Dimensions of Phosphorus Sustainability: Phosphorus Flows in a Rapidly Growing City and Field Tests of Potential Agricultural Prototypes

by

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ABSTRACT

Phosphorus (P) is a limiting nutrient in ecosystems and is mainly used as fertilizer to grow food. The demand for P is increasing due to the need for increased food supply to support a growing population. However, P is obtained from phosphate rock, a finite resource that takes millions of years to form. These phosphate rock deposits are found in only a few countries. This uneven distribution of phosphate rock leads to a potential imbalance in socio-economic systems, generating food security pressure due to unaffordability of P fertilizer. Thus, the first P-sustainability concern is a stable supply of affordable P fertilizer for agriculture. In addition, improper management of P from field to fork leaves an open end in the global P cycle that results in widespread water pollution. This eutrophication leads to toxic algal blooms and hypoxic "dead zones". Thus, the second P-sustainability concern involves P pollution from agriculture and cities. This thesis focuses on P flows in a city (Macau as a case study) and on potential strategies for improvements of sustainable P management in city and agriculture. Chapter 2 showed a P-substance-flow analysis for Macau from 1998-2016. Macau is a city with a unique economy build on tourism. The major P flows into Macau were from food, detergent, and sand (for land reclamation). P recovery from wastewater treatment could enhance Macau's overall P sustainability if the recovered P could be directed towards replacing mined P used to produce food. Chapters 3 and 4 tested a combination of P sustainability management tactics including recycling P from cities and enhancing P-use efficiency (PUE) in agriculture. Algae and biosolids were used as recycled-P fertilizers, and genetically transformed lettuce was used as the a PUE-enhanced crop. This P sustainable system was compared to the conventional agricultural system using commercial fertilizer

and the wild type lettuce. Chapters 3 and 4 showed that trying to combine a PUE-enhancement strategy with P recycling did not work well, although organic fertilizers like algae and biosolids may be more beneficial as part of longer-term agricultural practices. This would be a good area for future research.

DEDICATION

I dedicate my thesis to my Lord, Jesus Christ. Without His guidance, I would not be here at ASU. He gave me this opportunity to study phosphorus, which means the “light-bearer” in Greek. Jesus said in Matthew 5:14, “You are the light of the world—like a city on a mountain, glowing in the night for all to see.”

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CHAPTER 1

INTRODUCTION

The Importance of Phosphorus

Phosphorus (P) is the fifteenth element in the Periodic Table and ranks as the 11th most abundant element of abundance in Earth's crust (Lynch and Brown 2008). Phosphorus was discovered by Hennig Brandt in 1669 and was revealed to the public in 1675 (Ashley et al. 2011). Phosphorus is essential to all life because it is a component of major molecules that are involved in fundamental biochemical functions, including genetic materials DNA and RNA, as well as ATP for energy transfer (Westheimer 1987). Phosphorus is also a key element in biological structural materials, including phospholipids and bones. In ecosystems, P is important as a limiting nutrient that, along with nitrogen, constrains primary production in terrestrial, freshwater, and marine ecosystems (Elser et al. 2007).

The global P cycle includes the processes of tectonic uplift, weathering, atmospheric deposition, soil processing (occlusion, decomposition-mineralization, desorption-adsorption), erosion, leaching, and runoff (Brady and Weil 2012). These processes operate over disparate time scales. For example, P in the ocean is taken up by phytoplankton and enters the marine food chain. As phytoplankton and marine animals die, P is transferred to the bottom of the ocean on the time scale of years (Murray and Renard 1891). Over a much longer time, this deposited P forms phosphate rock through a process called phosphogenesis (Filippelli 2011). Then, through tectonic uplift (an even slower process that takes from thousands to millions of years), the P reappears mainly as sedimentary rocks. These are the rocks that make up about 80% of global phosphate rock reserves, with the rest found in igneous deposits (Brady and Weil 2012; Ruttenberg 2013; Sims and Sharpley 2005; Walker and Syers 1976). This global P cycle has been identified as one of the nine planetary boundaries within which human activity should be confined in order to maintain a safe operating space for humanity (Carpenter and Bennett 2011; Elser et al. 2014; Rockström et al. 2009; Steffen et al. 2015).

Humans mainly use P to grow food to feed a growing population, as P is one of the key elements in agricultural fertilizer and as a supplement in animal feed. Since the start of the Green Revolution in the 1940s, global food production has increased three-fold (Childers et al. 2011). To produce this increased food supply, more and more P-rich fertilizers have been produced by mining reserves of phosphate rock. Each year, around 16 Mt of P is applied as fertilizer globally (Cordell and White 2014), and the demand for P production is still rising (Cordell et al. 2009). This rising demand has amplified human contributions to the global P cycle to an intensity that is now four times larger than before the Industrial Revolution (Falkowski et al. 2000; Lavelle et al. 2005; Liu et al. 2008). This increased dependence of humanity on phosphorus extraction for food production has created major concerns about its long-term sustainability.

Phosphorus Sustainability Concern 1: Supply

It has been estimated that global food production will need to double by 2050 to establish food security for 9 billion people (Godfray et al. 2010; Tilman et al. 2011). However, P for fertilizer production is obtained from phosphate rock, a finite resource that takes millions of years to form (Smil 2000). The largest phosphate rock deposits are found in northern Africa, China, the Middle East, and the United States (Jasinski 2013). Based on the US Geological Survey (USGS) report in 2013, Morocco and Western Sahara, China, Algeria, and Syria control 86% of global phosphate rock reserves; Morocco and Western Sahara alone control 75%. In terms of production, China, the United States, Morocco and Western Sahara, and Russia control 75% of global phosphate rock output (Jasinski 2013).

This uneven distribution of phosphate rock leads to a potential imbalance in socio-economic systems, generating food security pressure due to unaffordability of P fertilizer in Sub-Saharan Africa and subsequent impacts of hunger, particularly on the households of small farmers (Buresh et al. 1997; Cordell 2010; Scholz and Wellmer 2013). In 2008, the price of a ton of phosphate rock spiked from US$ 50 to US$ 430. The cause of this more than 800% increase in the price of phosphate rock was associated with several upward pressures on phosphate fertilizer markets, including increased crop production for biofuel, increases in the price of oil (Cordell, 2010), and dietary shifts to more meat (Metson et al. 2012). The spike in the price of phosphate rock, along with uncertainty about the P supply, was a cause for global food shortages and associated unrest (Elser and Bennett 2011). Recent statistical analyses (Elser et al. 2014) found strong evidence for non-linear volatility and critical transitions between 2007 and 2013 in the commodity price of phosphate rock (as well as less striking impacts from the prices of N and K components of fertilizer). While the price of phosphate rock had declined more recently, the first P-sustainability concern is a stable supply of affordable P fertilizer for agriculture.

Phosphorus Sustainability Concern 2: Pollution

The commodity price of phosphate rock does not reflect the full cost of P consumption. Insufficient management of P from field to fork leaves an open end in the global P cycle that results in widespread water pollution. This eutrophication leads to toxic algal blooms and hypoxic "dead zones" (Paerl and Huisman. 2009; Smith 2003; Smith and Schindler 2009). For example, in the United States, a bloom of cyanