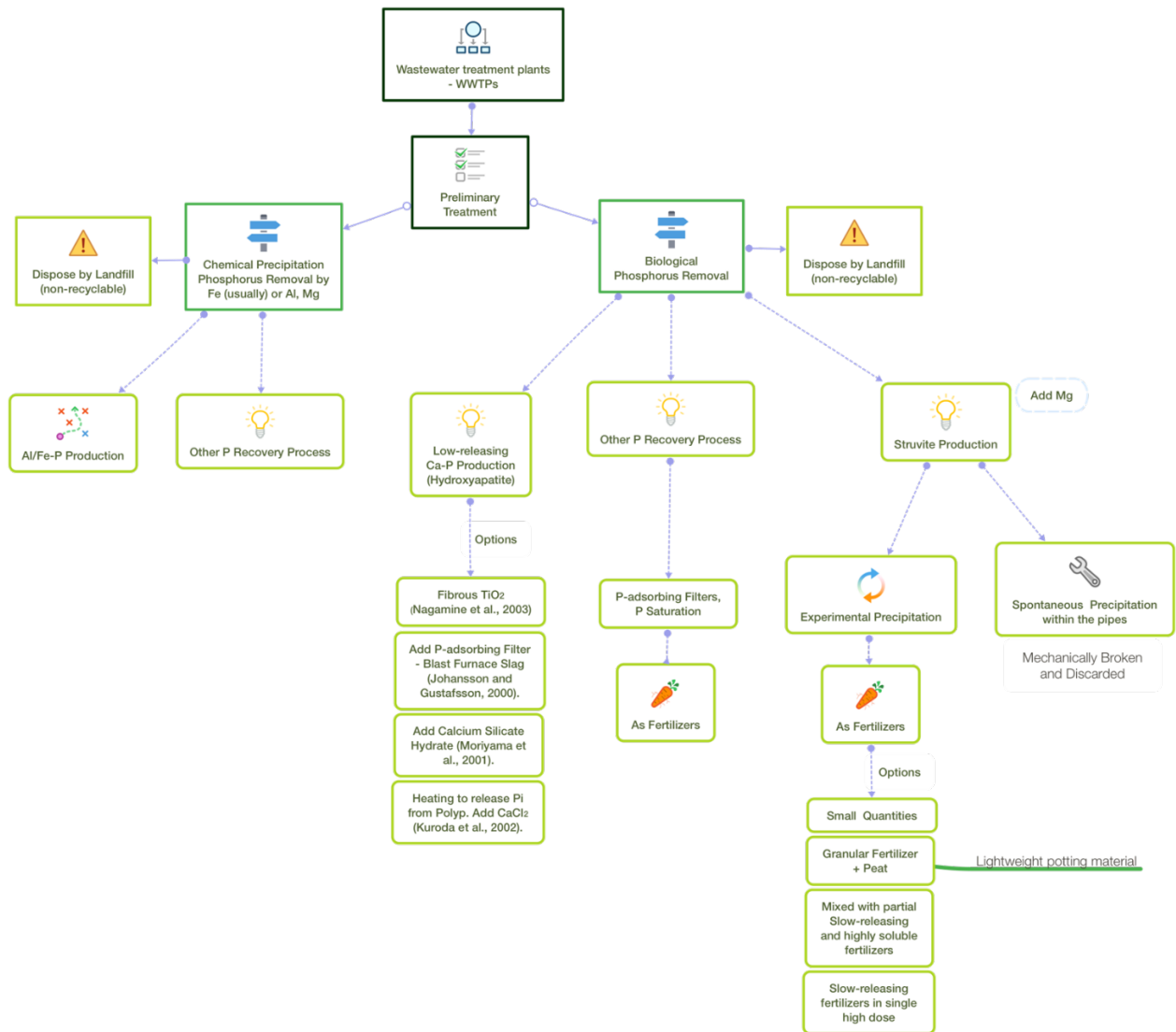


Figure 5-1 Basic Conceptual Process Diagram of selected Phosphorus Recovery processes from WWTPs.

5.3. Materials, Methodology, and Results

5.3.1. cAp mineral precipitation with solid CaCO_3 and acid-extracted P from TWAS

5.3.1.1. Introduction

The major phosphate mineral in PR that is used to produce P-fertilizer is cAp. A carbonate-free version of this mineral, hydroxyapatite (HAp), was precipitated from a municipal wastewater treatment plant (NETAWATER®) in Japan (Kabbe, 2017). At the laboratory scale, dissolved CaCl_2 used to precipitate calcium and P-rich solids with the hot-water-extractable polyP from the waste activated sludge from an EBPR process (Kuroda et al., 2002). No published literature was identified that reported the use of limestone to produce calcium phosphate minerals with P extracted from WWTPs (Ross, 2017). The first step towards precipitating cAp with acid-extracted P from TWAS as to add powdered, synthetic calcium carbonate and sodium bicarbonate to increase the pH and the cAp supersaturation, and include apatite seed to lower the supersaturation required for cAp precipitation (Kashchiev & Van Rosmalen, 2003) for cAp mineral growth.

5.3.1.2. Material and Methodology

Pi extraction of TWAS by acid digestion (Section 4.3.1.) produced a supernatant with 43.7 ± 13.6 mM Pi. Acid digestion of the TWAS-pellet (the TWAS with supernatant removed) generated 55.7 ± 13.0 mM Pi (back-calculated to the TWAS volume). After acid extraction, all of the samples were centrifuged and 0.5 mL the supernatants were decanted and transferred to 2 mL microcentrifuge tubes (TWAS-S) for further precipitation. The solution pH measured with pH paper (HYDRION® pH=2-10, Lot 208515). Synthetic, ACS-grade calcium carbonate powder (MP, CAT 191213), was added stepwise to neutralize the from pH less than 1 to 6. The stepwise addition was required for the carbon dioxide gas bubbling to subside between calcium carbonate dosing. At pH 6, 10 mg of synthetic hydroxyapatite (HAp) powder (Sigma) was added to the microtubes as seed crystals to avoid its dissolution at more acidic pH. The solution pH was increased from 6 to pH 7 with ACS-grade sodium bicarbonate (AMRESCO® CAS 144-558) to prevent undissolved calcium carbonate from being in the product. Once neutralized to pH 7, the samples were left to crystallize for 24 hours at room temperature. The samples were

centrifuged at 8000 rcf for 5 min. The supernatant Pi concentration of precipitated samples was measured with the vanadomolybdate assay, with protein solubilization with Triton X-100.

The solid products were characterized as powder on a quartz slide with Raman confocal spectroscopy. (WITec Alpha 300, 20x lens (WITec GmbH, Ulm, Germany)). The excitation laser was either 785 nm (XTRA High Power Single Frequency Diode Laser, Toptica Photonics, Victor, NY, USA), 488 nm or 532 nm (WITec GmbH, Ulm, Germany). Calibration was performed with Si. Data were collected with an integration time of 1 second for 45 seconds. Standard materials were synthetic hydroxyapatite, calcite, and demineralized bone powder. The spectra were filtered to remove cosmic ray spikes. Fourier Transform Infrared Spectroscopy (AT-IR) was performed with a Cary 630 FT-IR (ATR) (Agilent Technologies).

5.3.1.3. Result and Discussion

Figure 5-2 shows the Pi concentrations before P-extraction, after acidic P-extraction, and after neutralization/precipitation. The Pi concentrations in both groups increase significantly during the acidic P-extraction (Section 4.3.1). The Pi concentration for the TWAS-S samples (without removed supernatant) increased from 1.2 ± 1.0 mM (37.9 ± 31.7 mg/L) (per unit of initial TWAS volume) (n=8) to 55.7 ± 13.0 mM ($1,732 \pm 403.2$ mg/L) (n=12) by acid digestion. The Pi concentration for the supernatant-removed TWAS samples increased from 0.79 ± 0.86 mM (24.6 ± 26.8 mg/L) to 43.7 ± 16.3 mM ($1,359 \pm 507.1$ mg/L) (n=12) by acid digestion. In this experiment, the Pi concentrations after neutralization/precipitation decreased to 3.5 ± 2.5 mM (108.3 ± 78.1 mg/L) (n=11) for the supernatant of TWAS-samples, and to 0.24 ± 0.12 mM (7.5 ± 3.7 mg/L) (n=8) for the TWAS samples with the supernatant removed. Assuming that the reduced Pi concentration was due to phosphate precipitation, an average of 93.7 % (TWAS) and 99.5 % (TWAS-S) of Pi was removed from solution.

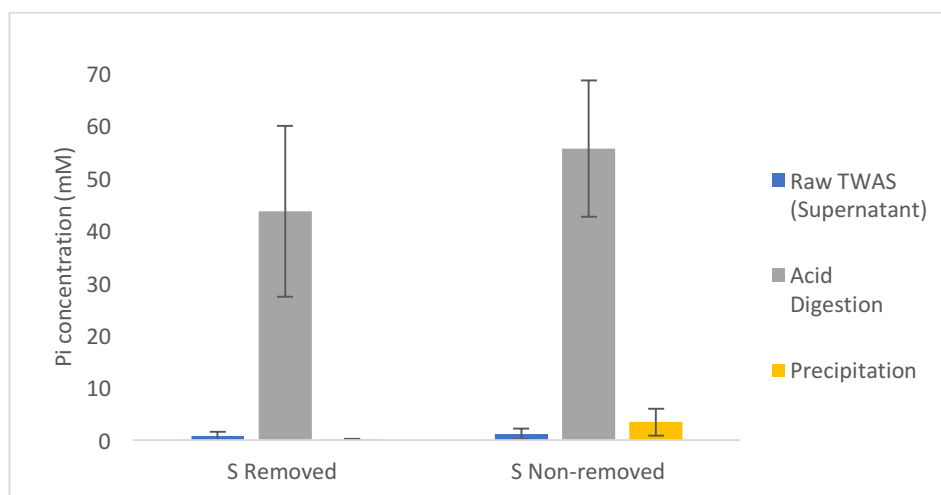


Figure 5-2 Pi concentration before acidic P-extraction, after P-extraction at Section 4.3.1 (Figure 4-6), and after neutralization/precipitation for S (n=11) and S-non-removed (n=8) at the removed supernatant group.

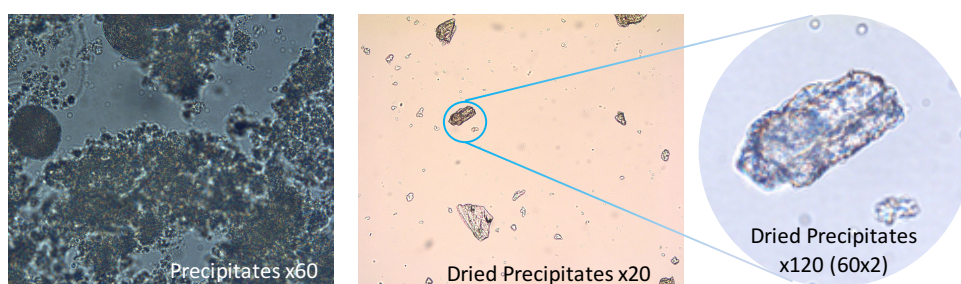


Figure 5-3 Optical microscope images without TBO staining of precipitates formed by extracted sludge with pure calcium carbonate solids for the feasibility test (experiment detail at section 4.3.1 for acid digestion and 5.3.1 for precipitation).

Figure 5-3 presents the optical microscopic images of the precipitates from the thickened waste activated sludge sample used for the precipitation feasibility test. During the acid extraction process, the acid has already hydrolyzed a significant fraction of the total phosphorus to Pi. During the precipitation process, free calcium ions reacted with extracted Pi to generate calcium phosphate solids, which are not dyed by TBO. The microscopic images for precipitates might be relatively meaningless, but in contrast, the absence of staining shows an absence of polyP, as polyP stains pink with TBO. However, the images support the conclusion of a solid precipitate produced by this process.

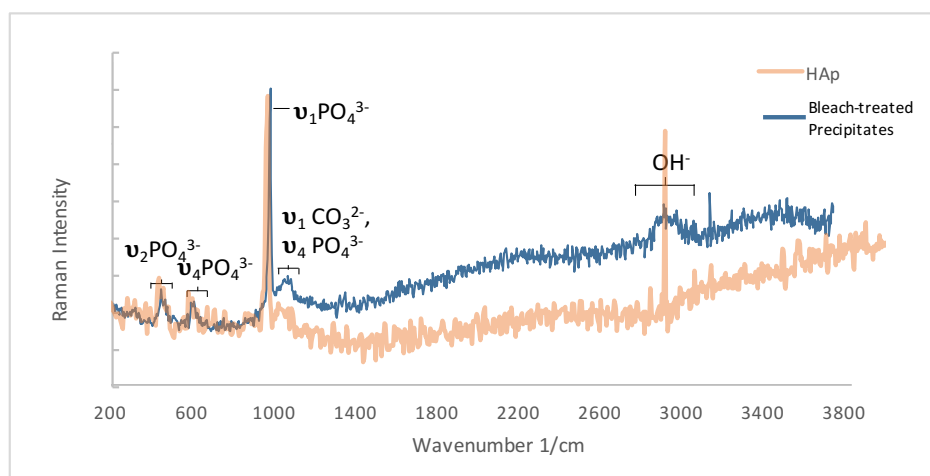


Figure 5-4 An example Raman spectrum of a precipitate after the acidified TWAS neutralization experiment with HAp seed.

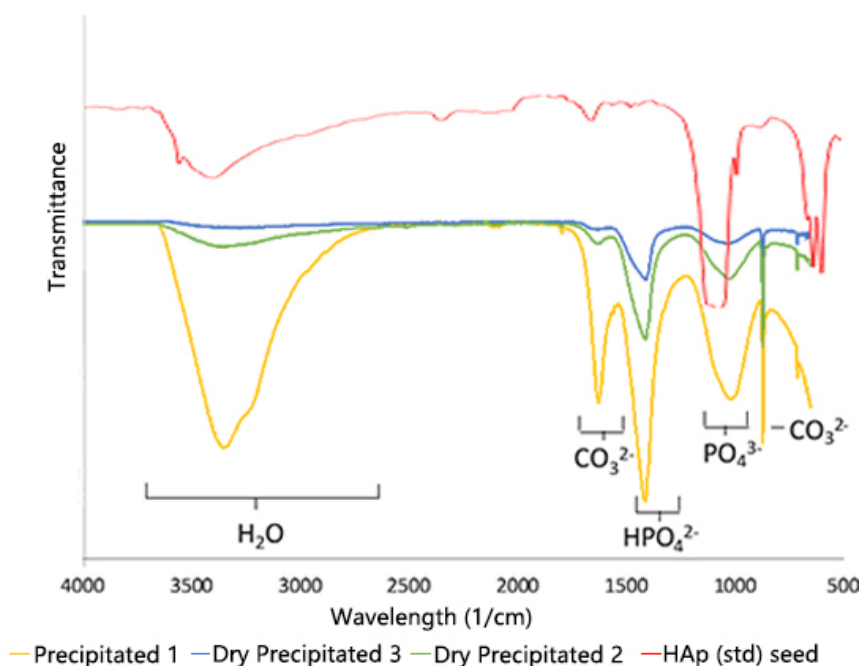


Figure 5-5 FT-IR spectra of precipitates and standard HAp seed. Sample 1 was air-dried, and sample 2 and 3 were dried at 118 °C for 1 hour after air-drying.

The precipitates and synthetic HAp were characterized by Raman spectroscopy (Figure 5-3) and FT-IR (Figure 5-4). According to Raman spectrum, the precipitate indicated the presence of apatite ($\nu_1 = 960 \text{ cm}^{-1}$) with carbonate substitutions ($\nu_1 = 1070 \text{ cm}^{-1}$). The carbonate substitution ($\nu_1 \text{ CO}_2$) of apatite at 1070 cm^{-1} differentiates this precipitate from the HAp seed, which does not have this shift. Similarly, the tested precipitates do not have a calcium carbonate (calcite) content because it would generate a shift at 1090 cm^{-1} . Therefore, it is concluded that cAp could be

precipitated from Pi acid-extracted from TWAS, and encouraged further experiments of cAp production by P-streams from acidified municipal waste sludge, calcium carbonate and sodium bicarbonate solids, and HAP seed.

The results of Chapter 4 suggest that the metal content in the TWAS could re-precipitate as metal phosphate during the neutralization process. It is possible that the inclusion of the HAP seeds in this neutralization-precipitation favored cAp precipitation over metal phosphate precipitation. It is also possible that the use of solid CaCO_3 as a neutralizing agent to pH 6 created a higher $\text{Ca}^{2+}/\text{Fe}^{3+}$ ratio in the solution, also favoring cAp precipitation.

The precipitates were pale in color, suggesting an apatite mineral, instead of black, which is the color of iron phosphate (Parfitt, Atkinson, & Smart, 1975), which is likely to be the predominate metal phosphate solid. TWAS contains iron phosphates, which contribute to its dark color. The visual observations, Raman and FT-IR spectroscopy results, and the reduction in Pi concentration after precipitation led to the conclusion that cAp precipitated from acid-extracted P from TWAS.

Future work is required to obtain Raman spectroscopic information for TWAS. The fluorescence generated by TWAS makes it difficult to obtain Raman-shift information. Wet TWAS, dried TWAS, dried TWAS after bleach treatment, and polyP-extracted TWAS samples were examined with Raman spectroscopy with a 785 nm laser. However, the strong fluorescence backgrounds generated by organic matter overwhelmed the signals from chemical shifts. Other sample preparation methods for reducing fluorescence, such as microwave and other chemical treatments, should be investigated.

5.3.2. Increasing Synthetic Calcium Carbonate and Limestone Solubility with Carbon Dioxide

5.3.2.1. Introduction

Due to the low solubility of calcium carbonate in water, it was previously determined that synthetic CaCO_3 does not produce a Ca-CO_3 solution with a high enough Ca-CO_3 concentration to precipitate cAp from a synthetic solution with 27 mM (840 mg/L) Pi (Ross, 2017). In order to

test the feasibility of a continuous cAp crystallizer for further development, the addition of a concentrated Ca-CO₃-rich solution is required to become the calcium source in the reaction and then to increase the cAp saturation in the P-rich waste stream. Based on the equilibria between calcium carbonate, water, and carbon dioxide (Figure 5-6), and knowing that CO₂ is available on-site at ROPEC, the feasibility of using CO₂ to increase the Ca-CO₃ concentration in solution for cAp precipitation was tested. This work was in collaboration with Meouch, who also measured some of these values (Meouch, 2016).

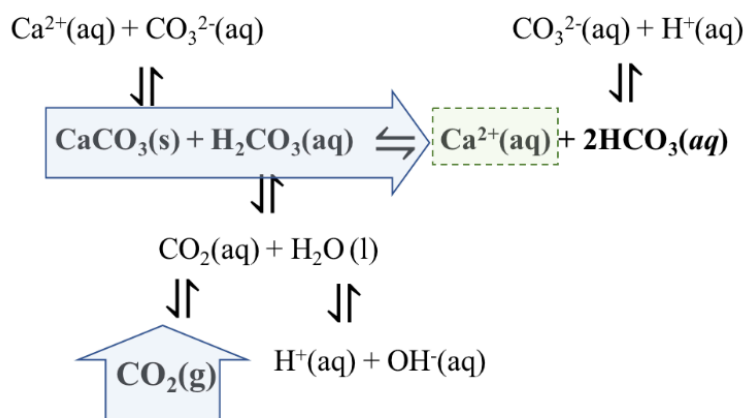


Figure 5-6 The equilibrium relationships between calcium carbonate, carbonic acid, water, and carbon dioxide (Ross, 2017).

The CO₂ gas concentrations in the anaerobic off-gas and the flue gas from the methane-powered power plant at ROPEC are not known. Therefore three CO₂ gas concentrations were selected for the study. Pure CO₂ represents the highest chemical potential, 15 % CO₂ is representative of flue gas from bio-fuel-fired power plant from another WWTP (Kuo, 2015) and 7 % CO₂ is representative of methane-fired power plant off-gas (M. Songolzadeh, Soleimani, Ravanchi, & Songolzadeh, 2014).

Synthetic calcium carbonate was first selected as a Ca-CO₃ source because of its purity.

However, synthetic CaCO₃ is not a reasonable Ca-CO₃ source at the industrial scale envisioned for this P-recovery process. Therefore, limestone was also tested as a Ca-CO₃ source. King Brand™ limestone screenings (Home Depot, Ottawa) were dried in an oven ~80 °C until they were dry. They were sieved in an 8” nest (710 µm – 4.75 mm) and shaken for 10 min in an RX-29 RO-TAP. The <710 µm screenings were used in these tests to increase the surface area.

Limestone includes organic contaminants, so the use of 20% sodium dodecyl sulfate (SDS) as a surfactant to solubilize organics was also tested.

5.3.2.2. Materials, Methodology, Results and Discussion

5.3.2.2.1. Synthetic calcium carbonate dissolution in potable water with CO₂ gas

1 gram of synthetic calcium carbonate (Sigma, ACS grade) dissolved in 1 L of potable water (City of Ottawa) overnight. The soluble calcium concentration in this potable water was 0.30 ± 0.02 mM Ca²⁺. The slurry was transferred into a 1.7 L stirred tank reactor. The slurry was continually agitated with a paddle mixer attached to a motor. The pH value measured by an AR10 pH probe (Orion®) was recorded initially and at each time point. At the initial and each subsequent, 2 mL of the slurry was taken and placed in a 2 mL microcentrifuge tube. The head space was minimal, and the cap closed, in order to reduce CO₂ exchange with the atmosphere.

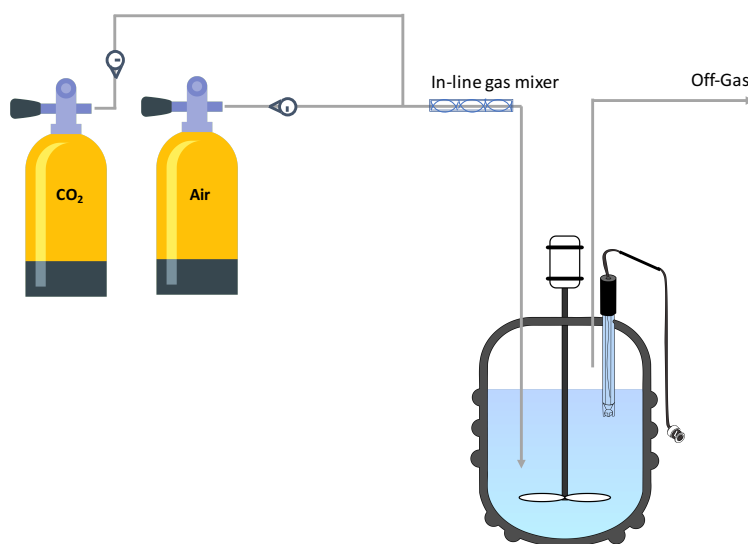


Figure 5-7 The schematic for bubbling CO₂ gas in a calcium carbonate slurry, in a closed environment.

The flow rates of compressed CO₂ and air (Linde®) were controlled with flow meters (Matheson®) to produce CO₂/air mixes of 100 %, 15 %, or 7 % CO₂ in the air, at a total flow rate of 1 L/min. The gas passed through an in-line mixer and entered the reactor through a bubbler located below the paddle mixer. The gas exited the reactor and was directed to the fume hood. The experiments were run until the calcium concentration did not change significantly, which was approximately 20 min. All of the 2 mL samples were centrifuged at 12,500 rcf for 5

min. The experiments were repeated at least four times; two repeats (synthetic CaCO_3 with 100 % CO_2 and 15 % CO_2) were completed by Ms. Meouch. The calcium concentration was measured by a colorimetric method, using o-cresolphthalein-complexone (o-CPC) (Schwarzenbach, 1955). The absorption was read with a UV-VIS plate reader (EpochMicroplate Spectrophotometer, BioTek® with Gen 5 software).

5.3.2.2.2. Commercial limestone dissolution in potable water with CO_2 gas

A similar protocol was used to measure the solubility of limestone exposed to 100 % CO_2 . Suspecting that the reactivity would be lower for limestone than synthetic calcium carbonate, two slurry densities were tested: 2 and 4 g/L limestone in potable water to increase the surface area of limestone in the reactor. To solubilize any organics included in the limestone, one test with 1 % SDS with 4 g/L limestone and 100% CO_2 was undertaken.

5.3.2.3. Result and Discussion

Figures 5-8 – 5-11 present the pH and calcium concentrations versus time for the synthetic calcium carbonate solids dissolved by CO_2 gas at different concentrations. This correlation between the increase in equilibrium average calcium concentration with a decrease in average pH is presented in Table 5-1. Figures 5.13 and 5.14 present the pH and calcium concentration for limestone dissolved at 2 or 4 g/L in contact with 100 % CO_2 gas, and 2 g/L limestone dissolved in contact with 1 % SDS in Table 5.15.

5.3.2.2.1. Synthetic calcium carbonate with CO_2

Figure 5-8 presents the pH and calcium concentration data for 25 min of gas-liquid-solid contact time for 1 g/L synthetic CaCO_3 and 100 % CO_2 at room temperature. The pH achieved a steady state value of 6.03 ± 0.15 after less than 5 min, and the calcium concentration value reached a steady state of 6.52 ± 0.77 mM after approximately 10 min. This experiment was repeated four times.

Figure 5-9 presents the pH and calcium concentration data for 25 min of gas-liquid-solid contact time at room temperature for 1 g/L synthetic CaCO_3 , and 15 % CO_2 . The pH achieved a steady state value of 6.38 ± 0.12 after less than 5 min, and the calcium concentration value reached a

steady state of 4.90 ± 0.60 mM after approximately 10 min. This experiment was repeated five times.

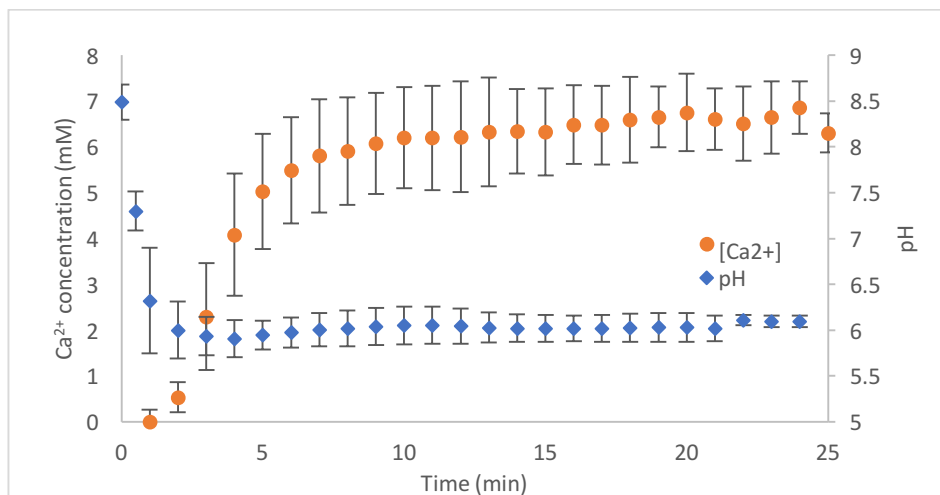


Figure 5-8 Calcium concentration and pH versus time for 1 g/L synthetic CaCO_3 exposed to 100 % CO_2 (n=4).

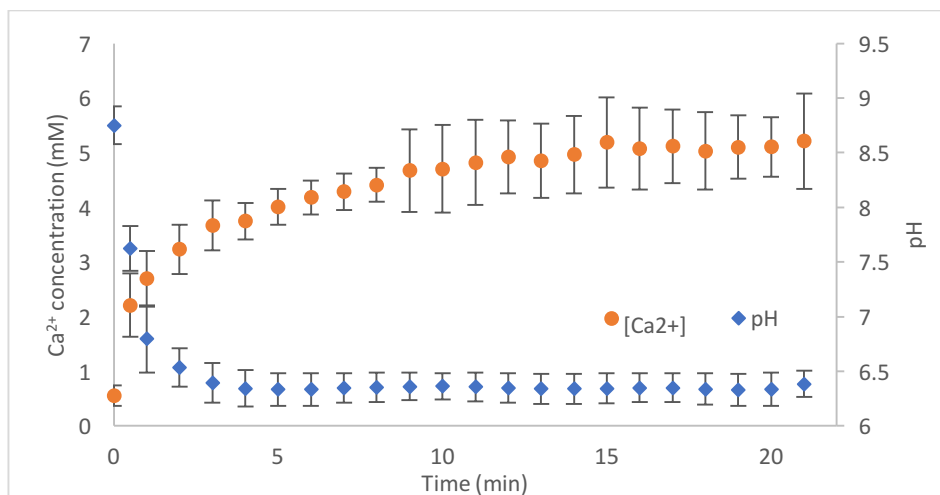


Figure 5-9 Calcium concentration and pH versus time for 1 g/L synthetic CaCO_3 in contact with 15 % CO_2 (n=5 for calcium assay, n=4 for pH).

Figures 5-10 and 5-11 present the pH and calcium concentration data for 20 min of gas-liquid-solid contact time at room temperature for 1 g/L synthetic CaCO_3 , and 7 % CO_2 . The process was interrupted by turning off the gas during the periods of 6 to 10 min. In Figure 5-11, the pH achieved a steady state value of 6.70 ± 0.08 before 5 min, and the calcium concentration value reached a steady state of 3.45 ± 0.48 mM after approximately 10 min. This experiment was repeated seven times.

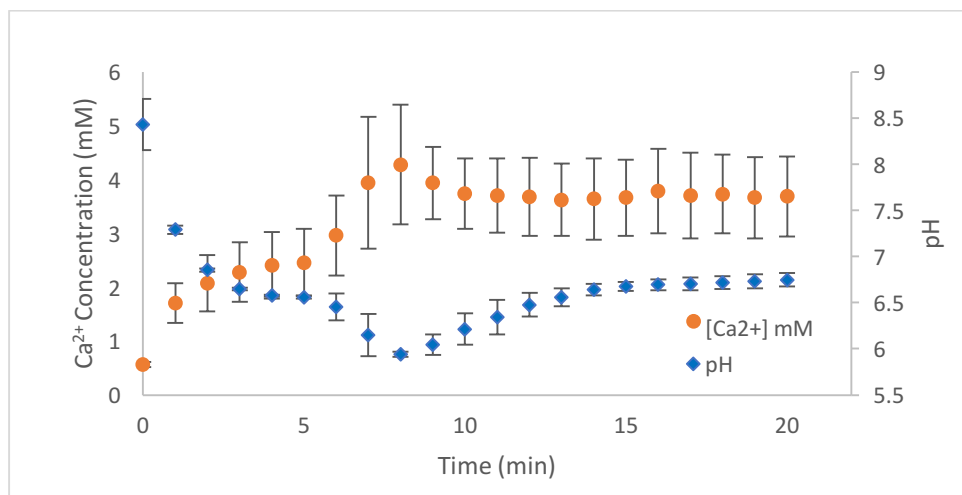


Figure 5-10 Calcium concentration and pH versus time for 1 g/L synthetic CaCO_3 in contact with 7% CO_2 ($n=3$) with a temporary gas shut-off between 6-10 min.

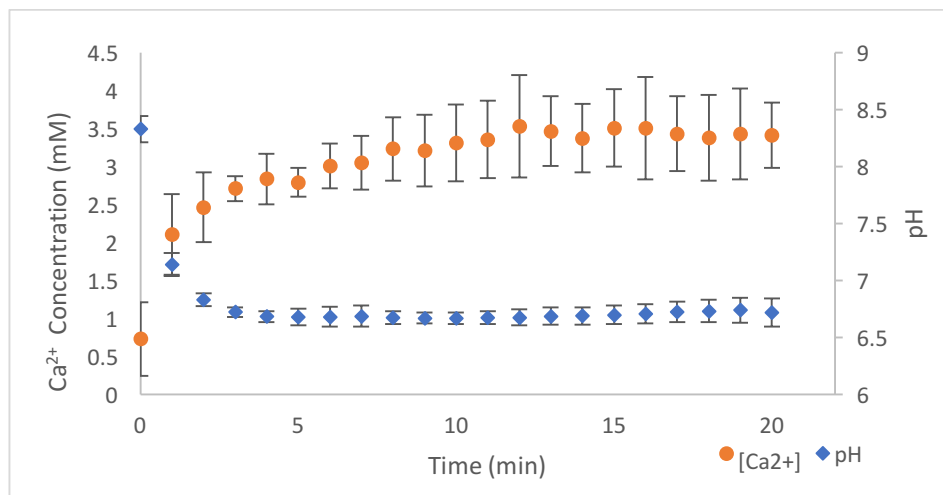


Figure 5-11 Calcium concentration and pH versus time for 1 g/L synthetic CaCO_3 in contact with 7 % CO_2 ($n=4$).

Bubbling CO_2 into the CaCO_3 -water slurry produces carbonic acid, which reduces the pH. The speciation of carbonate ions in solution is a function of pH (Figure 5-12). The reduction in pH caused by the spontaneous production of carbonic acid from CO_2 gas shifts the equilibria from $\text{CO}_3^{2-} / \text{HCO}_3^-$ species to $\text{HCO}_3^- / \text{H}_2\text{CO}_3^0$ species. The solubility product of CaCO_3 is a function of the concentration of Ca^{2+} and CO_3^{2-} ions. With the reduction in CO_3^{2-} concentration caused by the reduction in solution pH, at equilibrium with CaCO_3 solids, the calcium concentration must increase. The degree to which the calcium concentration increases is proportional to the pH decrease.

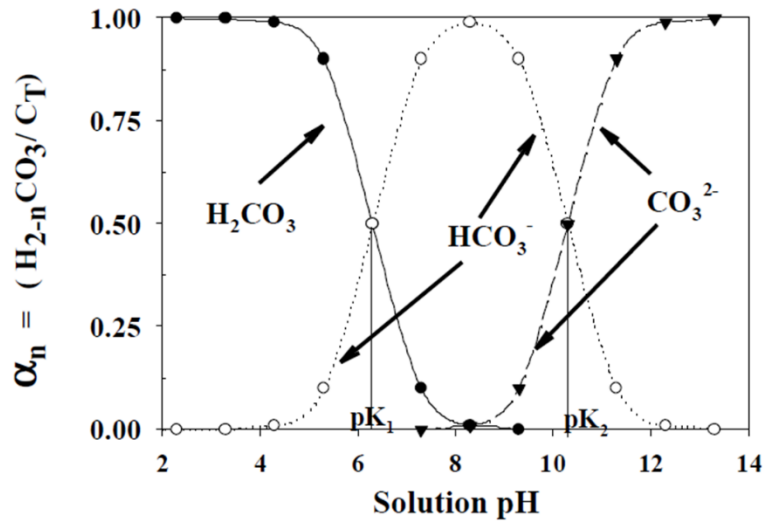


Figure 5-12 ion activity of carbonic acid, bicarbonate, and carbonate ions as a function of pH (n=the charge on the ion species) (reproduced from (Sparks, 2001)).

Table 5-1 Equilibrium calcium concentration and pH values of synthetic calcium carbonate dissolution in potable water, in contact with different CO₂ gas concentrations.

Gas % CO ₂	Calcium concentration (mM)	pH
100	6.52 ± 0.77	6.03 ± 0.15
15	4.90 ± 0.60	6.38 ± 0.12
7	3.45 ± 0.48	6.70 ± 0.08

5.3.2.2.2. Commercial limestone as a calcium carbonate source dissolved with 100 % CO₂

Figure 5-13 and Figure 5-14 show the soluble calcium concentration with contact time for 2 g/L limestone and 4 g/limestone with 100 % CO₂, respectively. The trends of calcium concentration and pH value with limestone solution are consistent with synthetic calcium carbonate (Section 5.3.2.2.1), and may not have reached a steady-state value after 120 min . It is reasonable result that limestone with impurities produces lower calcium concentration in the solution, even if the higher concentration of limestone solids was added compared with synthetic CaCO₃ to increase the surface area and reaction rate.

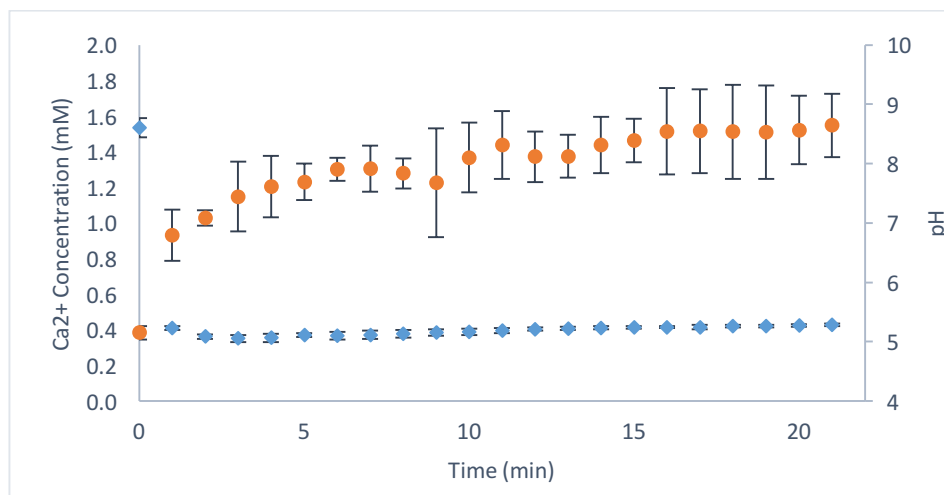


Figure -5-13 Calcium concentration and pH versus time for 2 g/L limestone in contact with 100 % CO₂ (n=3).

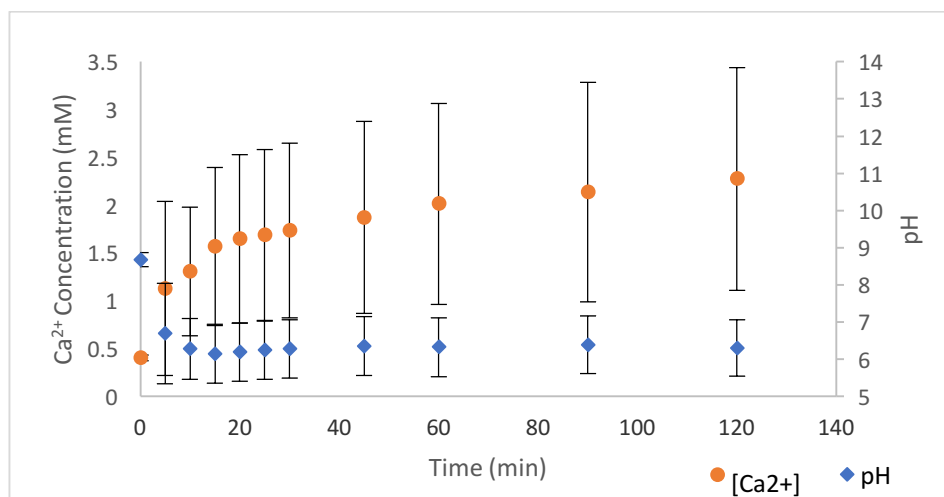


Figure 5-14 Calcium concentration and pH versus time for 4 g/L limestone in contact with 100 % CO₂ (n=3).

Moreover, in this experiment of Figure 5-13, within the 20 min of contact time, the increased mass of limestone in the solutions did not increase the soluble calcium concentration significantly (Appendix H, Table H-1) . However, there is a trend that the extended contact time could improve solubility of calcium ions in the solution in Figure 5-14 though there is no significant difference in the calcium concentration at 20 min and 120 min (Appendix H, Table H-2). Longer contact times are recommended for future work.

The surfactant was expected to increase the surface area of limestone solids by solubilizing organic impurities. Figure 5-15 shows the calcium concentration versus time for 4 g/L limestone

with 1% SDS in contact with 100 % CO₂. The maximum calcium concentration was 0.33 mM after 20 min. SDS as a surfactant may have formed micelles with organics, but they could also chelate with calcium and reduce the free calcium concentration (Meldrum, 2013). Therefore, undertaking repetitions were meaningless (n=1).

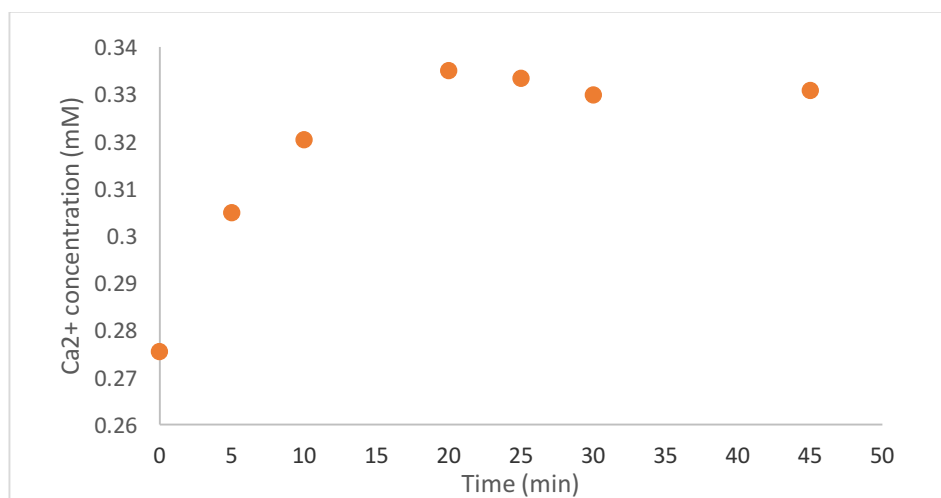


Figure 5-15 Calcium concentration from 100% CO₂ bubbled in a 4 g limestone in 1 L tap water with 1% SDS (n=1).

5.3.3. DIG-2 pretreatment with EDTA to precipitate calcium phosphate with a Ca-CO₃ solution from limestone

5.3.3.1. Introduction

As described in the introduction (Section 5.1), DIG-2 was chosen as the P-stream from ROPEC to evaluate the possibility of P-recovery as cAp by crystallization with a Ca-CO₃ solution produced from limestone and CO₂. However, the iron chloride addition in the chemical precipitation process and the aluminum content from the Lemieux water treatment sludge results in the majority of Pi in DIG-2 as metal phosphate solids, and the possibility of excess dissolved iron from chemical treatment. Powder x-ray diffraction results from dried DIG-2 solids are presented in Figure 5-16. Phase identification was undertaken (Rigaku Multiflex D3) and recorded in Table 5-2.

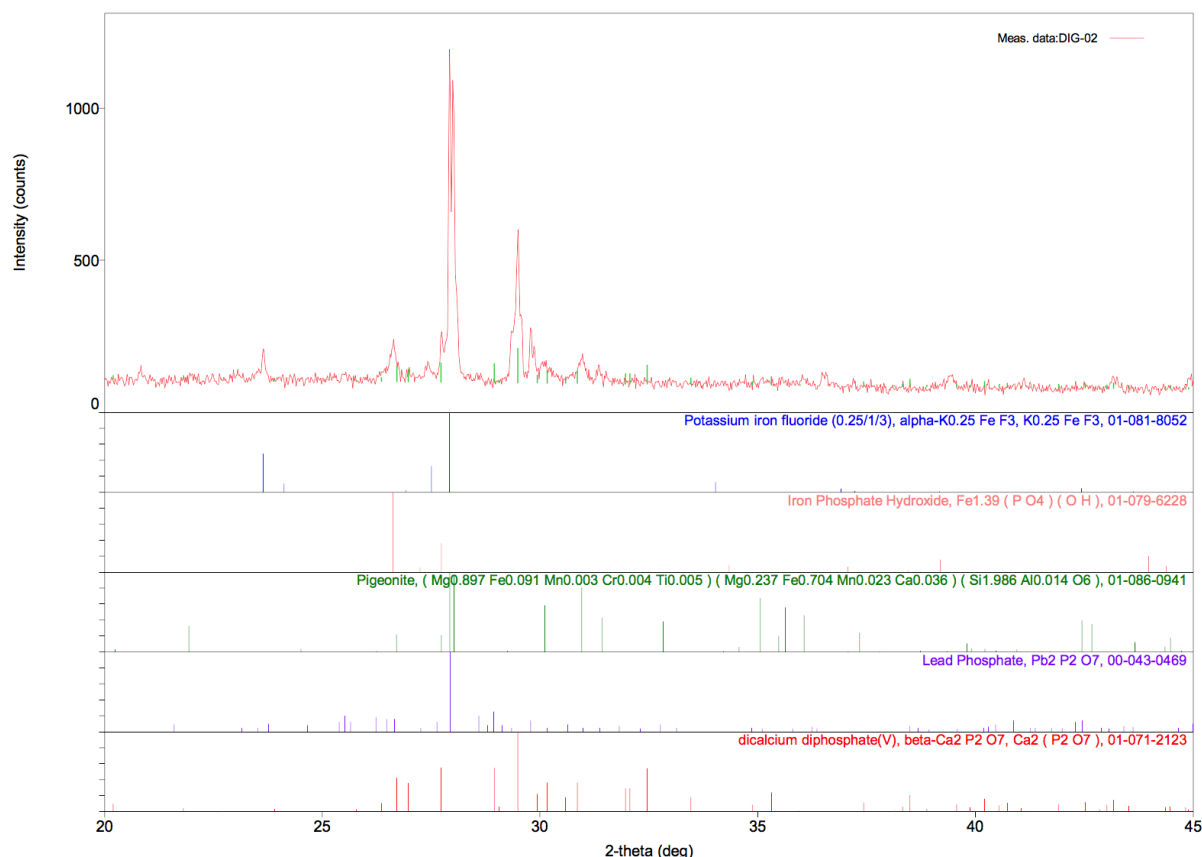


Figure 5-16 X-ray diffraction pattern of dried DIG-2 compared with potential minerals.

Table 5-2 Potential mineral phases compared with dried DIG-2 (Figure 5-16).

Mineral name	Chemical Formula	ICDD number
Potassium iron fluoride (0.25/1/3)	Alpha- $K_{0.25}FeF_3$, $K_{0.25}FeF_3$	01-081-8052
Iron phosphate hydroxide	$Fe_{1.39}(PO_4)(OH)$	01-079-6228
Pigeonite	$(Mg_{0.897}Fe_{0.091}Mn_{0.003}Cr_{0.004}Ti_{0.005})$ $(Mg_{0.237}Fe_{0.704}Mn_{0.023}Ca_{0.036})$ $(Si_{1.986}Al_{0.014}O_6)$	01-086-0941
Lead Phosphate	$Pb_2P_2O_7$	00-043-0469
Dicalcium diphosphate (V)	Beta- $Ca_2P_2O_7$, $Ca_2(P_2O_7)$	01-071-2123

In order to precipitate the Pi as cAp, a concentrated EDTA solution was added to chelate the metal ions in solution. Reduction of the free metal ion concentration would subsaturate the solution with respect to metal phosphate salts, and dissolve the iron phosphate to eliminate the effect of iron on Pi (Lucchesi & Hirn, 1960). A range of EDTA concentrations was mixed with DIG-2 to identify the maximum released Pi concentration after the dissolution of metal phosphates.

5.3.3.2. Materials and Methodology

X-ray diffraction was conducted to characterize the dried DIG-2 compared with potential minerals. The solids in DIG-2 were gravity-settled. A fraction of the settled solids was extracted with a 25 mL pipette. The solids were transferred to a watch glass and air-dried. The solids were ground in a mortar and pestle before analysis by powder x-ray diffraction. A Multiflex D3 (Rigaku Americas Corp., TX, USA) with $CuK_{\alpha 1}$ radiation ($1.5418 \text{ \AA}$) scanned the sample between $2\theta = 20^\circ$ - 45° at a rate of $1^\circ/2\theta/\text{min}$.

The method followed in this section follows the EDTA-extraction method used in Section of 4.3.2.3 (2) - PolyP Extraction – Combination of EDTA and Acid Hydrolysis. In this experiment, 200 mM EDTA (pH 8) was diluted by the series dilution method to generate 500 μL of 30, 60, 80, 90, 200 mM EDTA that was mixed with 500 μL DIG-2 in microcentrifuge tubes. DI water as 0 mM EDTA was performed as the control. The samples were shaken on a laboratory bench

shaker for 5 days at room temperature. The free Pi concentrations in the samples were measured after centrifugation at 10,000 rcf for 5 min. The EDTA extractions were repeated four times, except for 0, 80, and 200 mM EDTA, which were repeated eight times.

5.3.3.3. Results and Discussion

The free Pi concentration back-calculated to the initial DIG-2 volume in the control samples was 0.40 ± 0.11 mM Pi (12.44 ± 3.42 g/L Pi). Figure 5-17 shows the increase in free Pi concentration from 0 mM EDTA until approximately 90 mM EDTA, where the free Pi concentration does not change with increasing EDTA concentration. The same Pi-release efficiency from DIG-2 by 90 mM EDTA and 200 mM EDTA suggests that 90 mM EDTA as an optimum value to chelate the metal ions in DIG-2. This result indicates that the solid and liquid phases of DIG-2 might contain 20 mM Pi (620 mg/L P). This Pi could be dissolved in solution if metal ions were not present. Stream data from ROPEC showed that the total P as Pi concentration ranged from 20 to 25 mM Pi (621-781 mg/L P). However, 80 mM EDTA was proposed to conduct the pretreatment for cAp precipitation to avoid excess EDTA chelation of added calcium ions.

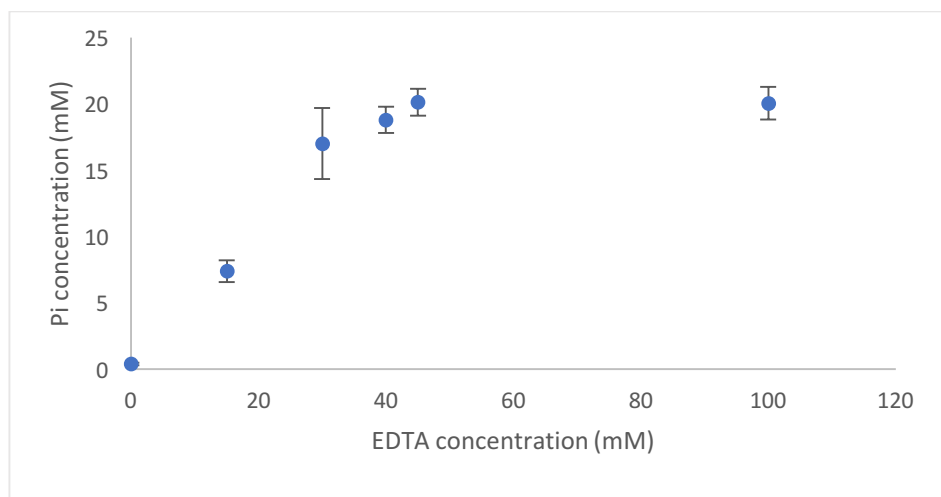


Figure 5-17 Pi concentration in the DIG-2 as a function of EDTA concentrations

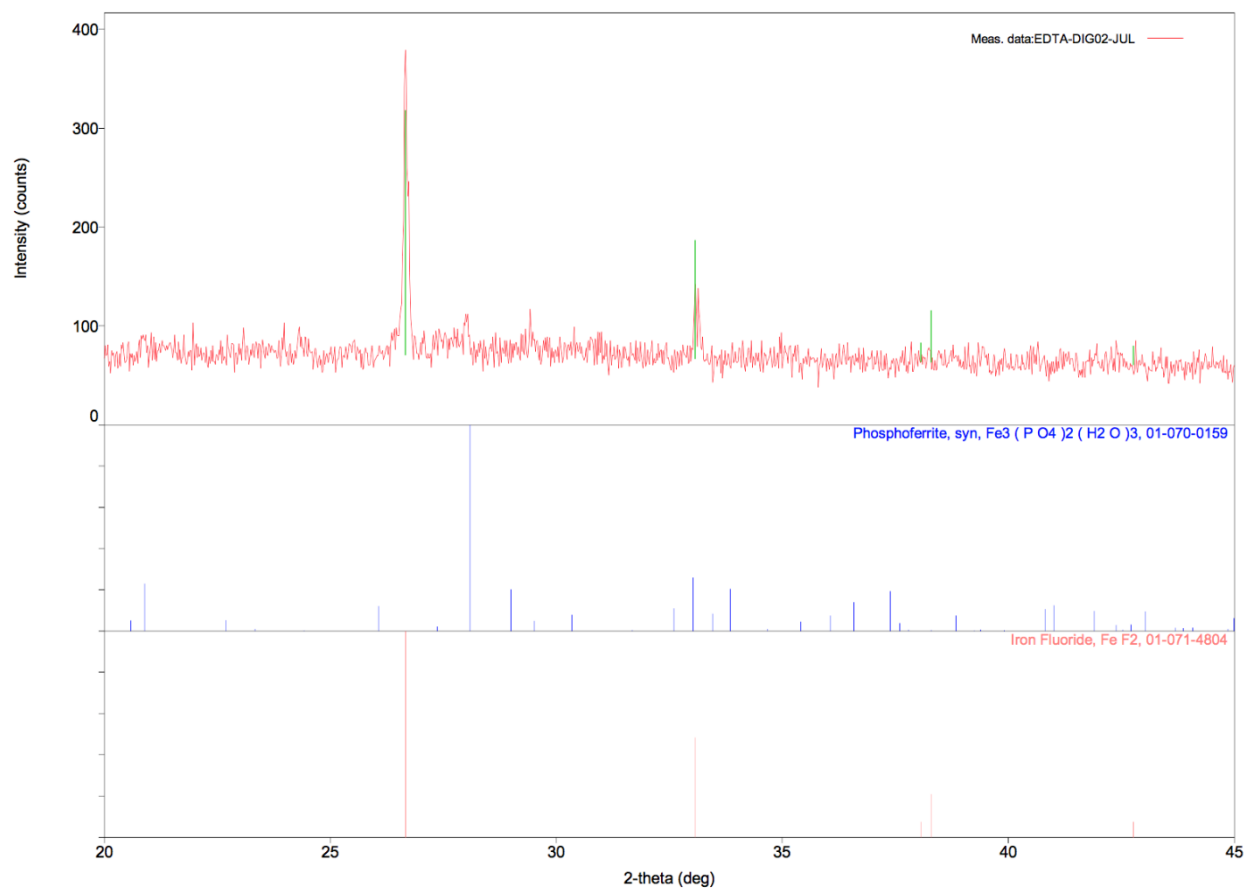


Figure 5-18 X-ray diffraction pattern of post-EDTA-treated DIG-2 compared with potential minerals.

Table 5-3 Potential mineral phases compared with dried DIG-2 (Figure 5-16).

Mineral name	Chemical Formula	ICDD number
Phosphoferrite, syn	$\text{Fe}_3(\text{PO}_4)_2(\text{H}_2\text{O})_3$	01-070-0159
Iron Fluoride	FeF_2	01-071-4804

5.3.4. Precipitation of cAp from EDTA-treated DIG-2 with Ca-CO_3 solutions produced from limestone and CO_2 gas.

5.3.4.1. Introduction

The initial work that examined the crystallization of cAp was undertaken with synthetic solutions by Ross (Ross, 2017). In her thesis, cAp was precipitated from synthetic phosphate solutions and

Ca-CO₃ solutions produced from dissolving synthetic calcium carbonate in potable water in contact with the CO₂ gas in semi-batch and a continuous stirred tank reactor.

This work reproduces the same semi-batch test, but instead of mixing Ca-CO₃ solutions with Pi-solutions of different Pi concentrations, different volume ratios of Ca-CO₃ solutions and EDTA-treated DIG-2 were mixed to evaluate cAp precipitation potential. For this precipitation test, the P-source is the EDTA-treated DIG-2 with a 50-50 by volume mixture of 80 mM EDTA and DIG-2 (Section 5.3.3). The Ca-CO₃ solution is the CO₂-treated limestone solution with a Ca²⁺ concentration of 1.52 ± 0.23 mM (2 g, 15-20 min contact time) (Section 5.3.2). The objective of this preliminary experiment was to identify if cAp could be precipitated from these two industrial-scaleable streams - assuming that iron addition before anaerobic digestion might be reduced in exchange for the potential benefits of cAp precipitation. The P-recovery efficiency is not included in the scope of this thesis, but it should be discovered in the future. For this preliminary work, HAP seed was not added in order to evaluate the most challenging precipitation conditions, which is precipitation through homogeneous nucleation (Kashchiev & Van Rosmalen, 2003).

5.3.4.2. Materials and Methodology

The initial Pi-solution was the 80 mM/L-EDTA-treated DIG-2 (1:1 volume) with 18.8 ± 1.0 mM Pi. The Ca-CO₃ solution was from 100 % CO₂ gas-treated limestone in potable water with a calcium concentration of 1.52 ± 0.23 mM Ca²⁺. The P-solution and Ca-solution were mixed with the following Ca:Pi volumetric ratios: 50/50, 60/40, 70/30, 80/20, 90/10 and 95/5, for a total final volume of 100 mL. The solutions were mixed by hand. After 20 min, the slurries were decanted into 50 mL centrifuge tubes, and centrifuged at 10,000 rcf for 5 min, because filtration was too slow. Soluble Pi and Ca concentrations were measured with the colorimetric assays. The supernatants were decanted, and the solids air-dried and hand-ground in a mortar in pestle before analysis with powder x-ray diffraction (Section 5.3.3.2). This experiment was repeated three times.

5.3.4.3. Results and Discussion

Figure 5-19 shows the theoretical and measured phosphate and calcium concentrations (equations 1, 2) if applying the effect of dilution only as a function of Ca-CO₃:Pi mixing volumes. Figure 5-20 indicates the relationship between the respective K_{sp} of [Ca] x [Pi] (mM²) and the Ca/Pi volume ratio based on the same results with Figure 5-19. The measured Ca²⁺ and Pi concentrations are lower than the theoretical values (Appendix H, Table H-13, H-14). The negative values of practical soluble calcium concentration in Figure 5-19 were caused by the calcium chelation by EDTA. It also caused the negative values of [Ca] x [Pi] (mM²) in Figure 5-20.

$$([Pi] \text{ or } [Ca])_{Theory} = ([Pi] \text{ or } [Ca])_{Initial} \times \partial_{Dilution} \quad (\text{Equation 1})$$

$$([Pi] \text{ or } [Ca])_{Measured} = ([Pi] \text{ or } [Ca])_{Colorimetry} \quad (\text{Equation 2})$$

This result supports the hypothesis that a solid calcium phosphate phase precipitated, although the phosphate concentration changes are very small at the highest Ca-CO₃:Pi ratios. In the Figure 5-19, the calcium concentrations were nearly zero when the Ca-/Pi-stream volume ratio is less than 4 (80/20). The 80 mM of EDTA was suspected to chelate almost all of the metals. However, the EDTA as Fe-EDTA in the treated DIG-2 has the potential to exchange with soluble calcium ion to form Ca-EDTA and release Fe ions (Xue, Sigg, & Kari, 1995). Then, these released Fe might re-precipitate the soluble Pi again.

After centrifugation, a thin layer of gray matter was observed at the top of the dark pellet. This was not seen in DIG-2, so it supported the possibility that a calcium phosphate solid precipitated. X-ray diffraction of these solids was complicated by the small amount of solid samples, and the presence of iron in the samples. Iron-containing samples fluoresce Cu-radiation (Gunn, 1957) which reduces the signal to noise ratio.

Preliminary phase identification was undertaken, with the caveat that the complexity of DIG-2 solution would be expected to precipitate a complex mix of poorly-crystalline solids, given the rapid precipitation process used to produce these solids. The possible mineral phases identified are listed in Table 5-4, and the diffraction data and ICDD phases presented in Figures 5-21 and 5-22.

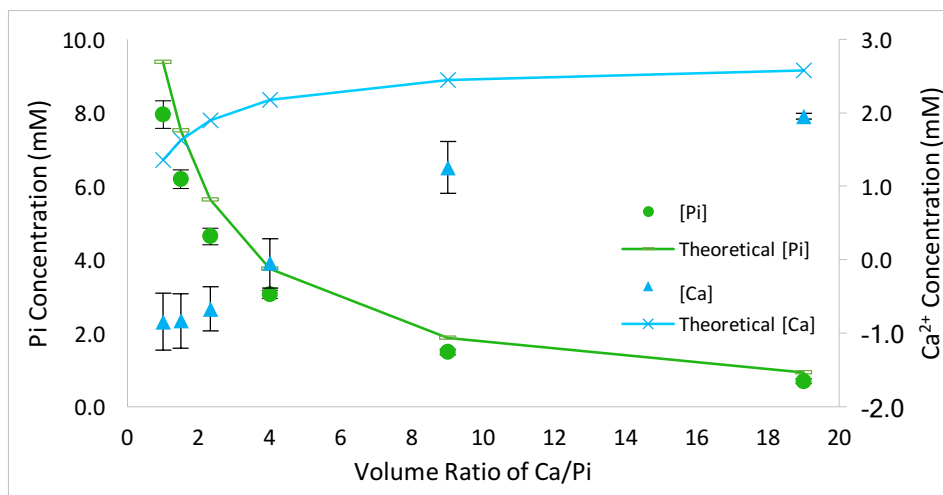


Figure 5-19 Measured (symbols) and calculated assuming dilution only (lines) calcium and phosphate concentrations (mM) in the second trial versus the volume ratio of Ca- and P-solutions. Volume ratios are mL of CaCO_3 solution (4 g/L limestone + 100 % CO_2) to mL of phosphate solution (DIG-2 + 80 mM EDTA) (n=3).

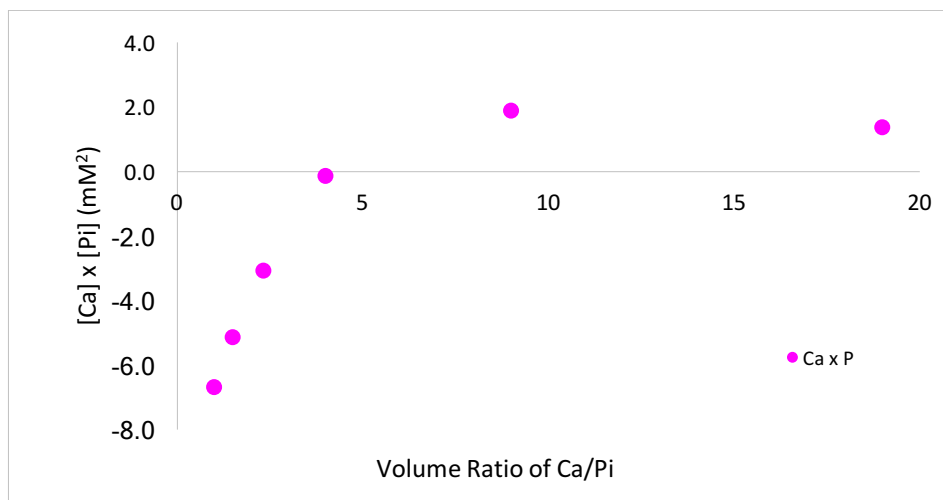


Figure 5-20 $[\text{Ca}] \times [\text{Pi}]$ (K_{sp}) at various volume ratios. Volume ratios are mL of CaCO_3 solution (4 g/L limestone + 100% CO_2) to mL of phosphate solution (DIG-2 + 80 mM EDTA).

Iron phosphate hydroxide is the only composition presenting in the two X-ray results of the dried DIG-2 sample and dried precipitates. Metal solids were identified, with a good match for iron phosphate hydroxide, and aluminum phosphate (Figure 5-21). Possible calcium-phosphate phases were calcium lead phosphate, and minor phases of Cd-Pb-hydroxylapatite, carbonate-hydroxylapatite, chloro-apatite and fluorapatite (Figure 5-22).

Table 5-4 Potential mineral phases of the precipitate based on Figure 5-21 and Figure 5-22.

Mineral name	Chemical Formula	ICDD number
Iron Phosphate Hydroxide	$\text{Fe}_4(\text{PO}_4)_3(\text{CO}_3)_3(\text{OH})_3$	01-080-0759
Aluminum Phosphate	$(\text{Al}(\text{PO}_3)_3$	00-015-0364
Calcium Lead Phosphate	$\text{Ca}_{10}\text{Pb}_{0.5}(\text{PO}_4)_7$	00-056-0249
Cd-Pb-Hydroxylapatite	$\text{Cd}_{2.24}\text{Pb}_{7.76}(\text{PO}_4)_6(\text{OH})_2$	01-071-2897
Carbonate-Hydroxylapatite	$\text{Ca}_{10}(\text{PO}_4)_3(\text{CO}_3)_3(\text{OH})_2$	00-019-0272
Chloro-Apatite	$\text{Ca}_{10}\text{Cl}_2(\text{PO}_4)_6$	00-001-1011
Fluorapatite	$(\text{CaF})\text{Ca}_4(\text{PO}_4)_3$	00-002-0845

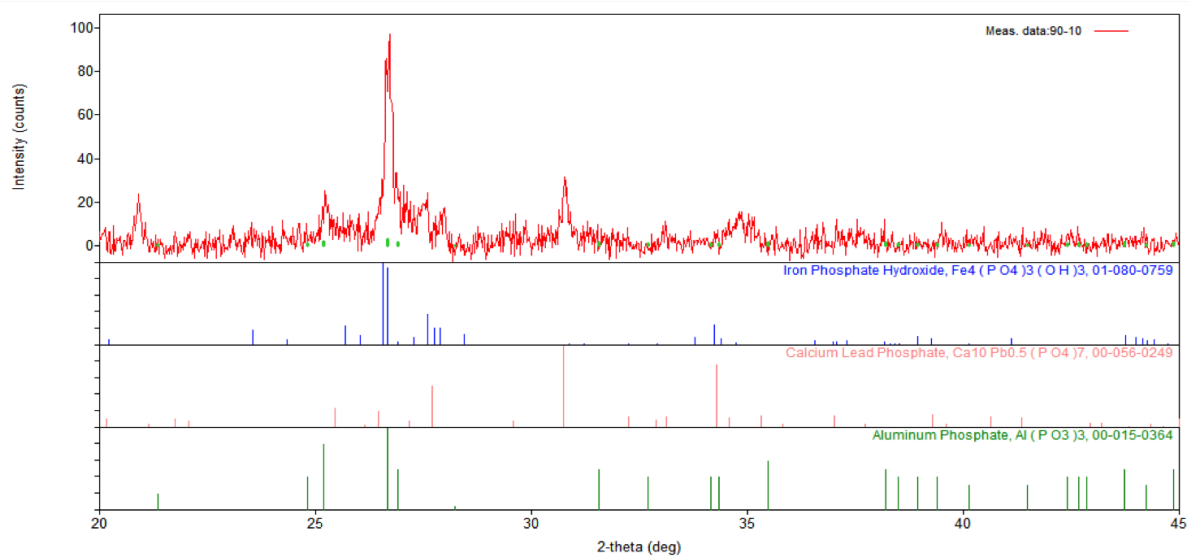


Figure 5-21 X-ray diffraction pattern of dried precipitates compared with standard minerals (I).

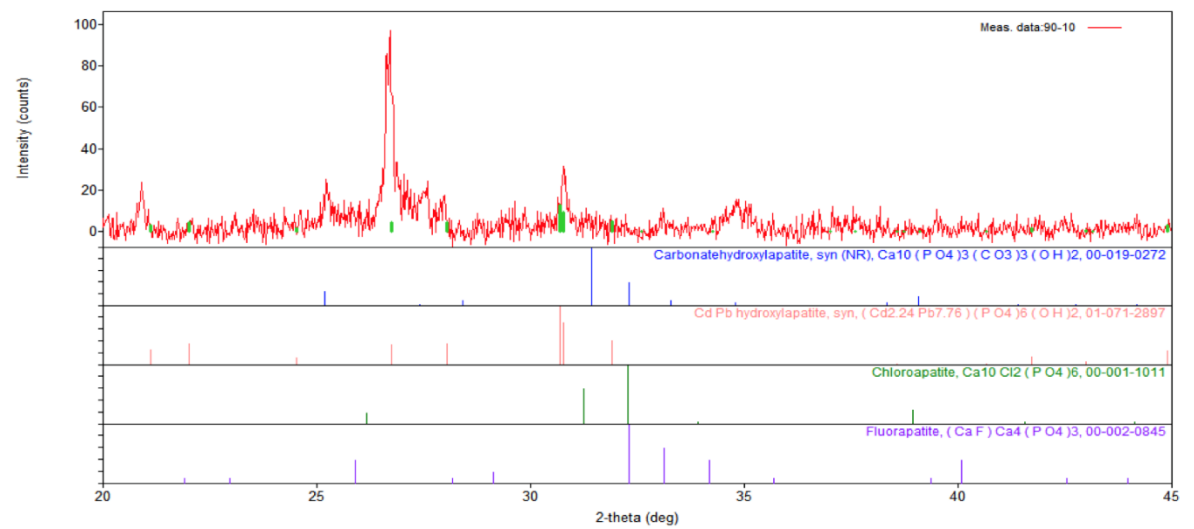


Figure 5-22 X-ray diffraction pattern of dried precipitates compared with other minerals (II).

5.4. Conclusions and Recommendations

In the feasibility test of calcium phosphate precipitation from acidic, Pi-extracted TWAS, Raman spectroscopy and FT-IR identified a carbonate apatite product by neutralization with synthetic calcium carbonate solids, and neutralization/precipitation sodium bicarbonate and synthetic hydroxyapatite seed.

Contacting gases with different CO₂ concentrations in a slurry with potable water and CaCO₃ (synthetic and limestone) solids decreased the pH and increased the calcium concentration with excess CaCO₃ solids. Limestone is an inexpensive Ca-CO₃ source for the Ca-CO₃ solution envisioned to precipitate cAp from Pi-containing solutions, but further work is required to increase its equilibrium calcium concentration to the values generated with synthetic calcite. The limestone experiment is expected to be run for longer time periods to determine the equilibrium calcium concentration and pH in the future. Besides, production of this Ca-CO₃ solution would also consume waste CO₂ that is the byproduct of anaerobic digestion and methane-fired power plants at WWTPs. Therefore, the compositions of the CO₂-containing streams from ROPEC should be determined. The further value of the most concentrated CO₂ gas stream should be evaluated with limestone to determine the potential feasibility of this process at a WWTP with anaerobic digesters.

Due to the presence of metal ions in the DIG-2 solution, the final concentration of 90 mM/L EDTA is suggested to chelate the metal ions to dissolve metal phosphates and chelate the metals during Ca-CO₃ solution addition and cAp precipitation. However, this Fe-EDTA is exchangeable with Ca to Ca-EDTA during the process of precipitation by adding the Ca-CO₃ solution to DIG-2. Therefore, a shorter precipitation time was undertaken to favor cAp precipitation before EDTA could exchange metal ions for calcium ions. X-ray diffraction results indicated a complex mixture of potassium iron fluoride (0.25/1/3), dicalcium diphosphate (V) and iron phosphate hydroxide in pre-EDTA-treated DIG-2, and phosphoferrite (syn) and iron fluoride in post-EDTA-treated DIG-2. Precipitation products included iron phosphate hydroxide, iron phosphate hydroxide, and Cd-Pb-hydroxylapatite. The complex composition of DIG-2 is expected, as is the range of products from this initial precipitation study. A technique for measuring iron concentration in the samples should be identified and adapted to these solutions.

The study of purposeful cAp precipitation from municipal wastewater is a long-term discovery project.

Future work includes the design and operation of a continuous process to precipitate cAp from EDTA-treated DIG-2 with a CO₂-boosted Ca-CO₃ solution from limestone. The optimal Ca-CO₃/DIG-2 ratio identified in the batch reactions should be applied to the design of a continuous stirred tank reactor as shown in Figure 5-23. The pH should be monitored during this process to evaluate the effect of pH on the Ca-P mineral formation by the P-transition from Fe-P (Oxmann & Schwendenmann, 2015). However, a limitation is that the metal content of samples from ROPEC should be chelated or otherwise reduced to precipitate the Pi as cAp.

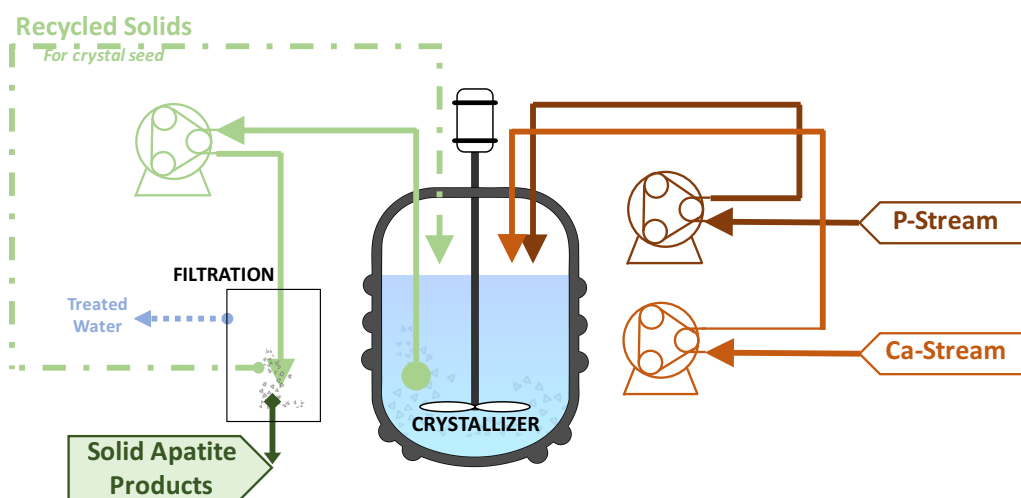


Figure 5-23 Schematic of a future continuous stirred tank reactor for cAp precipitation.

5.5. References

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6. Conclusions and Future Work

The known phosphate rock reserves are adequate for human demand for food security in the short-term. However, in the long-term, closing the P-loop is the key point related to recycling and sustainable P development. In particular, there are two main drivers P sustainability: reducing the P-loss from the excessive agricultural application, and improving the technologies to increase the P-recovery from human, animal or other organic waste P-rich streams. Currently, P-use is a one-pass flow, from phosphate rock to P-fertilizer, to food production, to food consumption, to P-waste, mostly as insoluble Fe- and Al-P if the waste is treated in a municipal wastewater treatment plant. If not treated, then P-rich waters contribute to the problem of eutrophication. Municipal wastewater plants have aerobic, and sometimes aerobic and anaerobic processes.

The first part of the thesis was the evaluation of P-recovery as polyP, or as Pi hydrolyzed from polyP in aerobic sludge. The second was to build on the work of Ross (2017). This preliminary precipitation work with EDTA-extracted anaerobic digested and limestone-CO₂-saturated solutions was performed to evaluate the possibility of using limestone and EDTA-treated samples from ROPEC to precipitation and determine the feasibility of precipitating calcium carbonate phosphate rock.

In fact, this strategy of a P recovery process by produce calcium carbonate phosphate was approached from two in this thesis. In this first stage of P-extraction, the total phosphorus in the thickened waste activated sludge was quantitated by the concentrated acid oxidation as soluble orthophosphate. Different acid and EDTA extraction were used to extract polyphosphate as an important P composition of TWAS. The preliminary estimate for the polyphosphate content in TWAS is ~ 30 % by alkaline extraction.

Ideally, the intracellular polyphosphate could be extracted; then the extracted polyphosphate could be broken down by the hydrolysis as orthophosphate as the working P-rich stream for the next stage of crystallization. However, waste activated sludge is too complicated. The interference of metal phosphates and the possibility of excess free metal ions from chemical precipitation for the ROPEC P-removal process convoluted the P-extraction results. Other

compositions in the sludge might also affect the extraction to varying degrees. It is also possible that unstable polyphosphate might have already been partially broken down during the extracted process. Further work is required to investigate the conditions required to extract polyP from TWAS.

The second direction was to produce P-containing solids by crystallization as a P-recovered product. It focused on the feasibility of generating calcium carbonate phosphate from P_i and $Ca-CO_3$ -rich industrial streams. The synthetic work performed to investigate another P-recovery options for municipal wastewater treatment plants with access to limestone rocks in this research group. Instead of using calcium chloride or calcium hydroxide as the Ca-streams as is reported in the literature, two reasons lead to the selection of limestone as the Ca-source for this P-recovery process. Limestone as the commercial calcium carbonate is cheap and sufficient in Canada. Dissolved carbonate in solution also favored the precipitation of carbonate apatite. To generate $Ca-CO_3$ streams with high enough calcium concentration to precipitate calcium carbonate phosphate, synthetic (calcite) and commercial (limestone) calcium carbonate solids were created by bubbling gas with different CO_2 concentrations through the potable water with different to increase the solubility and to dissolve more calcium and carbonate ions in the saturated solution. The effects of solids density, time, and a 1 % SDS solution were measured to optimize the conditions to produce the most concentrated $Ca-CO_3$ solution.

To reduce the complexity in the precipitation of calcium carbonate phosphate from ROPEC P-rich stream, another P-stream – the anaerobically digested slurry - was chosen. The major P-phase in this product is expected to be inorganic orthophosphate, as the polyphosphates found in the PAOs in TWAS are hydrolyzed by PAOs in the anaerobic digester. However, the complication of metal ions, as metal phosphates and hydroxides in the solid state, and also possibly in solution remains with this P-rich stream. To eliminate the effect of metal ions in the anaerobic digesate, EDTA was added to chelate the metals as a pretreatment for P-stream to free the inorganic phosphate ions in solution. Then, the variable volume ratios of P- and $Ca-CO_3$ solutions were mixed to induce a batch precipitation. Final calcium and inorganic phosphate concentrations were measured and compared with the theoretical dilution concentrations. There was concentration loss from solution, indicating the precipitation of calcium and phosphate. This precipitation work should be expanded, and pH measurement included as a variable to follow.

Characterization with Raman spectroscopy is a nondestructive technique to identify Raman-active chemical bonds. It is a beneficial technique for identify P-molecules such as polyphosphate, orthophosphate minerals such as carbonate apatite and calcium carbonate phases. However, this identification could help to explain the results clearly. However, the auto-fluorescence generated by WWTP samples was extremely strong, caused possibly by one or more of the complex organic components, and/or the iron. Therefore, it is necessary to investigate a sample pretreatment strategy to characterize WWTP-sourced samples with this technique.

X-ray diffraction was performed to characterize the WWTP-sourced precipitates; the small amount of material, high fluorescence background caused by the presence of iron, and homogeneous nucleation conditions made exact crystalline phase identification difficult. A number of metal, calcium, and phosphate phases are potential precipitates. Calcium carbonate phosphate was a candidate material, possible multiple substitutes of heavy metal ions such as lead. This suggests that the precipitates might be able to co-precipitate surrounding metal ions which are a component of municipal wastewaters.

These are the first known attempts to extract P from TWAS and anaerobic digestate with the goal of precipitating calcium carbonate phosphate minerals. The Ca-CO_3 solutions produced from limestone and CO_2 gas precipitated some potentially interesting phases from EDTA-treated anaerobic digestate to chelate metal ions. It is hoped that this work will provide a baseline of data from which future will optimize the precipitating conditions to generate calcium carbonate apatite minerals from municipal wastewater. The use of chemical precipitation to reduce the phosphate concentration conflicts with these attempts to precipitate calcium carbonate apatite. EDTA use as a chelating agent is a temporary method for reducing the effect of these metals on carbonate apatite precipitation. In the future, testing the potential for carbonate apatite precipitation from municipal wastewater may benefit from using sludge from a WWTP with effective enhanced biological phosphate removal that does not use chemical precipitation as a P-source for this P-recovery strategy.

7. Annexes and Appendices

A. Annex - The price of phosphate rock and others in the recent 20 years

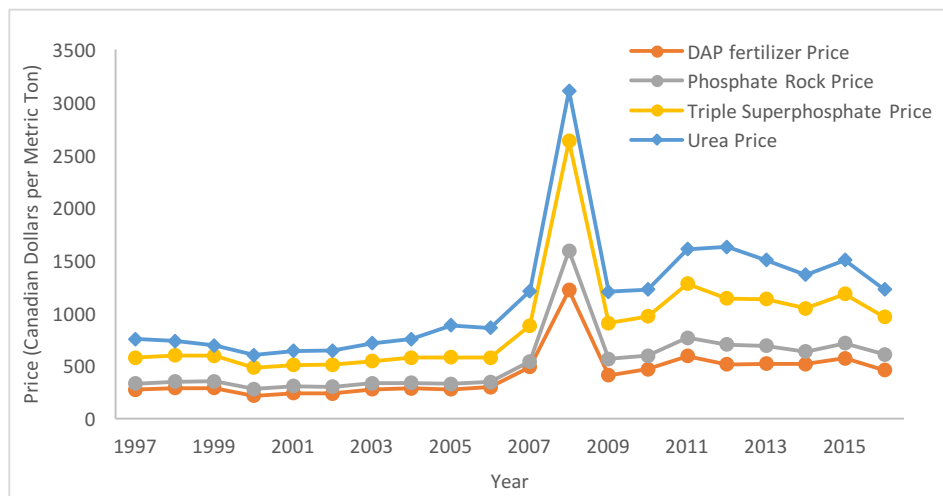


Figure A-1 The price of phosphate rock and P-fertilizers in recent 20 years (Data: world bank).

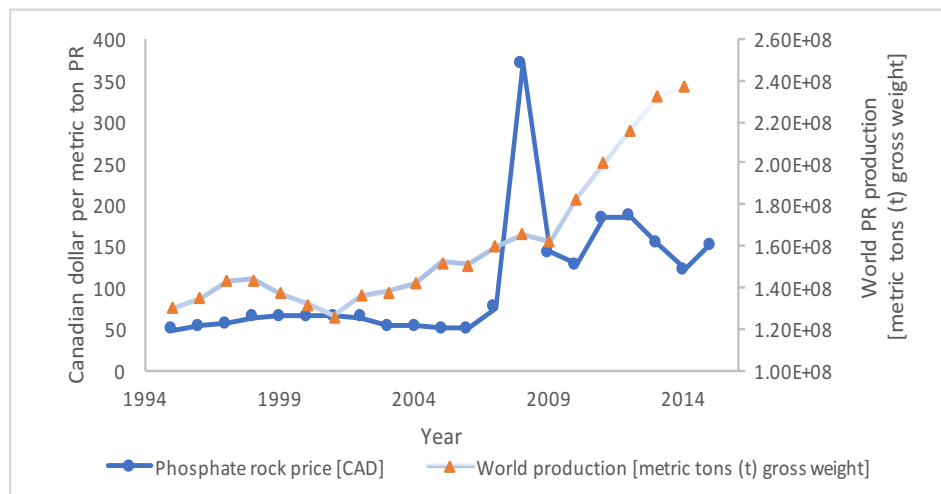


Figure A-2 The price of phosphate rock and the growth of world population in recent 20 years (Data: world bank).

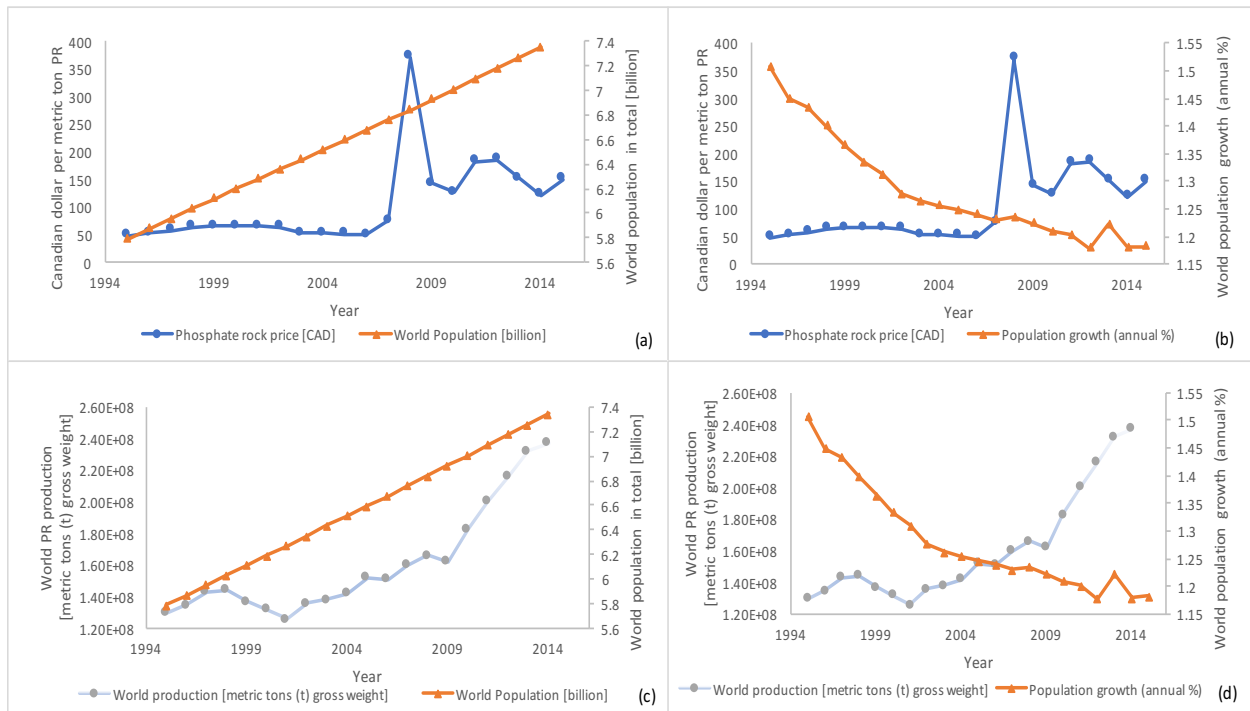


Figure A-3 Phosphate rock price and other factors in the recent 20 years (Data: world bank).

B. Annex – Supplements for Raman setup in Chapter 3

B.1. Optic-Mechanics

Concerning the metallic post can reflect the interference (Figure 8), others alternative options are considered.

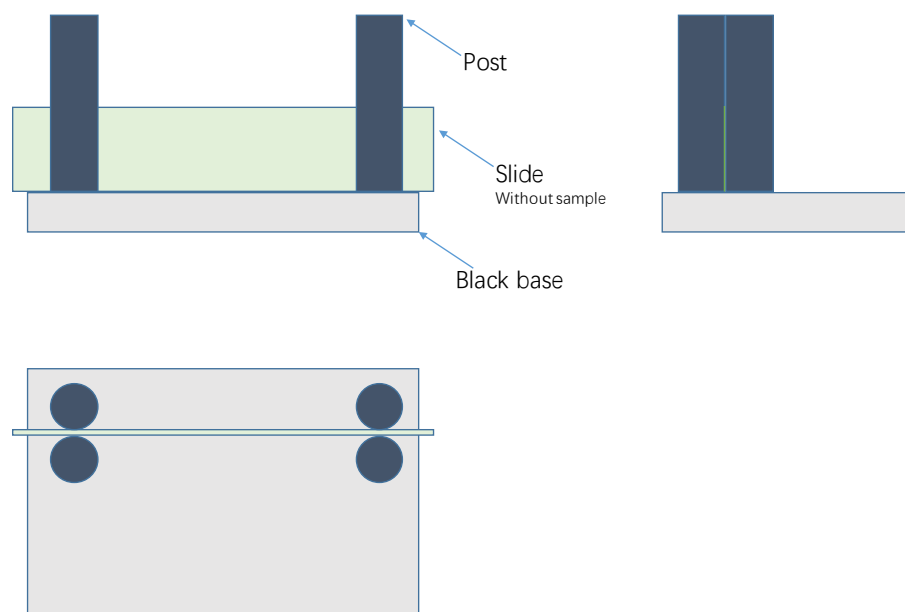


Figure B-1 The schematic layout of lab-made sample holder.

B.2. Past versions of layout

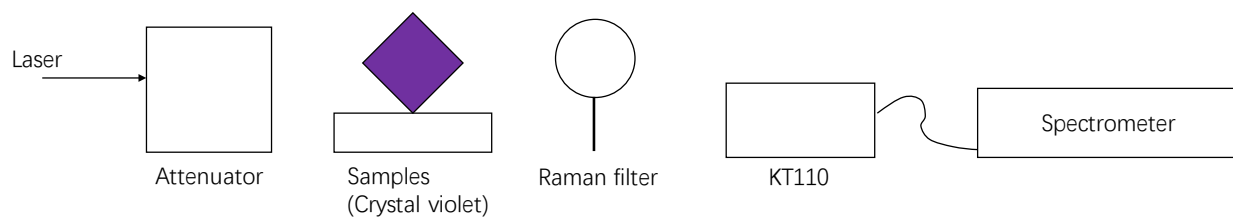


Figure B-2 Version I setup at June 2nd, 2016

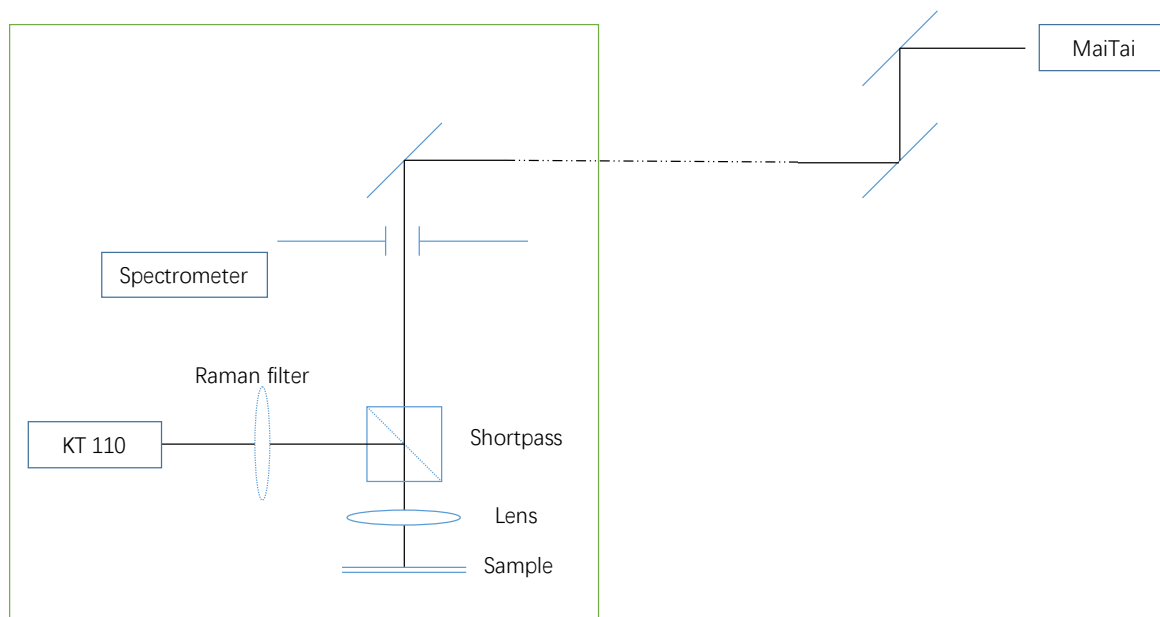


Figure B-3 Version II setup at June 14th, 2016

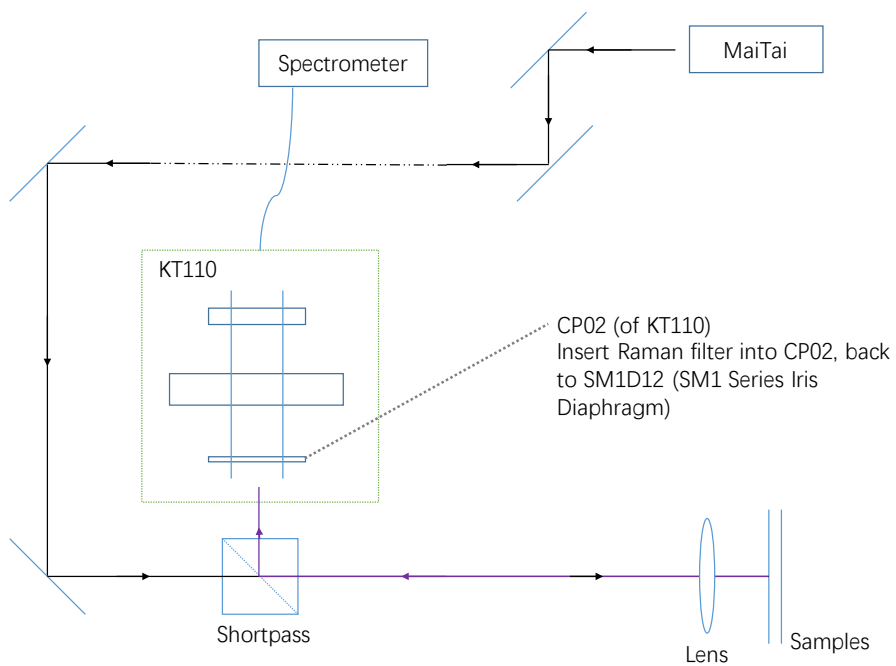


Figure B-4 Version III setup at July 21th, 2016

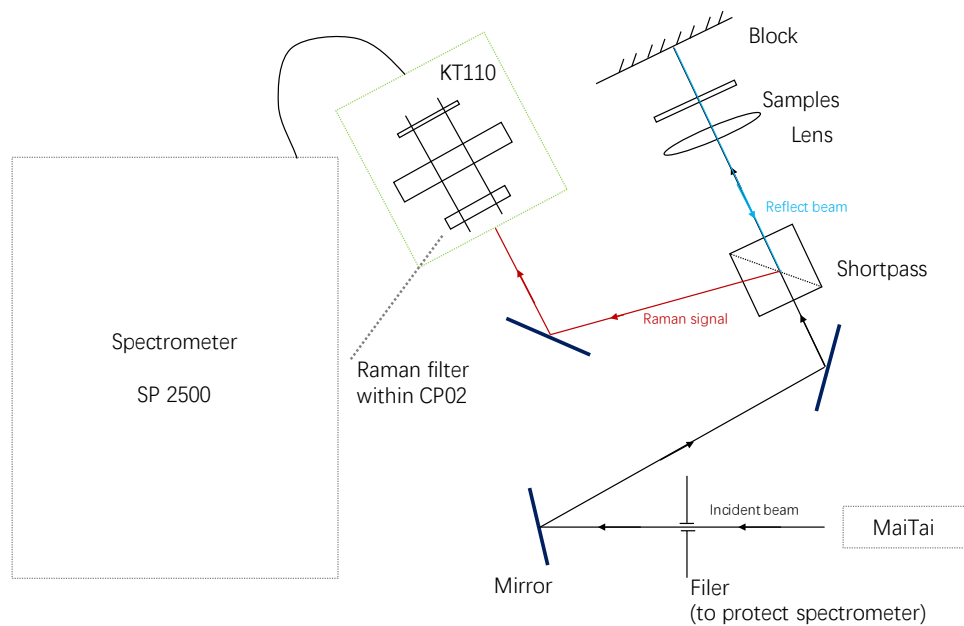


Figure B-5 Version V setup at August 10th, 2016

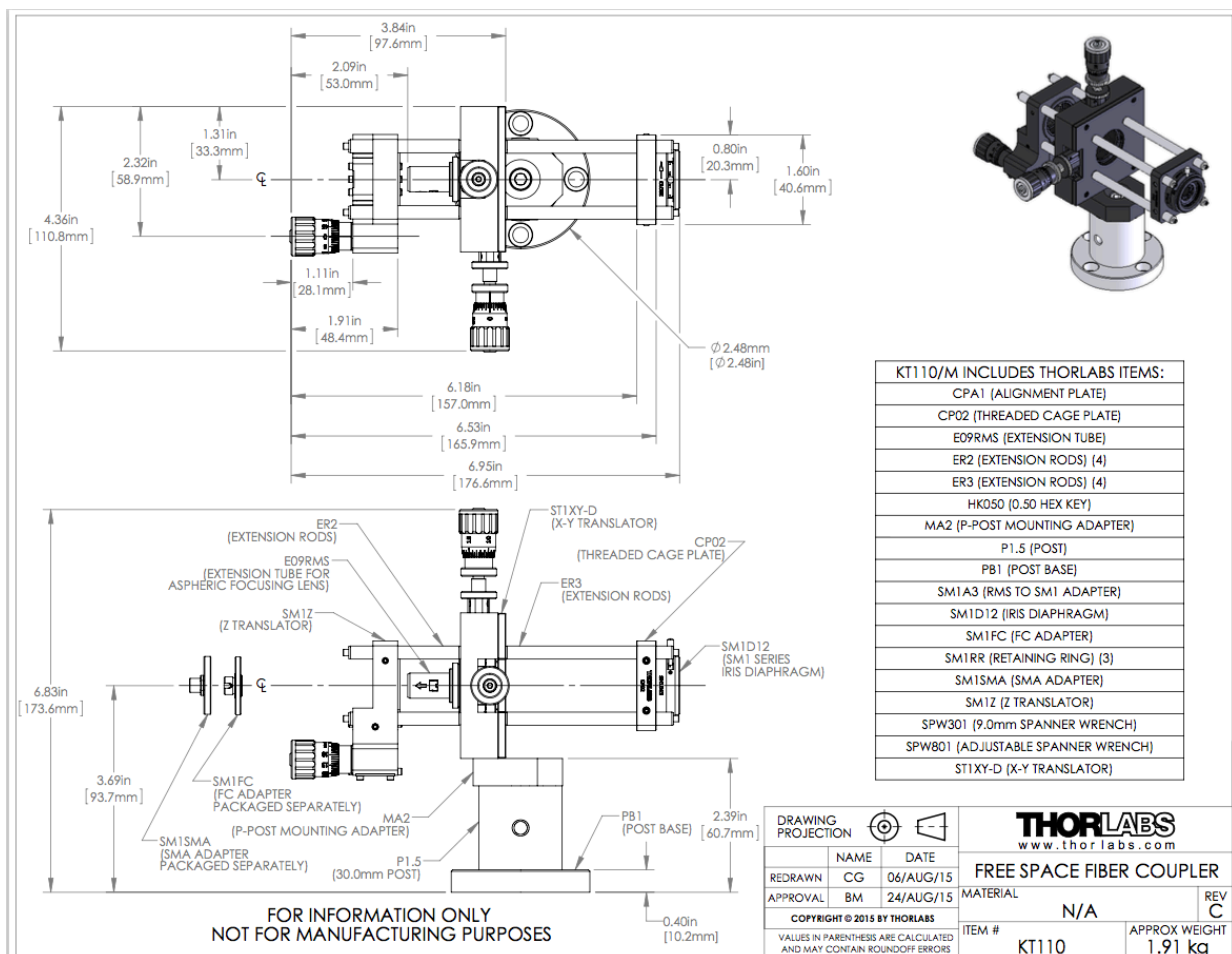


Figure B-6 AutoCAD dawning of KT110 free space launch (Available at <https://www.thorlabs.com/thorproduct.cfm?partnumber=KT110>)

C. Annex – TBO staining images

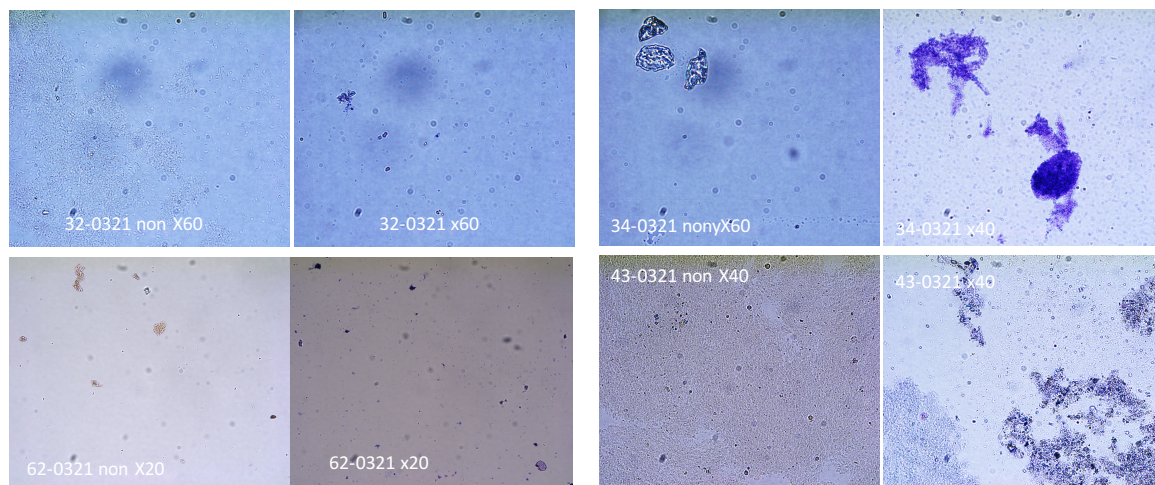


Figure C-1 The comparison without staining and with TBO staining.

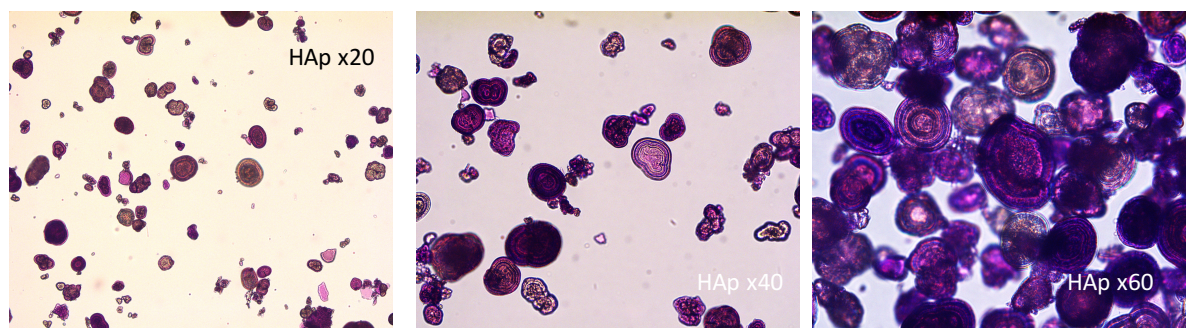


Figure C-2 Interesting results of TBO staining on the HAp(s).

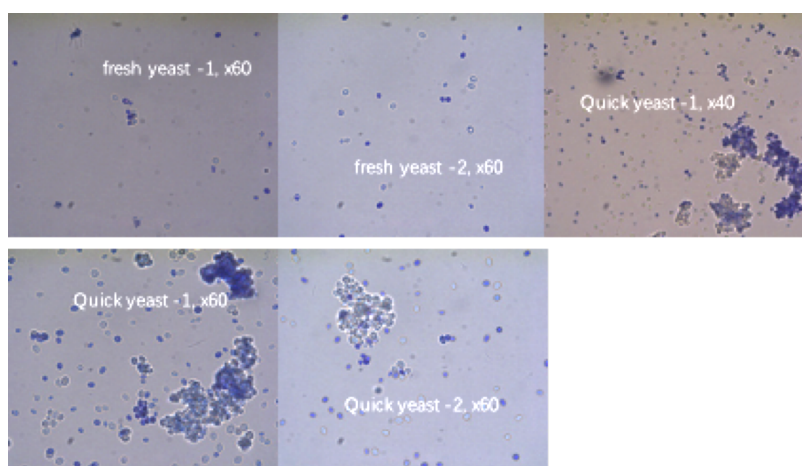


Figure C-3 The results of TBO staining on the yeasts.

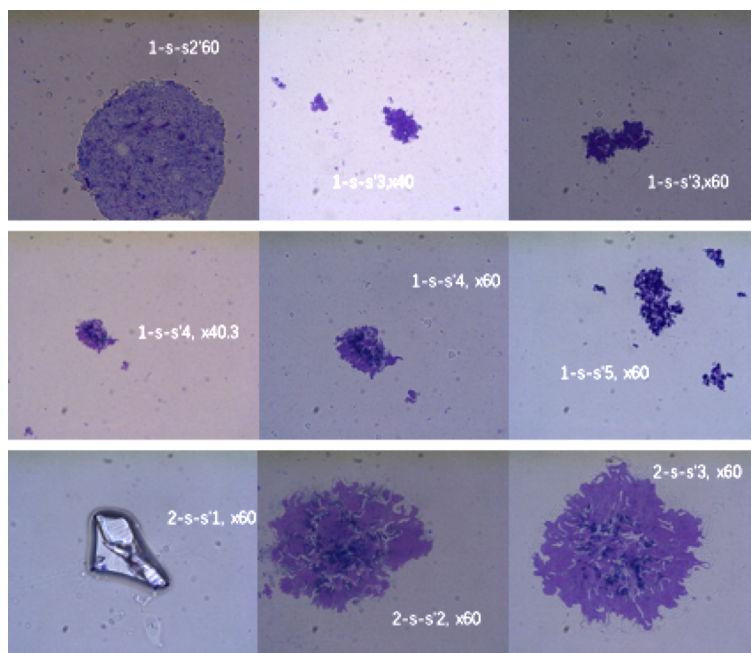


Figure C-4 The results of TBO staining on the alkaline extracted yeasts.

D. Annex – SEM images

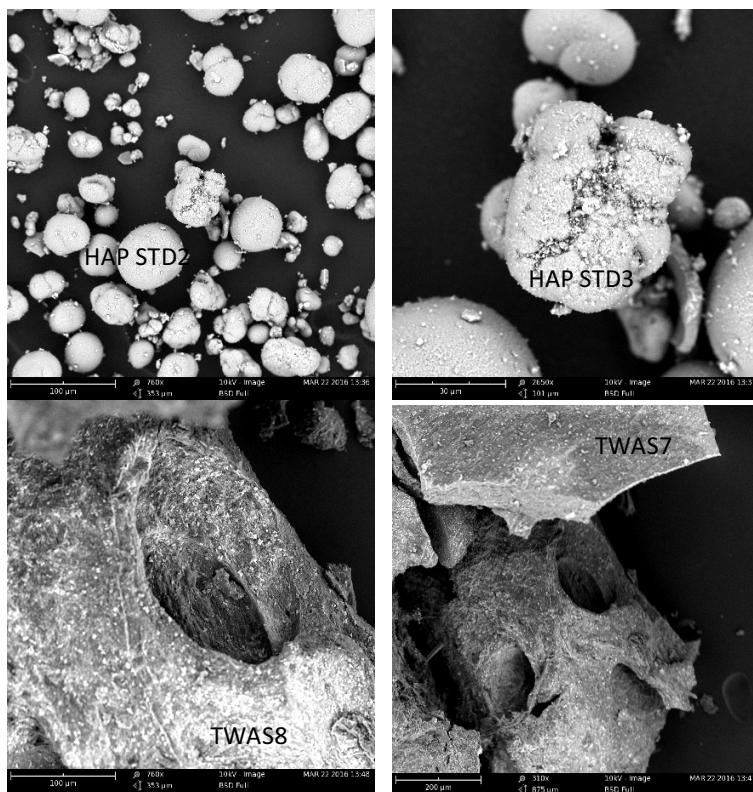


Figure D-1 SEM images of Hap and dried TWAS.

E. Annex – Colorimetric assay supplements

E.1. Dilution tables for colorimetric assay – Vanadomolybdate method

Table E-1 Standard w/ Triton X-100 (10%)

<i>STD [ρ_i] mM</i>	<i>V_p μL</i>	<i>V_{Triton} μL</i>	<i>V_{water} μL</i>	<i>V_{Total} μL</i>
0	0	25	25	50
0.025	2.5	25	22.5	50
0.050	5	25	20	50
0.100	10	25	15	50
0.150	15	25	10	50
0.200	20	25	5	50
0.250	25	25	0	50

Table E-2 Samples w/ Triton X-100 (10%)

<i>Dilution factor</i>	<i>V_{sample} μL</i>	<i>V_{Triton} μL</i>	<i>V_{water} μL</i>	<i>V_{Total} μL</i>
2	25	25	0	50
5	10	25	15	50
10	5	25	20	50
20	2.5	25	22.5	50

Note: The timing of requiring Triton X-100: there is floc in the plate after adding A&B&C Vanadomolybdate solutions.

For both Table 1 and Table 2, if there is no requirement for Triton X-100,

$$V_{\text{water (modified)}} = V_{\text{Total}} - V_{\text{sample}} = 50\mu\text{L} - V_{\text{sample}}$$

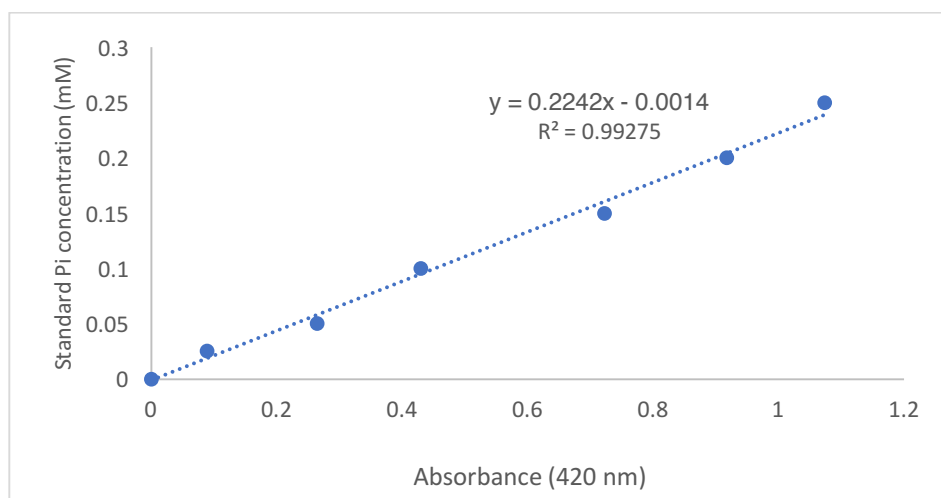
Procedure as SOP said: Mix 1:1:1 solution A:B:C of Vanadomolybdate assay. Added 250 μ L of colorimetric solution to each 50 μ L sample.

E.2. Example of calculation for Vanadomolybdate assay

Table E-3 C Example of calibration data of Vanadomolybdate assay.

Absorbance (420 nm)	Conc (mM)
0	0
0.089	0.025
0.265	0.05
0.43	0.1
0.723	0.15
0.918	0.2
1.074	0.25

Figure E-1 Graphing example of calibration curve of Vanadomolybdate assay.



Based on the Figure E-1, the linear least squares regression analysis gives a slope = 0.2242 and the y – intercept = -0.0014. According to Beer's law, the concentration of unknown samples could be calculated by this calibration equation by their the absorbance. Therefore,

$$[\text{unknown Pi}](mM) = 0.2242 \times (\text{unknown absorbance } (A, 420nm)) + y - \text{intercept}$$

For example, the absorbance (420 nM) of the sample 1 of unknown is 0.142 in Table E-4.

According to the above equation,

$$\begin{aligned} [\text{Sample 1 Pi}](mM) &= 0.2242 \times (\text{Sample 1 absorbance } (A, 420nm)) - 0.0014 \\ &= 0.2242 \times (0.142 (A, 420nm)) - 0.0014 = 0.06 \text{ mM} \end{aligned}$$

Table E-4 Example of results of Vanadomolybdate assay.

Initial Pi of TWAS (mM)	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
1	2	0.142	0.06
2	2	0.283	0.13
3	2	0.192	0.09
4	2	0.158	0.07
5	2	0.182	0.08
6	2	0.161	0.07
7	2	0.171	0.08
8	2	0.132	0.06

E.3. The effect of basic condition on Vanadomolybdate assay

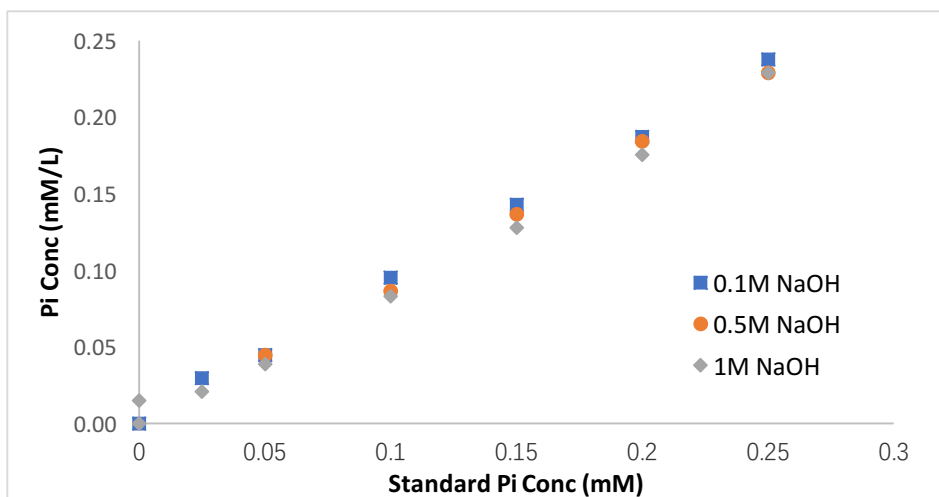


Figure E-2 The effect of basic condition on Vanadomolybdate assay by variable concentrations of NaOH with standard Pi solution.

The varying basic condition was suspected to affect the results from Vanadomolybdate assay for Pi assay. Figure C-1 implies the the effect might exist. In the future, this effect, even the certain “standard protocol” should be considered to verify to determine the proper condition to conduct before using.

F. Annex – Other supplements

F.1. Another potential P source from wastewater plants: biosolids cake

Biosolids cake samples were placed in glass tubes and 2mL of 96% H₂SO₄ (aq) added to each - The samples were place on a heating block at -140 degree for 1hourturned black - Samples were cooled to room Temp and then 1mL 30% H₂O₂(aq) was added to each one - Samples were then returned to heating block for 2hours, then cooled to room temp - Samples were transferred to 1.5mL Eppendorf tubes and centrifuged at 6000xg for 5mins - Samples diluted by a factor of 5 and analyzed.

Table F-1 The acidic digestion result for biosolids cake from ROPEC.

Sample - #	Weight (Gram)	Absorbance (420 Nm)	Pi Conc (mM/L)/gram	Average Concentration mM per Liter Per Gram Cake
2A+1H ₂ O ₂ -1	0.0168	0.082	964.615	991.201
2A+1H ₂ O ₂ -2	0.02	0.103	1017.787	
A+2H ₂ O ₂ -1	0.0121	0.108	1763.956	1804.244
A+2H ₂ O ₂ -2	0.0135	0.126	1844.532	
1A+1H ₂ O ₂ -1	0.0083	0.095	1508.008	1363.048
1A+1H ₂ O ₂ -2	0.0106	0.098	1218.087	

F.2. Raw data for acid hydrolysis on yeast with/ without heating

Table F-2 The results of acid digestion on yeasts (I)

	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)	DiLution factor on sample	Conc Pi [mM]
Control roomT&DI 1	5	0.133	1.730	8	13.843
Control roomT&DI 2	5	0.134	1.743	8	13.947
Ex_chamber+Acid 1	5	0.048	0.625	8	4.996
Ex_chamber+Acid 2	5	0.057	0.742	8	5.933
Ex_chamber+Acid 3	5	0.047	0.612	8	4.892
Ex_chamber+Acid 4	5	0.048	0.625	8	4.996
Ex_chamber+Acid 5	5	0.044	0.572	8	4.580
Ex_chamber+Acid 6	5	0.045	0.585	8	4.684
Control Heat&D*I 1	5	0.118	1.535	8	12.282
Control Heat&D*I 2	5	0.171	2.225	8	17.799
Ex_heat+Acid 1	5	0.069	0.898	8	7.182
Ex_heat+Acid 2	5	0.073	0.950	8	7.598
Ex_heat+Acid 3	5	0.074	0.963	8	7.702
Ex_heat+Acid 4	5	0.076	0.989	8	7.910
Ex_heat+Acid 5	5	0.072	0.937	8	7.494
Ex_heat+Acid 6	5	0.075	0.976	8	7.806

Table F-3 The results of acid digestion on yeasts (II)

	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
C_A(acid)_1*	1	10	0.376	11.42
C_A_2*	2	10	0.345	10.48
E_H(heat)A_1	3	10	0.094	2.86
E_HA_2	4	10	0.097	2.95
E_HA_3	5	10	0.092	2.79
E_HA_4	6	10	0.091	2.76
E_HA_5	7	10	0.093	2.83
E_HA_6	8	10	0.104	3.16

C_TA_1 **, ***	9	20	0.105	6.38
C_TA_2 ***	10	20	0.058	3.52
E_R(room)A_1	11	20	0.108	6.56
E_RA_2	12	20	0.109	6.62
E_RA_3	13	20	0.085	5.16
E_RA_4	14	20	0.096	5.83
E_RA_5	15	20	0.096	5.83
E_RA_6	16	20	0.126	7.65
C_A_1*	17	20	0.162	9.84
C_A_2*	18	20	0.196	11.91

F.3. The results for heating then 7-min acid hydrolysis on TWAS

Table F-4 The results of heating and then 7-min hydrolysis on TWAS

Time (min)		0	30	45	60	75	90	120
<i>Conc. Pi (mM)</i>	70 degree heat	0.82	1.30	0.93	1.12	0.68	1.65	0.70
	70 heat + 7min	15.27	29.69	41.11	16.56	39.97	41.68	31.98

G. Annex – Raw data and supplements for Chapter 4

G.1. Raw data and supplements for Section 4.3.1.

G.1.1. ANOVA and t-Test reports

Table G-1 t-Test report for acid digestion by 500 ul 1 M HCl + 500 ul TWAS, T> 100C

t-Test: Two-Sample Assuming Unequal Variances		
	LG	OM(2016)
Mean	43.680875	38.68916667
Variance	265.745801	7.476953788
Observations	16	12
Hypothesized Mean Difference	0	
df	16	
t Stat	1.202482981	
P(T<=t) one-tail	0.123335287	
t Critical one-tail	1.745883676	
P(T<=t) two-tail	0.246670574	
t Critical two-tail	2.119905299	

Table G-2 Two-way ANOVA report for Figure 4-6

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
S	1	185.63692	185.63692	1.61429	0.21057
HCl	1	28746.07998	28746.07998	249.97395	0
Model	2	30934.25614	15467.12807	134.50109	0
Error	44	5059.83721	114.9963	--	--
Corrected Total	46	35994.09335	--	--	--

At the 0.05 level, the population means of **S** are **not significantly** different.

At the 0.05 level, the population means of **HCl** are **significantly** different.

Means Comparisons

Bonferroni Test

S								
	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
1 0	13.80045	3.16369	4.36214	7.66165E-5	0.05	1	7.42445	20.17645

HCl

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0.5 0	51.16726	3.12911	16.352	2.58586E-20	0.05	1	44.86095	57.47358

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

G.1.2. Raw data

Table G-3 Raw data for the t-Test (H.1.1 with Table H-1)

0.5M HCl with oven heating Acid Digested [Pi] mM	
<i>OM 2016</i>	<i>LG (Figure4-6)</i>
38.93	39.49
42.65	27.96
42.36	27.59
39.21	43.65
40.36	30.11
39.79	25.65
34.63	36.25
38.07	29.83
39.5	53.94
33.49	42.74
36.64	32.29
38.64	45.96
	59.26
	62.30
	56.98
	84.90

Table G-4 Raw data of initial Pi results in the untreated TWAS sample with 20% triton X-100 for Chapter 4.3.1 at “Non-removed” series.

Date	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
Oct 26 2015	1	2	0.105	0.758
	2	2	0.099	0.715
	3	2	0.116	0.838
	4	2	0.058	0.419
Oct 27 2015	5	2	0.238	0.141
	6	2	-0.027	-0.016
	7	2	-0.049	-0.029
	8	2	0.02	0.012
Nov 05 2015	9	2	0.039	0.254
	10	2	0.057	0.371

	11	2	0.036	0.234
	12	2	0.043	0.280
Nov 10 2015	13	2	0.31	1.924
	14	2	0.373	2.315
	15	2	0.324	2.011
	16	2	0.387	2.401

Table G-5 Raw data of Pi results by acid digestion with 20% triton X-100 for Chapter 4.3.1 at “Non-removed” series, the dilution factor of digestion was 2.

Date	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
Oct 26 2015	1	20	0.209	39.490
	2	20	0.148	27.964
	3	20	0.146	27.586
	4	20	0.231	43.647
Oct 27 2015	5	30	0.108	30.112
	6	30	0.092	25.651
	7	30	0.13	36.245
	8	30	0.107	29.833
Nov 05 2015	9	20	0.284	53.941
	10	20	0.225	42.735
	11	20	0.17	32.289
	12	20	0.242	45.964
Nov 10 2015	13	20	0.312	59.259
	14	20	0.328	62.298
	15	20	0.3	56.980
	16	20	0.447	84.900

Table G-6 Raw data of initial Pi results in the untreated TWAS sample with 20% triton X-100 for Chapter 4.3.1 at “S Removed” series.

Date	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
11/06/2015	1	2	0.039	0.254
	2	2	0.057	0.371
	3	2	0.036	0.234
	4	2	0.043	0.280
11/12/2015	5	2	0.31	1.924

	6	2	0.373	2.315
	7	2	0.324	2.011
	8	2	0.387	2.401

Table G-7 Raw data of Pi results by acid digestion with 20% triton X-100 for Chapter 4.3.1 at “S Removed” series, the dilution factor of digestion was 2.

Date	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
11/06/2015	1	20	0.294	54.244
	2	20	0.272	50.185
	3	20	0.159	29.336
	4	20	0.251	46.310
11/12/2015	5	20	0.312	59.259
	6	20	0.328	62.298
	7	20	0.3	56.980
	8	20	0.447	84.900
11/26/2015	9	20	0.348	64.207
	10	20	0.295	54.428
	11	20	0.265	48.893
	12	20	0.31	57.196

G.2. Raw data for Chapter 4.3.2. Total phosphorus measurements

G.2.1. ANOVA and t-Test reports

Table G-8 The t-test for acid digestion with 0.01M HCl at Figure 4-8

t-Test: Two-Sample Assuming Unequal Variances - [HCl]=0.01M

	<i>Removed S - experimental group</i>	<i>Without S removal - control group</i>
Mean	2.572032726	2.374695199
Variance	0.051761697	0.057415087
Observations	4	4
Hypothesized Mean Difference	0	
df	6	
t Stat	1.194468025	
P(T<=t) one-tail	0.13868394	
t Critical one-tail	1.943180281	

P(T<=t) two-tail 0.27736788
t Critical two-tail 2.446911851

Table G-9 Two-way ANOVA reports for Figure 4-8

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
S	1	74.31621	74.31621	4.56814	0.03985
HCl	4	34584.64681	8646.1617	531.47097	0
Model	5	34658.96302	6931.7926	426.0904	0
Error	34	553.12428	16.26836	--	--
Corrected Total	39	35212.08731	--	--	--

At the 0.05 level, the population means of **S** are **significantly** different.

At the 0.05 level, the population means of **HCl** are **significantly** different.

Means Comparisons

Bonferroni Test

S

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0 1	2.7261	1.27547	2.13732	0.03985	0.05	1	0.13402	5.31818

HCl

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0.05 0.01	0.18512	2.0167	0.0918	1	0.05	0	-5.86892	6.23917
0.1 0.01	4.86313	2.0167	2.41142	0.2144	0.05	0	-1.19092	10.91717
0.1 0.05	4.678	2.0167	2.31963	0.265	0.05	0	-1.37605	10.73205
0.5 0.01	62.16163	2.0167	30.82339	1.96678E-25	0.05	1	56.10758	68.21567
0.5 0.05	61.9765	2.0167	30.7316	2.16927E-25	0.05	1	55.92245	68.03055
0.5 0.1	57.2985	2.0167	28.41197	2.83735E-24	0.05	1	51.24445	63.35255
1 0.01	61.02663	2.0167	30.26059	3.60212E-25	0.05	1	54.97258	67.08067
1 0.05	60.8415	2.0167	30.1688	3.97974E-25	0.05	1	54.78745	66.89555
1 0.1	56.1635	2.0167	27.84917	5.45144E-24	0.05	1	50.10945	62.21755
1 0.5	-1.135	2.0167	-0.5628	1	0.05	0	-7.18905	4.91905

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-10 The t-test for acid digestion with 0.05M HCl at Figure 4-8

t-Test: Two-Sample Assuming Unequal Variances - [HCl]=0.5M

	Removed S - experimental group	Without S removal - control group
Mean	59.90163745	69.36828708
Variance	58.17849537	0.959384617
Observations	4	4

Hypothesized Mean Difference	0
df	3
t Stat	-2.462030471
P(T<=t) one-tail	0.045351882
t Critical one-tail	2.353363435
P(T<=t) two-tail	0.090703764
t Critical two-tail	3.182446305

Table G-11 The t-test for 0.5M EDTA and 3.5M HCl at Figure 4-9.

t-Test: Two-Sample Assuming Unequal Variances

	<i>0.5M EDTA</i>	<i>3.5M HCl</i>
Mean	84.79805951	86.9752572
Variance	23.05234733	67.98247167
Observations	4	4
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.456377363	
P(T<=t) one-tail	0.333629077	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.667258153	
t Critical two-tail	2.570581836	

Table G-12 The t-test for 1M HCl and 3.5M HCl at Figure 4-9.

t-Test: Two-Sample Assuming Unequal Variances

	<i>1M HCl</i>	<i>3.5M HCl</i>
Mean	72.04946258	86.9752572
Variance	87.77394623	67.98247167
Observations	4	4
Hypothesized Mean Difference	0	
df	6	
t Stat	-2.391908057	
P(T<=t) one-tail	0.026942944	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.053885887	
t Critical two-tail	2.446911851	

Table G-13 The t-test for 0.25M EDTA and 0.5M EDTA at Figure 4-9.

t-Test: Two-Sample Assuming Unequal Variances

	<i>0.25M EDTA</i>	<i>0.5M EDTA</i>
Mean	54.94358099	84.79805951
Variance	167.0064687	23.05234733
Observations	4	4
Hypothesized Mean Difference	0	
df	4	
t Stat	-4.331072716	
P(T<=t) one-tail	0.006169643	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.012339287	
t Critical two-tail	2.776445105	

Table G-14 The t-test for 0.25M EDTA in Figure 4-9 and 0.2M EDTA at Figure 4-10.

t-Test: Two-Sample Assuming Unequal Variances

	<i>0.25M EDTA Figure 4-9</i>	<i>0.2M Figure4-10</i>
Mean	54.94358099	68.52938281
Variance	167.0064687	6.914569047
Observations	4	8
Hypothesized Mean Difference	0	
df	3	
t Stat	-2.08113018	
P(T<=t) one-tail	0.064431679	
t Critical one-tail	2.353363435	
P(T<=t) two-tail	0.128863357	
t Critical two-tail	3.182446305	

Table G-15 The t-test for dd water in Figure 4-9 and Figure 4-10.

t-Test: Two-Sample Assuming Unequal Variances

	<i>ddWater in Figure4-10</i>	<i>ddWater in Figure4-9</i>
Mean	3.47532905	3.261200124
Variance	0.339886895	0.269393693
Observations	8	4
Hypothesized Mean Difference	0	

df	7
t Stat	0.64610987
P(T<=t) one-tail	0.269402554
t Critical one-tail	1.894578605
P(T<=t) two-tail	0.538805109
t Critical two-tail	2.364624252

Table G-16 The t-test for 200 mM EDTA in Figure 4-11 and Figure 4-10.

t-Test: Two-Sample Assuming Unequal Variances

	<i>0.2M EDTA in Figure4-11</i>	<i>0.2M EDTA In Figure4-10</i>
Mean	73.32800699	68.52938281
Variance	70.39176617	6.914569047
Observations	6	8
Hypothesized Mean Difference	0	
df	6	
t Stat	1.352058704	
P(T<=t) one-tail	0.112547255	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.22509451	
t Critical two-tail	2.446911851	

Table G-17 The t-test for 200 mM and 250 mM EDTA in Figure 4-11 and Figure 4-9.

	<i>0.2M EDTA inFigure4- 11</i>	<i>0.25M EDTA in Figure 4-9</i>
Mean	73.32800699	54.94358099
Variance	70.39176617	167.0064687
Observations	6	4
Hypothesized Mean Difference	0	
df	5	
t Stat	2.51385282	
P(T<=t) one-tail	0.026787624	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.053575248	
t Critical two-tail	2.570581836	

Table G-18 One-way ANOVA report for Figure 4-10 ([EDTA]>160mM)

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
160	4	0	66.5	6.74537	3.37268
180	4	0	65.825	8.35878	4.17939
200	8	0	68.5375	2.63327	0.931

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	23.47375	11.73687	0.38662	0.6869
Error	13	394.64625	30.3574		
Total	15	418.12			

Null Hypothesis: The means of all levels are equal.

Alternative Hypothesis: The means of one or more levels are different.

At the 0.05 level, the population means are not significantly different.

Fit Statistics

	R-Square	Coeff Var	Root MSE	Data Mean
	0.05614	0.08181	5.50976	67.35

Means Comparisons

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
180 160	-0.675	3.89599	-0.17326	1	0.05	0	-11.37314	10.02314
200 160	2.0375	3.37402	0.60388	1	0.05	0	-7.22736	11.30236
200 180	2.7125	3.37402	0.80394	1	0.05	0	-6.55236	11.97736

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
180 160	-0.675	3.89599	0.24502	0.98361	0.05	0	-10.96208	9.61208
200 160	2.0375	3.37402	0.85401	0.8205	0.05	0	-6.87137	10.94637
200 180	2.7125	3.37402	1.13694	0.70722	0.05	0	-6.19637	11.62137

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-19 ANOVA report for Figure 4-10

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
0	8	0	3.475	0.58314	0.20617
40	4	0	11.7925	2.65943	1.32971
60	4	0	18.66	1.84707	0.92353
80	4	0	24.74	1.92068	0.96034
120	4	0	31.1825	9.49006	4.74503
160	4	0	66.485	6.7225	3.36125
180	4	0	65.8075	8.34072	4.17036
200	8	0	68.52875	2.63126	0.93029

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	7	28343.08674	4049.01239	183.05078	0
Error	32	707.82761	22.11961		
Total	39	29050.91435			

Null Hypothesis: The means of all levels are equal.

Alternative Hypothesis: The means of one or more levels are different.

At the 0.05 level, the population means are significantly different.

Fit Statistics

	R-Square	Coeff Var	Root MSE	Data Mean
	0.97563	0.12968	4.70315	36.2675

Means Comparisons

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
40 0	8.3175	2.88008	2.88794	0.19319	0.05	0	-1.49689	18.13189
60 0	15.185	2.88008	5.27242	2.51848E-4	0.05	1	5.37061	24.99939
60 40	6.8675	3.32563	2.06502		1	0.05	-4.46519	18.20019
80 0	21.265	2.88008	7.38348	5.95769E-7	0.05	1	11.45061	31.07939
80 40	12.9475	3.32563	3.89325	0.01322	0.05	1	1.61481	24.28019
80 60	6.08	3.32563	1.82823		1	0.05	-5.25269	17.41269
120 0	27.7075	2.88008	9.6204	1.62213E-9	0.05	1	17.89311	37.52189
120 40	19.39	3.32563	5.83048	4.96744E-5	0.05	1	8.05731	30.72269
120 60	12.5225	3.32563	3.76545	0.01886	0.05	1	1.18981	23.85519
120 80	6.4425	3.32563	1.93723		1	0.05	-4.89019	17.77519
160 0	63.01	2.88008	21.87787	2.28487E-19	0.05	1	53.19561	72.82439
160 40	54.6925	3.32563	16.44576	1.01754E-15	0.05	1	43.35981	66.02519
160 60	47.825	3.32563	14.38074	4.53002E-14	0.05	1	36.49231	59.15769
160 80	41.745	3.32563	12.55251	1.85767E-12	0.05	1	30.41231	53.07769
160 120	35.3025	3.32563	10.61529	1.4438E-10	0.05	1	23.96981	46.63519
180 0	62.3325	2.88008	21.64264	3.16132E-19	0.05	1	52.51811	72.14689
180 40	54.015	3.32563	16.24204	1.45475E-15	0.05	1	42.68231	65.34769
180 60	47.1475	3.32563	14.17702	6.7339E-14	0.05	1	35.81481	58.48019
180 80	41.0675	3.32563	12.34879	2.87466E-12	0.05	1	29.73481	52.40019
180 120	34.625	3.32563	10.41157	2.34474E-10	0.05	1	23.29231	45.95769
180 160	-0.6775	3.32563	-0.20372		1	0.05	-12.01019	10.65519
200 0	65.05375	2.35157	27.66391	1.8112E-22	0.05	1	57.04033	73.06717
200 40	56.73625	2.88008	19.69955	5.22994E-18	0.05	1	46.92186	66.55064
200 60	49.86875	2.88008	17.31506	2.30108E-16	0.05	1	40.05436	59.68314
200 80	43.78875	2.88008	15.20401	9.51396E-15	0.05	1	33.97436	53.60314
200 120	37.34625	2.88008	12.96709	7.75225E-13	0.05	1	27.53186	47.16064
200 160	2.04375	2.88008	0.70962		1	0.05	-7.77064	11.85814
200 180	2.72125	2.88008	0.94485		1	0.05	-7.09314	12.53564

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
40 0	8.3175	2.88008	4.08417	0.10889	0.05	0	-1.01195	17.64695
60 0	15.185	2.88008	7.45633	2.19655E-4	0.05	1	5.85555	24.51445
60 40	6.8675	3.32563	2.92038	0.45735	0.05	0	-3.90522	17.64022
80 0	21.265	2.88008	10.44181	6.49227E-7	0.05	1	11.93555	30.59445
80 40	12.9475	3.32563	5.50589	0.00989	0.05	1	2.17478	23.72022
80 60	6.08	3.32563	2.5855	0.6066	0.05	0	-4.69272	16.85272
120 0	27.7075	2.88008	13.60529	8.63418E-8	0.05	1	18.37805	37.03695
120 40	19.39	3.32563	8.24554	4.46535E-5	0.05	1	8.61728	30.16272
120 60	12.5225	3.32563	5.32516	0.01376	0.05	1	1.74978	23.29522
120 80	6.4425	3.32563	2.73965	0.53706	0.05	0	-4.33022	17.21522
160 0	63.01	2.88008	30.93998	4.5994E-9	0.05	1	53.68055	72.33945
160 40	54.6925	3.32563	23.25782	8.00575E-9	0.05	1	43.91978	65.46522
160 60	47.825	3.32563	20.33744	0	0.05	1	37.05228	58.59772
160 80	41.745	3.32563	17.75194	0	0.05	1	30.97228	52.51772
160 120	35.3025	3.32563	15.01228	0	0.05	1	24.52978	46.07522
180 0	62.3325	2.88008	30.60731	9.34484E-9	0.05	1	53.00305	71.66195
180 40	54.015	3.32563	22.96972	7.88862E-9	0.05	1	43.24228	64.78772
180 60	47.1475	3.32563	20.04933	0	0.05	1	36.37478	57.92022
180 80	41.0675	3.32563	17.46383	0	0.05	1	30.29478	51.84022
180 120	34.625	3.32563	14.72418	0	0.05	1	23.85228	45.39772
180 160	-0.6775	3.32563	0.2881	1	0.05	0	-11.45022	10.09522
200 0	65.05375	2.35157	39.12268	0	0.05	1	57.43629	72.67121
200 40	56.73625	2.88008	27.85937	1.7199E-6	0.05	1	47.4068	66.0657
200 60	49.86875	2.88008	24.4872	9.07252E-8	0.05	1	40.5393	59.1982
200 80	43.78875	2.88008	21.50172	7.21287E-9	0.05	1	34.4593	53.1182
200 120	37.34625	2.88008	18.33824	0	0.05	1	28.0168	46.6757
200 160	2.04375	2.88008	1.00355	0.99609	0.05	0	-7.2857	11.3732
200 180	2.72125	2.88008	1.33622	0.97885	0.05	0	-6.6082	12.0507

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-20 ANOVA report for Figure 4-11 (pellet)

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
0	6	0	79.46667	27.18291	11.09738
100	6	0	54.78333	1.18729	0.48471
120	6	0	44.8	5.56273	2.27098
140	6	0	36.75	7.73789	3.15898
150	6	0	27.25	2.58979	1.05728
160	6	0	31.45	4.83684	1.97463
200	6	0	36.91667	6.27484	2.56169

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	6	11483.62905	1913.93817	14.87602	2.27946E-8
Error	35	4503.075	128.65929		
Total	41	15986.70405			

Null Hypothesis: The means of all levels are equal.

Alternative Hypothesis: The means of one or more levels are different.

At the 0.05 level, the population means are significantly different.

Fit Statistics

	R-Square	Coeff Var	Root MSE	Data Mean
	0.71832	0.25496	11.34281	44.4881

Means Comparisons

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
100 0	-24.68333	6.54877	-3.76915	0.01271	0.05	1	-46.13551	-3.23116
120 0	-34.66667	6.54877	-5.29361	1.38488E-4	0.05	1	-56.11884	-13.21449
120 100	-9.98333	6.54877	-1.52446	1	0.05	0	-31.43551	11.46884
140 0	-42.71667	6.54877	-6.52285	3.34135E-6	0.05	1	-64.16884	-21.26449
140 100	-18.03333	6.54877	-2.7537	0.19491	0.05	0	-39.48551	3.41884
140 120	-8.05	6.54877	-1.22924	1	0.05	0	-29.50217	13.40217
150 0	-52.21667	6.54877	-7.9735	4.65745E-8	0.05	1	-73.66884	-30.76449
150 100	-27.53333	6.54877	-4.20435	0.00362	0.05	1	-48.98551	-6.08116
150 120	-17.55	6.54877	-2.67989	0.23415	0.05	0	-39.00217	3.90217
150 140	-9.5	6.54877	-1.45065	1	0.05	0	-30.95217	11.95217
160 0	-48.01667	6.54877	-7.33216	3.00531E-7	0.05	1	-69.46884	-26.56449
160 100	-23.33333	6.54877	-3.56301	0.02272	0.05	1	-44.78551	-1.88116
160 120	-13.35	6.54877	-2.03855	1	0.05	0	-34.80217	8.10217
160 140	-5.3	6.54877	-0.80931	1	0.05	0	-26.75217	16.15217
160 150	4.2	6.54877	0.64134	1	0.05	0	-17.25217	25.65217
200 0	-42.55	6.54877	-6.4974	3.60717E-6	0.05	1	-64.00217	-21.09783
200 100	-17.86667	6.54877	-2.72825	0.20769	0.05	0	-39.31884	3.58551
200 120	-7.88333	6.54877	-1.20379	1	0.05	0	-29.33551	13.56884
200 140	0.16667	6.54877	0.02545	1	0.05	0	-21.28551	21.61884
200 150	9.66667	6.54877	1.4761	1	0.05	0	-11.78551	31.11884
200 160	5.46667	6.54877	0.83476	1	0.05	0	-15.98551	26.91884

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
100 0	-24.68333	6.54877	5.33039	0.00986	0.05	1	-45.15441	-4.21226
120 0	-34.66667	6.54877	7.4863	1.2497E-4	0.05	1	-55.13774	-14.19559
120 100	-9.98333	6.54877	2.15591	0.72863	0.05	0	-30.45441	10.48774
140 0	-42.71667	6.54877	9.2247	3.16636E-6	0.05	1	-63.18774	-22.24559
140 100	-18.03333	6.54877	3.89431	0.11475	0.05	0	-38.50441	2.43774
140 120	-8.05	6.54877	1.7384	0.8779	0.05	0	-28.52108	12.42108
150 0	-52.21667	6.54877	11.27624	7.9412E-8	0.05	1	-72.68774	-31.74559
150 100	-27.53333	6.54877	5.94585	0.00299	0.05	1	-48.00441	-7.06226
150 120	-17.55	6.54877	3.78994	0.13369	0.05	0	-38.02108	2.92108
150 140	-9.5	6.54877	2.05153	0.7708	0.05	0	-29.97108	10.97108
160 0	-48.01667	6.54877	10.36924	3.29814E-7	0.05	1	-68.48774	-27.54559
160 100	-23.33333	6.54877	5.03885	0.01694	0.05	1	-43.80441	-2.86226
160 120	-13.35	6.54877	2.88294	0.40994	0.05	0	-33.82108	7.12108
160 140	-5.3	6.54877	1.14454	0.98243	0.05	0	-25.77108	15.17108
160 150	4.2	6.54877	0.90699	0.99483	0.05	0	-16.27108	24.67108
200 0	-42.55	6.54877	9.18871	3.41355E-6	0.05	1	-63.02108	-22.07892
200 100	-17.86667	6.54877	3.85832	0.12101	0.05	0	-38.33774	2.60441
200 120	-7.88333	6.54877	1.70241	0.88798	0.05	0	-28.35441	12.58774
200 140	0.16667	6.54877	0.03599	1	0.05	0	-20.30441	20.63774
200 150	9.66667	6.54877	2.08753	0.75655	0.05	0	-10.80441	30.13774
200 160	5.46667	6.54877	1.18053	0.97945	0.05	0	-15.00441	25.93774

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-21 ANOVA report for Figure 4-11 (Supernatant)

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
[EDTA]	6	38913.4523	6485.57538	55.87279	0
no AH: 0 7 min AH: 1	1	1385.02843	1385.02843	11.93193	9.41019E-4
Interaction	6	1465.60646	244.26774	2.10435	0.0635
Model	13	41764.08719	3212.62209	27.67652	0
Error	70	8125.42645	116.07752	--	--
Corrected Total	83	49889.51364	--	--	--

At the 0.05 level, the population means of [EDTA] are significantly different.

At the 0.05 level, the population means of no AH: 0 7 min AH: 1 are significantly different.

At the 0.05 level, the interaction between [EDTA] and no AH: 0 7 min AH: 1 is not significant.

Means Comparisons

Bonferroni Test

[EDTA]

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
100 0	18.6225	4.39844	4.23389	0.00144	0.05	1	4.7547	32.4903
120 0	26.88083	4.39844	6.11145	1.0448E-6	0.05	1	13.01303	40.74863
120 100	8.25833	4.39844	1.87756	1	0.05	0	-5.60947	22.12613
140 0	37.75	4.39844	8.58259	3.24438E-11	0.05	1	23.8822	51.6178
140 100	19.1275	4.39844	4.3487	9.5736E-4	0.05	1	5.2597	32.9953
140 120	10.86917	4.39844	2.47114	0.034	0.05	0	-2.99863	24.73697
150 0	47.83167	4.39844	10.87469	2.28823E-15	0.05	1	33.96387	61.69947
150 100	29.20917	4.39844	6.64081	1.17698E-7	0.05	1	15.34137	43.07697
150 120	20.95083	4.39844	4.76324	2.09514E-4	0.05	1	7.08303	34.81863
150 140	10.08167	4.39844	2.2921	0.52313	0.05	0	-3.78613	23.94947
160 0	62.97917	4.39844	14.31853	3.65149E-21	0.05	1	49.11137	76.84697
160 100	44.35667	4.39844	10.08464	5.90467E-14	0.05	1	30.48887	58.22447
160 120	36.09833	4.39844	8.20708	1.59396E-10	0.05	1	22.23053	49.96613
160 140	25.22917	4.39844	5.73594	4.77958E-6	0.05	1	11.36137	39.09697
160 150	15.1475	4.39844	3.44384	0.02042	0.05	1	1.2797	29.0153
200 0	62.33417	4.39844	14.17189	6.25546E-21	0.05	1	48.46637	76.20197
200 100	43.71167	4.39844	9.938	1.08612E-13	0.05	1	29.84387	57.57947
200 120	35.45333	4.39844	8.06044	2.9689E-10	0.05	1	21.58553	49.32113
200 140	24.58417	4.39844	5.5893	8.58679E-6	0.05	1	10.71637	38.45197
200 150	14.5025	4.39844	3.29719	0.03226	0.05	1	0.6347	28.3703
200 160	-0.645	4.39844	-0.14664	1	0.05	0	-14.5128	13.2228

no AH: 0 7 min AH: 1

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
1 0	-8.12119	2.35106	-3.45426	9.41019E-4	0.05	1	-12.81024	-3.43214

Tukey Test

[EDTA]

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
100 0	18.6225	4.39844	5.98762	0.00129	0.05	1	5.26999	31.97501
120 0	26.88083	4.39844	8.6429	1.08248E-6	0.05	1	13.52832	40.23335
120 100	8.25833	4.39844	2.65527	0.50176	0.05	0	-5.09418	21.61085
140 0	37.75	4.39844	12.13762	2.95282E-8	0.05	1	24.39749	51.10251
140 100	19.1275	4.39844	6.15	8.65551E-4	0.05	1	5.77499	32.48001
140 120	10.86917	4.39844	3.49472	0.18589	0.05	0	-2.48335	24.22168
150 0	47.83167	4.39844	15.37914	0	0.05	1	34.47915	61.18418
150 100	29.20917	4.39844	9.39152	1.30021E-7	0.05	1	15.85665	42.56168
150 120	20.95083	4.39844	6.73624	1.95648E-4	0.05	1	7.59832	34.30335
150 140	10.08167	4.39844	3.24152	0.26248	0.05	0	-3.27085	23.43418
160 0	62.97917	4.39844	20.24946	0	0.05	1	49.62665	76.33168
160 100	44.35667	4.39844	14.26184	4.20731E-8	0.05	1	31.00415	57.70918
160 120	36.09833	4.39844	11.60657	3.21736E-8	0.05	1	22.74582	49.45085
160 140	25.22917	4.39844	8.11184	4.70938E-6	0.05	1	11.87665	38.58168
160 150	15.1475	4.39844	4.87032	0.01613	0.05	1	1.79499	28.50001
200 0	62.33417	4.39844	20.04208	0	0.05	1	48.98165	75.68668
200 100	43.71167	4.39844	14.05445	4.27453E-8	0.05	1	30.35915	57.06418
200 120	35.45333	4.39844	11.39918	3.33148E-8	0.05	1	22.10082	48.80585
200 140	24.58417	4.39844	7.90446	8.37792E-6	0.05	1	11.23165	37.93668
200 150	14.5025	4.39844	4.66294	0.0246	0.05	1	1.14999	27.85501
200 160	-0.645	4.39844	0.20738	1	0.05	0	-13.99751	12.70751

no AH: 0 7 min AH: 1

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-8.12119	2.35106	4.88506	9.4105E-4	0.05	1	-12.81024	-3.43214

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.
Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-22 Two-way ANOVA report for Figure 4-8

ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
HCI	4	34584.59421	8646.14855	650.33056	0
S-rem-1 intact 1	1	74.39256	74.39256	5.59553	0.02467
Interaction	4	154.29552	38.57388	2.90138	0.03839
Model	9	34813.2823	3868.14248	290.94703	0
Error	30	398.85017	13.29501	--	--
Corrected Total	39	35212.13248	--	--	--

At the 0.05 level, the population means of **HCI** are **significantly** different.

At the 0.05 level, the population means of **S-rem-1 intact 1** are **significantly** different.

At the 0.05 level, the interaction between **HCI** and **S-rem-1 intact 1** is **significant**.

Means Comparisons

Bonferroni Test

HCI

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0.05 0.01	0.18625	1.82312	0.10216	1	0.05	0	-5.33742	5.70992
0.1 0.01	4.865	1.82312	2.66851	0.12171	0.05	0	-0.65867	10.38867
0.1 0.05	4.67875	1.82312	2.56635	0.15512	0.05	0	-0.84492	10.20242
0.5 0.01	62.16375	1.82312	34.09753	1.49277E-24	0.05	1	56.64008	67.68742
0.5 0.05	61.9775	1.82312	33.99537	1.62979E-24	0.05	1	56.45383	67.50117
0.5 0.1	57.29875	1.82312	31.42902	1.61341E-23	0.05	1	51.77508	62.82242
1 0.01	61.02625	1.82312	33.4736	2.56239E-24	0.05	1	55.50258	66.54992
1 0.05	60.84	1.82312	33.37144	2.80196E-24	0.05	1	55.31633	66.36367
1 0.1	56.16125	1.82312	30.80509	2.89332E-23	0.05	1	50.63758	61.68492
1 0.5	-1.1375	1.82312	-0.62393	1	0.05	0	-6.66117	4.38617

S-rem-1 intact 1

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
1 0	2.7275	1.15304	2.36549	0.02467	0.05	1	0.37268	5.08232

Tukey Test

HCI

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0.01	0.18625	1.82312	0.14448	0.99997	0.05	0	--	--
0.1 0.01	4.865	1.82312	3.77384	0.08304	0.05	0	--	--
0.1 0.05	4.67875	1.82312	3.62936	0.10273	0.05	0	--	--
0.5 0.01	62.16375	1.82312	48.22119	2.78181E-5	0.05	0	--	--
0.5 0.05	--	--	--	--	0.05	0	--	--
0.5 0.1	--	--	--	--	0.05	0	--	--
1 0.01	--	--	--	--	0.05	0	--	--
1 0.05	--	--	--	--	0.05	0	--	--
1 0.1	--	--	--	--	0.05	0	--	--
1 0.5	--	--	--	--	0.05	0	--	--

S-rem-1 intact 1

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	2.7275	1.15304	3.3453	0.02467	0.05	1	0.37267	5.08233

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

G.2.2. Raw data

Table G-23 Total phosphorus measurement by persulfuric acid in Chapter 4.3.2.1.

Time(min)-n	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
0-1	20	0.051	4.997
0-2	20	0.035	3.429
20-1	20	0.088	8.621
20-2	20	0.084	8.230
40-1	20	0.107	10.483
40-2	20	0.081	7.936
60-1	20	0.123	12.050
60-2	20	0.148	14.500
80-1	20	0.219	22.582
80-2	20	0.295	28.951
100-1	40	0.076	15.088
100-2	40	0.227	31.449
100-3	40	0.092	18.027
100-4	40	0.123	24.101

Table G-24 Total phosphorus measurement by variable concentrations of HCl (I) in Chapter 4.3.2.2 (the dilution factor of hydrolysis=2).

Sample	assay dilution	absorbance	m _{empty}	m _{tube+TWAS}	m _{TWAS/g}	Conc. Pi mM/L
F-H001-1	5	0.0542	0.9578	1.4595	0.5017	2.254
F-H001-2	5	0.0602	0.965	1.4248	0.4598	2.732
F-H005-1	5	0.0442	0.9954	1.4455	0.4501	2.049
F-H005-2	5	0.0552	0.9504	1.3977	0.4473	2.575
F-H01-1	5	0.1582	0.9571	1.1848	0.2277	14.499
F-H01-2	5	0.0722	0.958	1.4182	0.4602	3.274
F-H05-1	5	1.5252	0.9573	1.4115	0.4542	70.075
F-H05-2	5	1.5342	1.0022	1.4698	0.4676	68.468
F-H1-1	50	0.1462	0.9922	1.4448	0.4526	67.409
F-H1-2	50	0.1472	0.9571	1.4069	0.4498	68.292
N-001-1	5	0.0582	0.9671	1.4453	0.4782	2.540
N-001-2	5	0.0632	0.9304	1.4114	0.481	2.742
N-005-1	5	0.0502	0.9654	1.4228	0.4574	2.290
N-005-2	5	0.0582	0.9925	1.4781	0.4856	2.501
N-01-1	5	0.1182	0.9301	1.3854	0.4553	5.418
N-01-2	5	0.1002	0.9931	1.5176	0.5245	3.987

N-05-1	50	0.1392	0.9983	1.4597	0.4614	62.957
N-05-2	50	0.1552	0.9653	1.4671	0.5018	64.542
N-H1-1	50	0.1392	0.9666	1.4397	0.4731	61.400
N-H1-2	50	0.1412	0.9988	1.4531	0.4543	64.860

Table G-25 Total phosphorus measurement by variable concentrations of HCl (II) in Chapter 4.3.2.2 (the dilution factor of hydrolysis=2).

Sample	assay dilution	absorbance	m _(empty)	m _(+TWAS)	m _(TWAS) /g	Conc. Pi [mM/L]
F-H001-1	5	0.054	1.0281	1.5289	0.5008	2.286
F-H001-2	5	0.052	1.0487	1.5441	0.4954	2.226
F-H005-1	5	0.076	0.9571	1.461	0.5039	3.198
F-H005-2	5	0.069	0.9968	1.4787	0.4819	3.036
F-H01-1	10	0.076	0.9751	1.4601	0.485	6.646
F-H01-2	10	0.166	0.9744	1.451	0.4766	14.771
F-H05-1	10	0.593	0.9646	1.3221	0.3575	70.345
F-H05-2	10	0.687	0.9972	1.422	0.4248	68.585
F-H1-1	100	0.065	0.9662	1.4626	0.4964	55.531
F-H1-2	100	0.086	0.9587	1.5525	0.5938	61.421
F-H2O-1	5	0.045	0.9345	1.4761	0.5416	1.781
NF-H001-1	5	0.065	1.0067	1.509	0.5023	2.744
NF-H001-2	5	0.054	1.001	1.5071	0.5061	2.262
NF-H005-1	5	0.064	0.9899	1.4826	0.4927	2.754
NF-H005-2	5	0.067	0.9969	1.4929	0.496	2.864
NF-H01-1	10	0.059	0.9735	1.4662	0.4927	5.078
NF-H01-2	10	0.06	1.0022	1.5093	0.5071	5.018
NF-H05-1	10	0.675	0.9497	1.5399	0.5902	48.502
NF-H05-2	10	0.776	0.9918	1.5092	0.5174	63.605
NF-H1-1	100	0.077	1.0231	1.5429	0.5198	62.822
NF-H1-2	100	0.084	0.9744	1.512	0.5376	66.264
NF-H2O-2	2	0.145	0.9701	1.4753	0.5052	2.539

Table G-26 Total phosphorus measurement by concentrated HCl and EDTA in Chapter 4.3.2.3.

Sample	abs	Dilute (experi)	Dilute (Assay)	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
H2O-1	0.088	2	2	1.1192	1.5869	0.4677	2.893
H2O-2	0.094	2	2	1.1006	1.4572	0.3566	4.031
H2O-3	0.129	2	2	1.1138	1.7443	0.6305	3.059

H2O-4	0.156	2	2	1.1136	1.8675	0.7539	3.062
H2O+EDTA-1	0.255	2	20	1.1088	1.7246	0.6158	60.065
H2O+EDTA-2	0.16	2	20	1.1007	1.5898	0.4891	48.342
H2O+EDTA-3	0.18	2	20	1.1133	1.7582	0.6449	41.019
H2O+EDTA-4	0.219	2	20	1.1254	1.5793	0.4539	70.349
EDTA-1	0.373	2	20	1.1118	1.7961	0.6843	78.273
EDTA-2	0.343	2	20	1.1195	1.6743	0.5548	88.949
EDTA-3	0.367	2	20	1.1065	1.7328	0.6263	84.177
EDTA-4	0.315	2	20	1.1065	1.6238	0.5173	87.794
1M HCL-1	0.304	2	20	1.1253	1.6642	0.5389	81.408
1M HCL-2	0.456	2	20	1.1066	1.9408	0.8342	78.184
1M HCL-3	0.344	2	20	1.1406	1.948	0.8074	61.294
1M HCL-4	0.338	2	20	1.1097	1.8324	0.7227	67.312
3.5M HCL-1	0.42	2	20	1.1404	1.8827	0.7423	81.051
3.5M HCL-2	0.475	2	20	1.1193	1.8038	0.6845	99.182
3.5M HCL-3	0.41	2	20	1.1069	1.8057	0.6988	84.087
3.5M HCL-4	0.411	2	20	1.1095	1.8142	0.7047	83.582

Table G-27 Total phosphorus measurement by variable concentrations of EDTA in Chapter 4.3.2.3.

	abs	dilute sample	dilute plate	m(empty) gram	M(tube+s) gram	m(TWAS) gram	Pi (mM)
Control-1	0.455	2	1	1.11	1.7063	0.5963	2.641
Control-2	0.455	2	1	1.1137	1.6377	0.524	3.005
Control-3	0.537333333	2	1	1.1405	1.7323	0.5918	3.144
Control-4	0.510333333	2	1	1.1101	1.691	0.5809	3.042
(conto2)-1	0.554	2	1	0.9933	1.5131	0.5198	3.760
(conto2)-2	0.545	2	1	0.9969	1.4826	0.4857	3.959
(conto2)-3	0.559	2	1	0.9292	1.4121	0.4829	4.084
(conto2)-4	0.481	2	1	0.9928	1.4001	0.4073	4.168
20mM-1	0.337	2	5	1.1064	1.8347	0.7283	8.000
20mM-2	0.440333333	2	5	1.1196	1.6559	0.5363	14.208
20mM-3	0.404333333	2	5	1.1404	1.7075	0.5671	12.334
20mM-4	0.362	2	5	1.1098	1.6056	0.4958	12.626
30mM-1	0.546333333	2	5	0.9424	1.4734	0.531	18.150

30mM-2	0.520666667	2	5	0.997	1.5255	0.5285	17.381
30mM-3	0.515333333	2	5	0.9556	1.4688	0.5132	17.716
30mM-4	0.553	2	5	0.9541	1.4101	0.456	21.392
40mM-1	0.735	2	5	1.114	1.6576	0.5436	23.425
40mM-2	0.804666667	2	5	1.1097	1.6212	0.5115	27.259
40mM-3	0.758333333	2	5	1.1134	1.6347	0.5213	25.204
40mM-4	0.663333333	2	5	1.1102	1.6083	0.4981	23.068
60mM-1	0.105666667	2	50	0.9925	1.5145	0.522	36.049
60mM-2	0.079	2	50	0.9298	1.5841	0.6543	21.586
60mM-3	0.081	2	50	0.9638	1.5411	0.5773	25.075
60mM-4	0.11	2	50	0.9847	1.4508	0.4661	42.008
80mM-1	0.1945	2	50	0.9346	1.3967	0.4621	74.555
80mM-2	0.18	2	50	0.9168	1.3867	0.4699	67.887
80mM-3	0.169	2	50	0.975	1.4888	0.5138	58.318
80mM-4	0.186	2	50	0.9295	1	0.5057	65.169
90mM-1	0.200666667	2	50	1.1198	1.5619	0.4421	78.260
90mM-2	0.187666667	2	50	1.1134	1.6288	0.5154	62.752
90mM-3	0.189666667	2	50	1.1106	1.6431	0.5325	61.389
90mM-4	0.187666667	2	50	1.1133	1.645	0.5317	60.828
100mM-1	0.196	2	50	1.1223	1.6326	0.5103	66.213
100mM-2	0.191666667	2	50	1.1118	1.5824	0.4706	70.201
100mM-3	0.203	2	50	1.1071	1.6424	0.5353	65.391
100mM-4	0.210333333	2	50	1.1102	1.6599	0.5497	65.993
(1002)-1	0.206	2	50	0.9417	1.4707	0.529	68.953
(1002)-2	0.200666667	2	50	0.9931	1.4841	0.491	72.377
(1002)-3	0.207333333	2	50	0.9171	1.4312	0.5141	71.408
(1002)-4	0.206	2	50	0.9416	1.4804	0.5388	67.699

Table G-28 Total phosphorus measurement by 7-min hydrolysis after the variable concentrations of EDTA extraction in Chapter 4.3.2.3.

mM EDTA	Sample	abs	Dilute (Assay)	Dilute (Test)	m(empty) gram	M(tube+s) gram	m(TWAS) gram	Pi (mM)
0mM-1	1	0.521	20	2	1.1118	1.674	0.5622	72.791
0mM-2	2	0.222	20	2	1.1133	1.6482	0.5349	32.873
0mM-3	3	0.51	20	2	1.1404	1.6549	0.5145	77.871
0mM-4	4	0.565	20	2	1.1131	1.6673	0.5542	80.039
0mM-5	5	0.744	20	2	1.1005	1.6385	0.538	108.420

0mM-6	6	0.653	20	2	1.1065	1.5952	0.4887	104.823
100mM-1	7	0.37	20	2	1.1133	1.6416	0.5283	55.151
100mM-2	8	0.379	20	2	1.1133	1.6676	0.5543	53.832
100mM-3	9	0.388	20	2	1.122	1.6923	0.5703	53.553
100mM-4	10	0.38	20	2	1.1195	1.6463	0.5268	56.790
100mM-5	11	0.401	20	2	1.1219	1.6949	0.573	55.072
100mM-6	12	0.353	20	2	1.1323	1.6458	0.5135	54.157
120mM-1	13	0.28	20	2	1.1407	1.6265	0.4858	45.515
120mM-2	14	0.298	20	2	1.114	1.615	0.501	46.939
120mM-3	15	0.345	20	2	1.101	1.6814	0.5804	46.838
120mM-4	16	0.311	20	2	1.1201	1.6562	0.5361	45.758
120mM-5	17	0.202	20	2	1.114	1.5862	0.4722	33.932
120mM-6	18	0.344	20	2	1.1095	1.6523	0.5428	49.939
140mM-1	19	0.337	20	2	1.1006	1.6888	0.5882	45.156
140mM-2	20	0.306	20	2	1.1132	1.654	0.5408	44.639
140mM-3	21	0.282	20	2	1.1009	1.6403	0.5394	41.282
140mM-4	22	0.276	20	2	1.1133	1.7451	0.6318	30.228
140mM-5	23	0.2	20	2	1.1209	1.5955	0.4746	29.121
140mM-6	24	0.217	20	2	1.1198	1.6178	0.498	30.123
150mM-1	25	0.212	20	2	1.1134	1.7515	0.6381	22.965
150mM-2	26	0.227	20	2	1.1404	1.7067	0.5663	27.716
150mM-3	27	0.206	20	2	1.1069	1.6347	0.5278	26.975
150mM-4	28	0.229	20	2	1.1132	1.6286	0.5154	30.722
150mM-5	29	0.2	20	2	1.1252	1.6498	0.5246	26.345
150mM-6	30	0.219	20	2	1.1117	1.6378	0.5261	28.778
160mM-1	31	0.206	20	2	1.1404	1.7248	0.5844	24.362
160mM-2	32	0.209	20	2	1.1194	1.6361	0.5167	27.957
160mM-3	33	0.217	20	2	1.1196	1.6235	0.5039	29.770
160mM-4	34	0.291	20	2	1.1194	1.6896	0.5702	35.320
160mM-5	35	0.264	20	2	1.1096	1.6414	0.5318	34.345
160mM-6	36	0.243	20	2	1.1091	1.5648	0.4557	36.881
200mM-1	37	0.26	20	2	1.1135	1.6583	0.5448	33.016
200mM-2	38	0.354	20	2	1.113	1.6553	0.5423	45.204
200mM-3	39	0.26	20	2	1.1067	1.6563	0.5496	32.727
200mM-4	40	0.289	20	2	1.1113	1.7301	0.6188	32.322
200mM-5	41	0.268	20	2	1.1092	1.6622	0.553	33.531

200mM-6	42	0.319	20	2	1.1129	1.6054	0.4925	44.840
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Table G-29 Total phosphorus measurement by 7-min hydrolysis on supernatant after the variable concentrations of EDTA extraction in Chapter 4.3.2.3 (the dilution factor on extraction =2).

mM EDTA	Sample	abs	dilute	m(empty)	M(tube+s)	m(TWAS)	Pi (mM/L)
0mM-1	1	0.113	2	1.1118	1.674	0.5622	1.400
0mM-2	2	0.366	2	1.1133	1.6482	0.5349	4.786
0mM-3	3	0.199	2	1.1404	1.6549	0.5145	2.701
0mM-4	4	0.228	2	1.1131	1.6673	0.5542	2.874
0mM-5	5	0.184	2	1.1005	1.6385	0.538	2.388
0mM-6	6	0.152	2	1.1065	1.5952	0.4887	2.170
100mM-1	7	0.215	10	1.1133	1.6416	0.5283	14.214
100mM-2	8	0.245	10	1.1133	1.6676	0.5543	15.444
100mM-3	9	0.227	10	1.122	1.6923	0.5703	13.905
100mM-4	10	0.265	10	1.1195	1.6463	0.5268	17.580
100mM-5	11	0.233	10	1.1219	1.6949	0.573	14.206
100mM-6	12	0.278	10	1.1323	1.6458	0.5135	18.922
120mM-1	13	0.299	10	1.1407	1.6265	0.4858	21.515
120mM-2	14	0.348	10	1.114	1.615	0.501	24.289
120mM-3	15	0.298	10	1.101	1.6814	0.5804	17.948
120mM-4	16	0.289	10	1.1201	1.6562	0.5361	18.843
120mM-5	17	0.374	10	1.114	1.5862	0.4722	27.699
120mM-6	18	0.318	10	1.1095	1.6523	0.5428	20.482
140mM-1	19	0.341	10	1.1006	1.6888	0.5882	20.271
140mM-2	20	0.354	10	1.1132	1.654	0.5408	22.890
140mM-3	21	0.372	10	1.1009	1.6403	0.5394	24.118
140mM-4	22	0.3115	10	1.1133	1.7451	0.6318	34.815
140mM-5	23	0.423	10	1.1209	1.5955	0.4746	62.856
140mM-6	24	0.349	10	1.1198	1.6178	0.498	49.461
150mM-1	25	0.175	10	1.1134	1.7515	0.6381	19.439
150mM-2	26	0.357	10	1.1404	1.7067	0.5663	44.488
150mM-3	27	0.274	10	1.1069	1.6347	0.5278	36.682
150mM-4	28	0.424	10	1.1132	1.6286	0.5154	58.017
150mM-5	29	0.389	10	1.1252	1.6498	0.5246	52.311
150mM-6	30	0.429	10	1.1117	1.6378	0.5261	57.505
160mM-1	31	0.532	10	1.1404	1.7248	0.5844	64.154

160mM-2	32	0.513	10	1.1194	1.6361	0.5167	69.975
160mM-3	33	0.503	10	1.1196	1.6235	0.5039	70.358
160mM-4	34	0.564	10	1.1194	1.6896	0.5702	69.695
160mM-5	35	0.534	10	1.1096	1.6414	0.5318	70.763
160mM-6	36	0.484	10	1.1091	1.5648	0.4557	74.870
200mM-1	37	0.166	10	1.1135	1.6583	0.5448	21.607
200mM-2	38	0.616	10	1.113	1.6553	0.5423	80.019
200mM-3	39	0.475	10	1.1067	1.6563	0.5496	60.928
200mM-4	40	0.468	10	1.1113	1.7301	0.6188	53.319
200mM-5	41	0.492	10	1.1092	1.6622	0.553	62.713
200mM-6	42	0.441	10	1.1129	1.6054	0.4925	63.140

Table G-30 Total phosphorus measurement by 7-min hydrolysis on pellet after the variable concentrations of EDTA extraction in Chapter 4.3.2.3 (the dilution factor on extraction =2).

mM EDTA	Sample	abs	Dilute	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	Pi mM/L
0mM-1	1	0.521	20	1.1118	1.674	0.5622	145.582
0mM-2	2	0NA	20	1.1133	1.6482	0.5349	NA
0mM-3	3	0.51	20	1.1404	1.6549	0.5145	155.742
0mM-4	4	0.565	20	1.1131	1.6673	0.5542	160.079
0mM-5	5	0.744	20	1.1005	1.6385	0.538	216.841
0mM-6	6	0.653	20	1.1065	1.5952	0.4887	209.646
100mM-1	7	0.37	20	1.1133	1.6416	0.5283	110.303
100mM-2	8	0.379	20	1.1133	1.6676	0.5543	107.663
100mM-3	9	0.388	20	1.122	1.6923	0.5703	107.107
100mM-4	10	0.38	20	1.1195	1.6463	0.5268	113.580
100mM-5	11	0.401	20	1.1219	1.6949	0.573	110.144
100mM-6	12	0.353	20	1.1323	1.6458	0.5135	108.313
120mM-1	13	0.28	20	1.1407	1.6265	0.4858	91.030
120mM-2	14	0.298	20	1.114	1.615	0.501	93.877
120mM-3	15	0.345	20	1.101	1.6814	0.5804	93.677
120mM-4	16	0.311	20	1.1201	1.6562	0.5361	91.516
120mM-5	17	0.202	20	1.114	1.5862	0.4722	67.864
120mM-6	18	0.344	20	1.1095	1.6523	0.5428	99.878
140mM-1	19	0.337	20	1.1006	1.6888	0.5882	90.311
140mM-2	20	0.306	20	1.1132	1.654	0.5408	89.278
140mM-3	21	0.282	20	1.1009	1.6403	0.5394	82.563

140mM-4	22	0.276	20	1.1133	1.7451	0.6318	60.455
140mM-5	23	0.2	20	1.1209	1.5955	0.4746	58.241
140mM-6	24	0.217	20	1.1198	1.6178	0.498	60.245
150mM-1	25	0.212	20	1.1134	1.7515	0.6381	45.930
150mM-2	26	0.227	20	1.1404	1.7067	0.5663	55.432
150mM-3	27	0.206	20	1.1069	1.6347	0.5278	53.949
150mM-4	28	0.229	20	1.1132	1.6286	0.5154	61.445
150mM-5	29	0.2	20	1.1252	1.6498	0.5246	52.690
150mM-6	30	0.219	20	1.1117	1.6378	0.5261	57.555
160mM-1	31	0.206	20	1.1404	1.7248	0.5844	48.724
160mM-2	32	0.209	20	1.1194	1.6361	0.5167	55.915
160mM-3	33	0.217	20	1.1196	1.6235	0.5039	59.540
160mM-4	34	0.291	20	1.1194	1.6896	0.5702	70.640
160mM-5	35	0.264	20	1.1096	1.6414	0.5318	68.690
160mM-6	36	0.243	20	1.1091	1.5648	0.4557	73.761
200mM-1	37	0.26	20	1.1135	1.6583	0.5448	66.031
200mM-2	38	0.354	20	1.113	1.6553	0.5423	90.408
200mM-3	39	0.26	20	1.1067	1.6563	0.5496	65.454
200mM-4	40	0.289	20	1.1113	1.7301	0.6188	64.643
200mM-5	41	0.268	20	1.1092	1.6622	0.553	67.061
200mM-6	42	0.319	20	1.1129	1.6054	0.4925	89.680

G.3. Raw data for Chapter 4.3.4. Sequence Extraction

Table G-31 Sequence extraction

Process	Sample	[Pi] mM	Process	Sample	[Pi] mM
Hot-Bath, 7-min	Ex9-P	0.936	initial	Ex5-s	11.273
Hot-Bath, 7-min	Ex10-P	0.511	initial	Ex6-s	3.656
initial	Ex5-s	11.273	initial	Ex7-s	3.013
initial	Ex6-s	3.656	initial	Ex8-s	4.113
initial	Ex7-s	3.013	initial	Ex(1)-s	4.103
initial	Ex8-s	4.113	initial	Ex(2)-s	3.162
initial	Ex(1)-s	4.103	initial	Ex1-s	8.382
initial	Ex(2)-s	3.162	initial	Ex2-s	8.222
initial	Ex1-s	8.382	initial	Ex3-s	7.509
initial	Ex2-s	8.222	initial	Ex4-s	8.175
initial	Ex3-s	7.509	initial	ReEx1-s	3.208

initial	Ex4-s	8.175	initial	ReEx2-s	3.938
initial	ReEx1-s	3.208	initial	ReEx3-s	3.903
initial	ReEx2-s	3.938	initial	ReEx4-s	3.623
initial	ReEx3-s	3.903	Wash, hot-bath	Ex(1)-ws	0.856
initial	ReEx4-s	3.623	Wash, hot-bath	Ex(2)-ws	0.746
NaOH, 7-min	Ex11-p	3.372	Wash, hot-bath	Ex1-ws	0.780
NaOH, 7-min	Ex12-P	5.404	Wash, hot-bath	Ex2-ws	0.914
Wash, hot-bath	Ex(1)-ws	0.856	Wash, hot-bath	Ex3-ws	0.875
Wash, hot-bath	Ex(2)-ws	0.746	Wash, hot-bath	Ex4-ws	1.040
Wash, hot-bath	Ex1-ws	0.780	Wash, hot-bath, 7-min	Ex5-P	0.618
Wash, hot-bath	Ex2-ws	0.914	Wash, hot-bath, 7-min	Ex6-P	2.833
Wash, hot-bath	Ex3-ws	0.875	Wash, hot-bath, NaOH, 7-min	Ex(1)-P	24.713
Wash, hot-bath	Ex4-ws	1.040	Wash, hot-bath, NaOH, 7-min	Ex(2)-P	23.786
Wash, hot-bath, 7-min	Ex5-P	0.618	Wash, hot-bath, NaOH, 7-min	Ex1-p	23.002
Wash, hot-bath, 7-min	Ex6-P	2.833	Wash, hot-bath, NaOH, 7-min	Ex2-p	23.550
Wash, hot-bath, NaOH, 7-min	Ex(1)-P	24.713	Wash, hot-bath, NaOH, 7-min	Ex3-P	21.376
Wash, hot-bath, NaOH, 7-min	Ex(2)-P	23.786	Wash, hot-bath, NaOH, 7-min	Ex4-P	32.509
Wash, hot-bath, NaOH, 7-min	Ex1-p	23.002	Wash, NaOH	ReEx1-AS	28.412
Wash, hot-bath, NaOH, 7-min	Ex2-p	23.550	Wash, NaOH	ReEx2-AS	31.123
Wash, hot-bath, NaOH, 7-min	Ex3-P	21.376	Wash, NaOH	ReEx3-As	32.415
Wash, hot-bath, NaOH, 7-min	Ex4-P	32.509	Wash, NaOH	ReEx4-As	35.759
Wash, NaOH	ReEx1-AS	28.412	Wash, NaOH, 7-min	Ex7-P	34.437
Wash, NaOH	ReEx2-AS	31.123	Wash, NaOH, 7-min	Ex8-P	36.174
Wash, NaOH	ReEx3-As	32.415	Wash, NaOH, hot-bath, 7-min	ReEx1-P	9.867
Wash, NaOH	ReEx4-As	35.759	Wash, NaOH, hot-bath, 7-min	ReEx2-P	10.357

Wash, NaOH, 7-min	Ex7-P	34.437	Wash, NaOH, hot-bath, 7-min	ReEx3-P	12.117
Wash, NaOH, 7-min	Ex8-P	36.174	Wash, NaOH, hot-bath, 7-min	ReEx4-P	12.580
Wash, NaOH, hot-bath, 7-min	ReEx1-P	9.867	Hot-Bath, 7-min	Ex9-P	0.936
Wash, NaOH, hot-bath, 7-min	ReEx2-P	10.357	Hot-Bath, 7-min	Ex10-P	0.511
Wash, NaOH, hot-bath, 7-min	ReEx3-P	12.117	NaOH, 7-min	Ex11-p	3.372
Wash, NaOH, hot-bath, 7-min	ReEx4-P	12.580	NaOH, 7-min	Ex12-P	5.404

Table G-32 20-hour extraction

<i>Time hour</i>	<i>HotWater (mM)</i>	<i>Hotwater +AD (mM)</i>	<i>3.5MHC l(mM)</i>	<i>3.5MHCl +AD(mM)</i>	<i>0.2MNaO H (mM)</i>	<i>0.2MNaOH +AD (mM)</i>
24	4.382	3.871	53.869	33.014	59.869	42.451
24	4.539	4.221	62.439	33.626	47.708	29.078
0.25	4.430	4.211	72.702	30.919	30.652	19.150
0.25	4.729	3.607	57.782	27.470	23.027	90.449
0.5	1.733	2.917	89.560	35.958	23.433	7.929
0.5	2.756	3.284	83.031	31.458	21.623	10.723
1	2.939	2.771	72.200	28.310	33.693	14.762
1	4.389	4.330	63.260	27.448	16.184	17.246
1.5	4.378	4.824	96.002	40.933	27.364	15.051
1.5	4.640	4.038	85.726	45.940	48.671	26.427
2	2.828	3.683	102.699	43.392	41.581	19.737
2	3.103	3.870	72.965	34.445	49.819	28.139
5	3.030	4.245	95.147	36.168	39.709	20.029
5	4.406	7.740	92.357	38.344	35.858	20.060
8	4.172	2.774	84.027	29.048	25.546	14.130
8	3.367	4.375	81.605	33.416	30.814	18.245
12	5.245	4.296	67.092	36.601	28.651	31.597
12	4.239	3.172	102.908	41.690	36.523	43.301
16	5.400	4.884	84.326	13.438	24.269	26.712
16	6.576	4.540	70.133	34.269	34.414	21.819
20	4.917	5.268	77.636	35.227	63.976	48.032
20			75.858	31.917	24.667	20.480

G.4. Supplements for Chapter 4.3.4. Sequence Extraction

G.4.1. Detail procedure and recorded photos of sequence extraction

Without Washing single series:

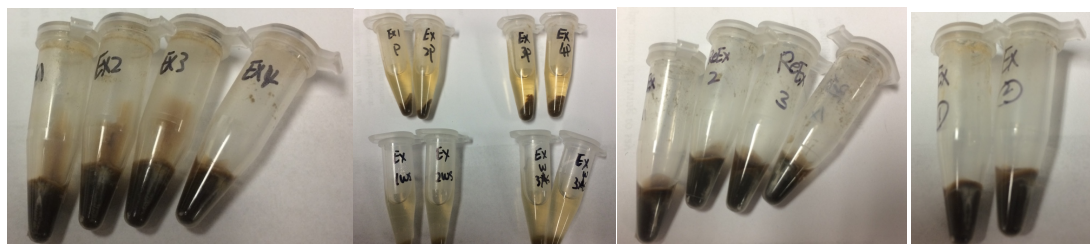
Procedure: (1) Mixing sample by shaking. (2) Extract –a, Hot-water Bath, 10min; Cooling down by ice/cooling water bath, -b, 0.2N NaOH at room temp 30min. (3) Phosphate and Polyphosphate. **Pellet**: Washing by water, phosphate assay. **Supernatant**: 7-min by 1.5N HCl 7min at Hot Water Bath

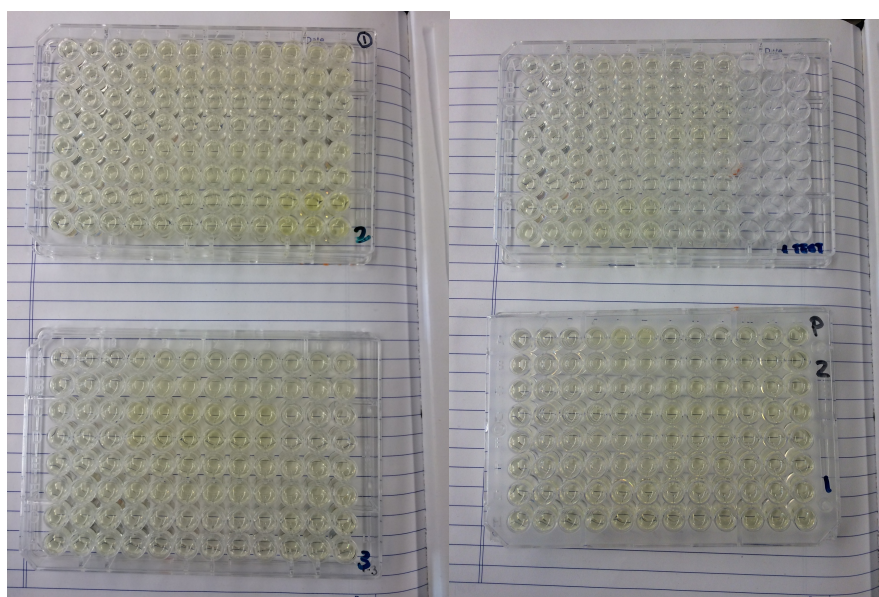
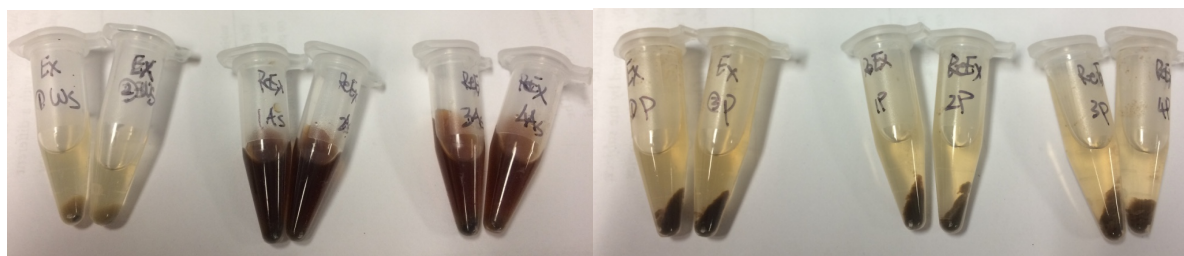
With Washing single series:

Procedure: (1) Mixing sample by shaking. (2) Washing several times by DI water for clear **supernatant**, the 1st **supernatant** remains as initial phosphate sample, the other mix together. (3) Extract, -a, Hot-water Bath, 10min; Cooling down by ice/cooling water bath, -b, 0.2N NaOH at room temp 30min. (4) Phosphate and Polyphosphate: **Pellet**: Washing by water, phosphate assay. **Supernatant**: 7-min by 1.5N HCl 7min at Hot Water Bath

Prior Order series:

Procedure: (1) Mixing sample by shaking: Washing several times by DI water for clear supernatant; 1st **supernatant** remains as initial phosphate sample, the other mix together. (2) Extraction. –a, Weimeberg Extract Hot-water Bath, 10min; Cooling down by ice/cooling water bath; Centrifugation. **Supernatant**: Keep it; **Pellet**: 0.2N NaOH at room temp 30min; Centrifugation. **Supernatant**: 7-min by 1.5N HCl 7min at Hot Water Bath; **Pellet**: washing for test the remain. –b, The reverse Weimeberg Extract: 0.2N NaOH at room temp 30min. Centrifugation: **Supernatant**: Keep it; **Pellet**: Hot-water Bath, 10min; Cooling down; the same.





G.5. Raw data for Chapter 4.3.5. Alkaline Extraction

G.5.1. ANOVA and t-Test reports

Table G-33 ANOVA report for Figure 4-17

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
[OH-]	4	11770.69308	2942.67327	271.44114	0
NaOH 1 / KOH 0	1	126.55806	126.55806	11.6741	0.00184
Interaction	4	255.17053	63.79263	5.88443	0.00128
Model	9	12152.42166	1350.26907	124.55293	0
Error	30	325.22778	10.84093	--	--
Corrected Total	39	12477.64944	--	--	--

At the 0.05 level, the population means of [OH-] are **significantly** different.

At the 0.05 level, the population means of NaOH 1 / KOH 0 are **significantly** different.

At the 0.05 level, the interaction between [OH-] and NaOH 1 / KOH 0 is **significant**.

Means Comparisons

Bonferroni Test

[OH-]

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0.2 0	3.63125	1.64628	2.20573	0.35204	0.05	0	-1.35664	8.61914
0.6 0	27.2425	1.64628	16.54793	1.25351E-15	0.05	1	22.25461	32.23039
0.6 0.2	23.61125	1.64628	14.3422	5.73884E-14	0.05	1	18.62336	28.59914
0.8 0	38.5025	1.64628	23.3876	8.12822E-20	0.05	1	33.51461	43.49039
0.8 0.2	34.87125	1.64628	21.18187	1.34632E-18	0.05	1	29.88336	39.85914
0.8 0.6	11.26	1.64628	6.83967	1.37097E-6	0.05	1	6.27211	16.24789
1 0	40.71125	1.64628	24.72927	1.65181E-20	0.05	1	35.72336	45.69914
1 0.2	37.08	1.64628	22.52353	2.37064E-19	0.05	1	32.09211	42.06789
1 0.6	13.46875	1.64628	8.18133	3.92592E-8	0.05	1	8.48086	18.45664
1 0.8	2.20875	1.64628	1.34166	1	0.05	0	-2.77914	7.19664

NaOH 1 / KOH 0

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
1 0	-3.5575	1.0412	-3.41674	0.00184	0.05	1	-5.68391	-1.43109

Tukey Test

[OH-]

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.2 0	3.63125	1.64628	3.11938	0.20517	0.05	0	-1.14396	8.40646
0.6 0	27.2425	1.64628	23.40231	7.44946E-7	0.05	1	22.46729	32.01771
0.6 0.2	23.61125	1.64628	20.28293	2.76003E-8	0.05	1	18.83604	28.38646
0.8 0	38.5025	1.64628	33.07507	0	0.05	1	33.72729	43.27771
0.8 0.2	34.87125	1.64628	29.95569	0	0.05	1	30.09604	39.64646
0.8 0.6	11.26	1.64628	9.67276	1.29136E-6	0.05	1	6.48479	16.03521
1 0	40.71125	1.64628	34.97246	0	0.05	1	35.93604	45.48646
1 0.2	37.08	1.64628	31.85309	0	0.05	1	32.30479	41.85521
1 0.6	13.46875	1.64628	11.57015	4.6961E-8	0.05	1	8.69354	18.24396
1 0.8	2.20875	1.64628	1.8974	0.66811	0.05	0	-2.56646	6.98396

NaOH 1 / KOH 0

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-3.5575	1.0412	4.832	0.00184	0.05	1	-5.68392	-1.43108

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table G-34 ANOVA report for Figure 4-18

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
[OH-]	1	1814.86057	1814.86057	62.41543	1.71148E-8
NaOH 1 - KOH 0	1	292.29892	292.29892	10.05254	0.00377
Interaction	1	116.78587	116.78587	4.01642	0.05519
Model	3	2259.32065	753.10688	25.90033	4.20558E-8
Error	27	785.08202	29.07711	--	--
Corrected Total	30	3044.40267	--	--	--

At the 0.05 level, the population means of [OH-] are significantly different.

At the 0.05 level, the population means of NaOH 1 - KOH 0 are significantly different.

At the 0.05 level, the interaction between [OH-] and NaOH 1 - KOH 0 is not significant.

Means Comparisons

Bonferroni Test

[OH-]

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
1 0.5	15.40383	1.93799	7.94837	1.52391E-8	0.05	1	11.42741	19.38025

NaOH 1 - KOH 0

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0 1	6.79217	1.93799	3.50475	0.00161	0.05	1	2.81575	10.76859

Tukey Test

[OH-]

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0.5	15.40383	1.93799	11.24069	8.90724E-8	0.05	1	11.4274	19.38027

NaOH 1 - KOH 0

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0 1	6.79217	1.93799	4.95647	0.00161	0.05	1	2.81573	10.7686

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

G.5.1. Raw Data

Table G-35 The results of alkaline extraction in Chapter 4.3.5.2 (2) – Run1.

	ABS	Dilute (test)	Dilute (assay)	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
Control-DI-1	0.652	2	1	0.9794	1.5762	0.5968	2.956
Control-DI-2	0.959666667	2	1	0.9923	1.628	0.6357	4.353
Control-DI-3s	0.355333333	2	1	0.9178	1.588	0.6702	1.188
Control-DI-4s	0.384666667	2	1	0.914	1.5327	0.6187	1.442
0.5M NaOH-1	0.706666667	2	5	0.9544	1.5224	0.568	17.097
0.5M NaOH-2	0.595333333	2	5	0.9427	1.6414	0.6987	11.301
0.5M NaOH-3s	0.640333333	2	5	0.93	1.4787	0.5487	15.728
0.5M NaOH-4s	0.633333333	2	5	0.9923	1.4928	0.5005	17.014
0.5M KOH-1	0.834666667	2	5	0.9927	1.5689	0.5762	20.476
0.5M KOH				0.9299	1.4549	0.525	
0.5M KOH-3s	0.632333333	2	5	0.9978	1.5713	0.5735	14.820
0.5M KOH-4s	0.887666667	2	5	0.9345	1.4654	0.5309	23.851
1M NaOH-1	1.239666667	2	5	0.9815	1.634	0.6525	28.203
1M NaOH-2	1.259666667	2	5	0.9565	1.4163	0.4598	40.732
1M NaOH-3s	1.079333333	2	5	0.9918	1.6893	0.6975	22.635
1M NaOH-4s	1.211666667	2	5	0.9935	1.6306	0.6371	28.168
1M KOH-1	1.418666667	2	5	0.9127	1.4953	0.5826	36.597
1M KOH-2	1.374666667	2	5	0.9406	1.4536	0.513	40.163

1M KOH-3s	1.382	2	5	0.9971	1.5414	0.5443	38.073
1M KOH-4s	1.252333333	2	5	0.9938	1.5241	0.5303	35.091

Table G-36 The result of the alkaline extraction in Chapter 4.3.5.2 (the dilution factor of extraction=2) –Run1.

	ABS	Dilute Assay	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
Control-DI-1s	0.147	2	0.9794	1.5762	0.5968	0.765
Control-DI-2s	0.144	2	0.9923	1.628	0.6357	0.657
Control-DI-3	NA	2	0.9178	1.588	0.6702	NA
Control-DI-4	NA	2	0.914	1.5327	0.6187	NA
0.5M NaOH-1s	0.296333333	5	0.9544	1.5224	0.568	10.578
0.5M NaOH-2s	0.3	5	0.9427	1.6414	0.6987	8.770
0.5M NaOH-3	1.582	5	0.93	1.4787	0.5487	87.307
0.5M NaOH-4	1.554333333	5	0.9923	1.4928	0.5005	93.914
0.5M KOH-1s	0.370333333	5	0.9927	1.5689	0.5762	14.612
0.5M KOH	NA		0.9299	1.4549	0.525	NA
0.5M KOH-3	1.669333333	5	0.9978	1.5713	0.5735	88.494
0.5M KOH-4s	NA		0.9345	1.4654	0.5309	NA
1M NaOH-1s	0.549	5	0.9815	1.634	0.6525	21.827
1M NaOH-2s	0.598333333	5	0.9565	1.4163	0.4598	34.471
1M NaOH-3	1.579333333	5	0.9918	1.6893	0.6975	68.557
1M NaOH-4	1.486666667	5	0.9935	1.6306	0.6371	70.317
1M KOH-1s	0.689333333	5	0.9127	1.4953	0.5826	32.295
1M KOH-2s	0.652	5	0.9406	1.4536	0.513	34.305
1M KOH-3	1.234666667	5	0.9971	1.5414	0.5443	67.217
1M KOH-4	1.209333333	5	0.9938	1.5241	0.5303	67.435

Table G-37 The result of 7-min hydrolysis after the alkaline extraction (NaOH, KOH) in Chapter 4.3.5.2. (the dilution factor of extraction=2) – Run2.

	abs	Dilute Assay	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
Control-DI-1s	0.052	2	0.9796	1.717	0.7374	0.563
Control-DI-2s	0.052666667	2	0.9158	1.736	0.8202	0.512
Control-DI-3	#DIV/0!	2	0.9972	1.7436	0.7464	0.000
Control-DI-4	#DIV/0!	2	1.0171	1.9478	0.9307	0.000
0.5M NaOH-1s	0.216	5	0.9188	1.8261	0.9073	4.591
0.5M NaOH-2s	0.26	5	0.9342	1.703	0.7688	6.510

0.5M NaOH-3	5.379	5	0.9936	1.7619	0.7683	133.605
0.5M NaOH-4	4.846	5	0.9921	1.7361	0.744	124.304
0.5M KOH-1s	0.334333333	5	0.9789	1.8016	0.8227	7.807
0.5M KOH-2s	0.361666667	5	0.9927	1.9291	0.9364	7.416
0.5M KOH-3	5.074	5	0.9436	1.833	0.8894	108.872
0.5M KOH-4	#DIV/0!	5	0.9918	2.0286	1.0368	0.000
1M NaOH-1s	0.586666667	5	1.01	2.0302	1.0202	11.014
1M NaOH-2s	0.559333333	5	0.9784	2.017	1.0386	10.316
1M NaOH-3	5.371	5	0.9971	2.1589	1.1618	88.222
1M NaOH-4	5.196	5	0.9846	1.985	1.0004	99.118
1M KOH-1s	0.771666667	5	0.9877	1.7781	0.7904	18.680
1M KOH-2s	0.802666667	5	0.9918	1.9885	0.9967	15.407
1M KOH-3	2.492666667	5	0.9853	1.7595	0.7742	61.473
1M KOH-4	4.958	5	0.9934	2.1254	1.132	83.585

Table G-38 The result of alkaline extraction (NaOH, KOH) in Chapter 4.3.5.2. (1-1) (the dilution factor of extraction=2) – Run2.

	abs	Dilute (Assay)	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
Control-DI-1	0.225666667	2	0.9796	1.717	0.7374	1.537
Control-DI-2	0.222	2	0.9158	1.736	0.8202	1.338
Control-DI-3s	0.223666667	2	0.9972	1.7436	0.7464	1.492
Control-DI-4s	0.289666667	2	1.0171	1.9478	0.9307	1.887
0.5M NaOH-1	0.397	5	0.9188	1.8261	0.9073	7.715
0.5M NaOH-2	0.522	5	0.9342	1.703	0.7688	13.060
0.5M NaOH-3s	0.532666667	5	0.9936	1.7619	0.7683	13.406
0.5M NaOH-4s	0.539	5	0.9921	1.7361	0.744	14.051
0.5M KOH-1	0.602	5	0.9789	1.8016	0.8227	14.569
0.5M KOH-2	0.571333333	5	0.9927	1.9291	0.9364	12.004
0.5M KOH-3s	0.66	5	0.9436	1.833	0.8894	15.063
0.5M KOH-4s	0.568333333	5	0.9918	2.0286	1.0368	10.771
1M NaOH-1	0.819	5	1.01	2.0302	1.0202	16.922
1M NaOH-2	1.035666667	5	0.9784	2.017	1.0386	21.697
1M NaOH-3s	0.986333333	5	0.9971	2.1589	1.1618	18.363
1M NaOH-4s	1.103666667	5	0.9846	1.985	1.0004	24.178
1M KOH-1	1.343333333	5	0.9877	1.7781	0.7904	37.977
1M KOH-2	1.373666667	5	0.9918	1.9885	0.9967	30.857

1M KOH-3s	1.328333333	5	0.9853	1.7595	0.7742	38.301
1M KOH-4s	1.233666667	5	0.9934	2.1254	1.132	24.161

Table G-39 The result of the alkaline extraction (NaOH, KOH) in Chapter 4.3.5.2. (the dilution factor of extraction=2).

sample	abs	Dilute (assay)	M _{Tube} Gram	M _{+samp} Gram	M _{TWAS} Gram	[Pi] (mM/L)
Control-1	0.380666667	1	1.1133	1.621	0.5077	2.669
Control-2	0.737	1	1.1009	1.5751	0.4742	5.522
Control-3	0.266333333	1	1.1402	1.6365	0.4963	1.914
Control-4	0.307666667	1	1.107	1.6925	0.5855	1.873
0.2M Na-1	0.431333333	2	1.119	1.6129	0.4939	6.215
0.2M Na-2	0.413	2	1.1136	1.5951	0.4815	6.105
0.2M Na-3	0.372666667	2	1.1405	1.758	0.6175	4.297
0.2M Na-4	0.396666667	2	1.1135	1.6681	0.5546	5.092
0.6M Na-1	0.361333333	10	1.1151	1.5906	0.4755	27.059
0.6M Na-2	0.399	10	1.12	1.5868	0.4668	30.424
0.6M Na-3	0.237333333	10	1.1094	1.6678	0.5584	15.169
0.6M Na-4	0.284333333	10	1.1117	1.6377	0.526	19.271
0.8M Na-1	0.657	10	1.1125	1.6189	0.5064	46.106
0.8M Na-2	0.567333333	10	1.1097	1.6232	0.5135	39.278
0.8M Na-3	0.589333333	10	1.1196	1.6758	0.5562	37.665
0.8M Na-4	0.540333333	10	1.1005	1.6125	0.512	37.524
1M Na-1	0.673333333	10	1.1153	1.6683	0.553	43.268
1M Na-2	0.603333333	10	1.1255	1.6278	0.5023	42.695
1M Na-3	0.592333333	10	1.1196	1.6363	0.5167	40.750
1M Na-4	0.634	10	1.1405	1.7197	0.5792	38.903

H. Annex – Raw data and supplements for Chapter 5

H.1. ANOVA and t-test in the chapter 5

Table H-1 T-test for 4g/L limestone solution in Figure 5-14.

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
2g 1-4g 0	1	0.00198	0.00198	0.00698	0.93424
time	4	5.50407	1.37602	4.84124	0.00678
Interaction	4	0.06159	0.0154	0.05418	0.99407
Model	9	5.56764	0.61863	2.17652	0.0708
Error	20	5.68456	0.28423	--	--
Corrected Total	29	11.2522	--	--	--

At the 0.05 level, the population means of **2g 1-4g 0** are **not significantly** different.

At the 0.05 level, the population means of **time** are **significantly** different.

At the 0.05 level, the interaction between **2g 1-4g 0** and **time** is **not significant**.

Means Comparisons

Bonferroni Test

2g 1-4g 0

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
0 1	0.01627	0.19467	0.08356	0.93424	0.05	0	-0.38981	0.42234

time

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
5 0	0.7855	0.3078	2.55196	0.18997	0.05	0	-0.18513	1.75613
10 0	0.94217	0.3078	3.06094	0.06167	0.05	0	-0.02846	1.91279
10 5	0.15667	0.3078	0.50898	1	0.05	0	-0.81396	1.12729
15 0	1.1235	0.3078	3.65006	0.01592	0.05	1	0.15287	2.09413
15 5	0.338	0.3078	1.09811	1	0.05	0	-0.63263	1.30863
15 10	0.18133	0.3078	0.58912	1	0.05	0	-0.78929	1.15196
20 0	1.19067	0.3078	3.86828	0.00957	0.05	1	0.22004	2.16129
20 5	0.40517	0.3078	1.31632	1	0.05	0	-0.56546	1.37579
20 10	0.2485	0.3078	0.80733	1	0.05	0	-0.72213	1.21913
20 15	0.06717	0.3078	0.21821	1	0.05	0	-0.90346	1.03779

Tukey Test

2g 1-4g 0

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0 1	0.01627	0.19467	0.11817	0.93424	0.05	0	-0.38981	0.42235

time

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
5 0	0.7855	0.3078	3.60901	0.11847	0.05	0	-0.13555	1.70655
10 0	0.94217	0.3078	4.32883	0.04342	0.05	1	0.02111	1.86322
10 5	0.15667	0.3078	0.71981	0.98549	0.05	0	-0.76439	1.07772
15 0	1.1235	0.3078	5.16197	0.01228	0.05	1	0.20245	2.04455
15 5	0.338	0.3078	1.55296	0.80546	0.05	0	-0.58305	1.25905
15 10	0.18133	0.3078	0.83314	0.97517	0.05	0	-0.73972	1.10239
20 0	1.19067	0.3078	5.47057	0.00757	0.05	1	0.26961	2.11172
20 5	0.40517	0.3078	1.86156	0.68463	0.05	0	-0.51589	1.32622
20 10	0.2485	0.3078	1.14174	0.92531	0.05	0	-0.67255	1.16955
20 15	0.06717	0.3078	0.3086	0.99945	0.05	0	-0.85389	0.98822

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.

Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Table H-2 T-test for 4g/L limestone solution in Figure 5-14.

t-Test: Two-Sample Assuming Unequal Variances

	20min	120min
Mean	1.646666667	2.276666667
Variance	0.771433333	1.354533333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.748381342	
P(T<=t) one-tail	0.247916854	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.495833708	
t Critical two-tail	2.776445105	

H.2. Raw data for Chapter 5.3.1.

Table H-3 Precipitation result with 20% Triton X-100 for “Non-removed S” series in the Chapter 5.3.1.

Date	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
Oct262015	1	2	0.019	0.236
	2	2	0.024	0.298
	3	2	0.02	0.248
	4	2	0.022	0.273
Oct272015	5	2	0.039	0.484
	6	2	0.046	0.571
	7	2	0.026	0.323
	8	2	0.034	0.422

Table H-4 Precipitation result with 20% Triton X-100 for “Removed S” series in the Chapter 5.3.1.

	Sample	Dilution factor (assay)	Absorbance (420 nm)	Conc. Pi (mM)
12/07/2015	1	2	0.295	3.669
	2	2	0.464	5.771
	3	2	0.14	1.741
	4	2	0.082	1.020
12/07/2015	5	20	0.3703	46.057
	6	2	0.129	1.604
	7	2	0.144	1.791

	8	2	0.403	5.012
12/07/2015	9	2	0.286	3.557
	10	2	0.12	1.493
	11	2	0.761	9.465
	12	2	0.257	3.197

H.3. Raw data for Chapter 5.3.2.

Table H-5 7% Result of CO₂ bubbled into synthetic calcium carbonate (I).

time/min	1 st [Ca ²⁺] mM/L	1st pH	2 nd [Ca ²⁺]	2nd pH	3 rd [Ca ²⁺]	3 rd pH
0	0.546	8.58	0.622	8.11	0.526	8.6
1	1.373	7.24	1.647	7.32	2.097	7.31
2	1.711	6.87	1.841	6.85	2.674	6.84
3	1.872	6.66	2.054	6.65	2.913	6.63
4	2.024	6.59	2.077	6.57	3.120	6.57
5	2.067	6.58	2.134	6.55	3.176	6.55
6	2.190	6.6	3.031	6.31	3.667	6.45
7	2.918	6.4	3.601	5.95	5.299	6.1
8	3.695	5.97	3.576	5.93	5.557	5.92
9	3.619	5.96	3.481	6	4.706	6.17
10	3.367	6.1	3.365	6.13	4.498	6.41
11	3.321	6.23	3.285	6.24	4.503	6.56
12	3.280	6.37	3.249	6.44	4.514	6.62
13	3.181	6.5	3.291	6.5	4.402	6.67
14	3.121	6.62	3.285	6.59	4.509	6.71
15	3.218	6.67	3.292	6.63	4.473	6.73
16	3.271	6.68	3.401	6.64	4.686	6.76
17	3.123	6.68	3.371	6.65	4.605	6.78
18	3.230	6.68	3.392	6.67	4.574	6.8
19	3.168	6.69	3.287	6.68	4.529	6.82
20	3.227	6.71	3.291	6.7	4.544	6.83
					4.509	6.83

Table H-6 7% Result of CO₂ bubbled into synthetic calcium carbonate (II).

time/min	5 th [Ca ²⁺]	5 th pH	6 th [Ca ²⁺]	6 th pH	7 th [Ca ²⁺]	7 th pH	8 th [Ca ²⁺]	8 th pH
0	0.507	8.16	1.454	8.35	0.488	8.42	0.467	8.38
1	1.816	7.08	2.875	7.04	1.712	7.27	2.013	7.17

2	2.316	6.78	3.100	6.81	2.011	6.91	2.412	6.81
3	2.614	6.71	2.921	6.7	2.551	6.78	2.744	6.69
4	2.470	6.68	3.251	6.65	2.691	6.75	2.923	6.65
5	2.603	6.67	2.909	6.64	2.653	6.78	2.990	6.62
6	2.702	6.68	3.168	6.63	2.807	6.8	3.326	6.61
7	2.795	6.7	3.432	6.62	2.698	6.81	3.259	6.61
8	2.638	6.71	3.515	6.63	3.236	6.73	3.531	6.62
9	2.511	6.73	3.477	6.63	3.318	6.68	3.514	6.63
10	2.572	6.74	3.707	6.63	3.443	6.65	3.527	6.65
11	2.605	6.76	3.740	6.64	3.474	6.64	3.592	6.65
12	2.598	6.78	4.200	6.64	3.596	6.64	3.710	6.64
13	2.815	6.8	3.855	6.65	3.481	6.65	3.698	6.64
14	2.706	6.8	3.663	6.66	3.588	6.65	3.529	6.64
15	2.768	6.82	3.951	6.67	3.637	6.66	3.658	6.64
16	2.584	6.83	4.084	6.69	3.433	6.66	3.918	6.65
17	2.708	6.85	3.580	6.72	3.642	6.67	3.791	6.65
18	2.579	6.87	3.397	6.73	3.685	6.66	3.840	6.66
19	2.538	6.89	3.829	6.74	3.648	6.66	3.693	6.66
20	2.771	6.9	3.646	6.65	3.518	6.65	3.689	6.66
	2.877	6.92	4.258	6.76	3.771	6.65	3.788	6.67

Table H-7 15% Result of CO₂ bubbled into synthetic calcium carbonate (I).

Time	1 st pH	1 st Absorbance	1 st Ca (mM/L)	2 nd pH	2 nd Absorbance	2 nd Ca (mM/L)
0	8.77	0.069	0.401	8.98	0.142	0.826
0.5	7.48	0.31	1.803	7.77	0.45	2.618
1	6.9	0.43	2.501	6.45	0.498	2.897
2	6.51	0.549	3.193	6.37	0.594	3.455
3	6.38	0.59	3.432	6.22	0.602	3.502
4	6.32	0.661	3.845	6.19	0.615	3.577
5	6.32	0.696	4.048	6.19	0.702	4.083
6	6.34	0.693	4.031	6.18	0.733	4.264
7	6.37	0.725	4.217	6.2	0.744	4.328
8	6.38	0.723	4.205	6.21	0.777	4.520
9	6.4	0.735	4.275	6.22	0.789	4.589
10	6.4	0.749	4.357	6.23	0.798	4.642
11	6.4	0.778	4.525	6.21	0.811	4.717
12	6.38	0.804	4.677	6.2	0.841	4.892
13	6.37	0.794	4.618	6.19	0.85	4.944
14	6.37	0.784	4.560	6.19	0.873	5.078

15	6.38	0.808	4.700	6.19	0.888	5.165
16	6.39	0.822	4.781	6.2	0.898	5.223
17	6.39	0.847	4.927	6.2	0.893	5.194
18	6.37	0.852	4.956	6.18	0.902	5.247
19	6.36	0.824	4.793	6.17	0.905	5.264
20	6.36	0.847	4.927	6.17	0.898	5.223

Table H-8 15% Result of CO₂ bubbled into synthetic calcium carbonate (II).

Time	pH	3rd Ca (mM/L)	4th pH	4th Ca (mM/L)	5th pH	5th Ca (mM/L)
0	8.67	0.495	8.58	0.481	8.53	0.686
1		3.272	7.04	2.098	6.45	2.849
2		3.659	6.72	2.618	6.39	3.756
3		4.353	6.58	3.402	6.4	3.881
4		4.160	6.52	3.403	6.41	3.545
5		4.342	6.49	3.560	6.43	4.276
6		4.567	6.48	3.857	6.44	4.149
7		4.699	6.47	3.892	6.46	4.185
8		4.795	6.47	4.114	6.47	4.411
9		5.760	6.46	4.058	6.47	4.480
10		5.834	6.46	3.978	6.48	4.477
11		5.928	6.46	4.115	6.48	4.470
12		5.855	6.46	4.281	6.49	4.541
13		5.740	6.46	4.120	6.49	4.673
14		5.904	6.46	4.311	6.49	4.607
15		6.357	6.46	4.530	6.5	4.587
16		6.026	6.46	4.264	6.5	4.563
17		5.980	6.46	4.361	6.5	4.551
18		5.814	6.46	4.107	6.5	4.411
19		5.840	6.46	4.511	6.51	4.659
20		5.791	6.47	4.493	6.51	4.391
21	6.3	5.829	6.47	4.590	6.51	4.443
HAP		0.982	HAP	1.027	HAP	1.010

Table H-9 100% Result of CO₂ bubbled into synthetic calcium carbonate (I).

Time	1st pH	1st Ca (mM/L)	2nd pH	2nd Ca (mM/L)
0	8.5	0.314	8.75	0.422
0.5	7.15	2.064	7.45	2.530
1	6.78	2.845	6.85	3.399

2	6.28	3.601	6.25	4.199
3	6.16	4.128	6.06	4.726
4	6.13	4.510	6.01	5.138
5	6.14	4.383	6.01	5.419
6	6.17	4.782	6.05	5.384
7	6.21	4.892	6.1	5.674
8	6.25	4.876	6.12	5.709
9	6.27	4.943	6.15	5.674
10	6.29	5.003	6.16	5.613
11	6.29	5.037	6.16	5.639
12	6.27	5.241	6.14	5.929
13	6.22	5.419	6.12	5.955
14	6.19	5.555	6.11	5.938
15	6.18	5.631	6.1	6.113
16	6.18	5.640	6.1	6.069
17	6.19	5.861	6.1	6.623
18	6.2	5.606	6.11	6.192
19	6.21	5.657	6.12	6.430
20	6.21	5.614	6.12	6.368
21	6.18	5.861	6.1	6.623
22	6.15	5.895	6.07	6.702
23	6.14	6.107	6.05	6.711
24	6.14	6.039	6.05	6.456

Table H-10 100% Result of CO₂ bubbled into synthetic calcium carbonate (II).

time/min	3 rd pH	3 rd [Ca]	4 th pH	4 th [Ca]
0	8.39	0.488	8.31	0.931
1	5.93	4.721	5.73	5.363
2	5.8	6.082	5.67	6.238
3	5.81	6.416	5.71	6.694
4	5.83	6.723	5.67	6.836
5	5.85	6.877	5.79	6.943
6	5.87	6.845	5.81	7.259
7	5.88	7.028	5.83	7.190
8	5.88	6.927	5.84	7.260
9	5.89	6.751	5.85	7.510
10	5.9	7.138	5.86	7.532
11	5.9	7.368	5.87	7.319
12	5.9	6.833	5.87	7.316

13	5.9	7.264	5.88	7.302
14	5.91	7.240	5.88	7.163
15	5.91	7.431	5.88	7.192
16	5.91	7.374	5.89	7.516
17	5.91	7.232	5.89	7.283
18	5.91	7.289	5.89	7.328
19	5.91	6.686	5.89	7.264
20	5.91	7.385	5.89	7.193
21	5.91	7.383	5.9	7.576
	HAP	1.050	HAP	1.059

Table H-11 100% Result of CO₂ bubbled into 2g/L limestone solution.

Time (min)	[Ca] (mM) Trial 1	[Ca] (mM) Trial 2	[Ca] (mM) Trial 3	pH Trial 1	pH Trial 2	pH Trial 3
0	0.364	0.428	0.359	8.61	8.44	8.77
1	0.891	1.093	0.813	5.25	5.25	5.19
2	0.990	1.074	1.021	5.1	5.12	5.04
3	1.003	1.371	1.069	5.08	5.1	4.99
4	1.048	1.390	1.180	5.09	5.12	4.99
5	1.177	1.348	1.169	5.11	5.14	5.08
6	1.274	1.377	1.253	5.12	5.15	5.03
7	1.195	1.447	1.275	5.14	5.17	5.04
8	1.182	1.315	1.338	5.15	5.19	5.06
9	0.883	1.474	1.321	5.17	5.2	5.09
10	1.188	1.574	1.342	5.18	5.22	5.11
11	1.301	1.657	1.361	5.2	5.23	5.14
12	1.223	1.508	1.385	5.21	5.24	5.19
13	1.252	1.493	1.377	5.22	5.25	5.2
14	1.337	1.620	1.359	5.23	5.26	5.21
15	1.352	1.592	1.448	5.24	5.27	5.22
16	1.240	1.700	1.604	5.24	5.27	5.23
17	1.281	1.748	1.518	5.25	5.28	5.21
18	1.266	1.791	1.483	5.26	5.29	5.24
19	1.246	1.773	1.511	5.26	5.29	5.24
20	1.342	1.725	1.498	5.27	5.3	5.26
21	1.397	1.744	1.505	5.27	5.31	5.27

Table H-12 100% Result of CO₂ bubbled into 4g/L limestone solution (I).

Time (min)	[Ca] mM_1st	pH_1st	[Ca]_2nd	pH_2nd	[Ca] (mM)_3rd	pH_3rd
0	0.41	8.52	0.43	8.89	0.37	8.61
5	0.69	7.17	0.52	7.73	2.17	5.17
10	0.87	6.89	0.96	6.59	2.08	5.34
15	0.90	7.00	1.31	6.02	2.50	5.42
20	0.94	7.01	1.37	6.12	2.63	5.45
25	1.00	7.04	1.37	6.21	2.69	5.48
30	1.02	7.07	1.43	6.27	2.76	5.50
45	1.07	7.14	1.54	6.35	3.00	5.56
60	1.19	7.16	1.65	6.21	3.20	5.59
90	1.20	7.19	1.79	6.34	3.42	5.63
120	1.23	7.13	2.07	6.11	3.53	5.66
135	1.49	6.26	2.13	6.15		
140	1.54	6.26	2.14	6.09		
145	1.55	6.25				

Table H-13 Data for precipitation result from DIG-2 with limestone solution (!).

	Volume Ratio	Volume Ratio	[Pi]	[Ca]	Ca x P
Set 1	1.00	50/50	7.6980	-0.3943	-3.0355
	1.50	60/40	6.1766	-0.4012	-2.4783
	2.33	70/30	4.7614	-0.3286	-1.5647
	4.00	80/20	3.0278	0.3354	1.0155
	9.00	90/10	1.5655	1.6590	2.5972
Set 2	19.00	95/5	0.7671	1.9105	1.4656
	1.00	50/50	8.3840	-1.0639	-8.9198
	1.50	60/40	5.9536	-1.0437	-6.2135
	2.33	70/30	4.3789	-0.8817	-3.8609
	4.00	80/20	3.1756	-0.2440	-0.7750
Set 3	9.00	90/10	1.5022	1.0768	1.6176
	19.00	95/5	0.6225	1.9827	1.2342
	1.00	50/50	7.7850	-1.0690	-8.3218
	1.50	60/40	6.4499	-1.0538	-6.7968
	2.33	70/30	4.7726	-0.7957	-3.7974
Average of the Sets	4.00	80/20	2.9634	-0.2390	-0.7082
	9.00	90/10	1.4132	1.0313	1.4574
	19.00	95/5	0.6943	1.9625	1.3626
	1.00	50/50	7.9557	-0.8424	-6.7018
	1.50	60/40	6.1934	-0.8329	-5.1584
	2.33	70/30	4.6376	-0.6687	-3.1010
	4.00	80/20	3.0556	-0.0492	-0.1504
	9.00	90/10	1.4936	1.2557	1.8756
	19.00	95/5	0.6946	1.9519	1.3559

<i>Mass of Ca and P in Ppt</i>	Volume Ratio		[Pi]	[Ca]	Ca x P
	1.00	50/50	0.1427	0.2200	1.5411
	1.50	60/40	0.1313	0.2461	1.8747
	2.33	70/30	0.0992	0.2569	2.5890
	4.00	80/20	0.0698	0.2221	3.1833
	9.00	90/10	0.0383	0.1187	3.0999
	19.00	95/5	0.0244	0.0627	2.5718
Volume Ratio			Pi Lost (mmol)	Ca Lost (mmol)	Ca/P Lost

Table H-14 Data for precipitation result from DIG-2 with limestone solution (!).

Theoretical Mass Ca+Pi in Ppt (mg)		STD of [Pi]	STD of [Ca]
Dilution Factor for Pi		Theoretical [Pi] (Dilute)	Dilution Factor for Ca
22.37		0.374	0.388
22.33		0.249	0.374
19.72		0.224	0.298
15.52		0.109	0.333
8.39		0.076	0.350
4.83		0.072	0.037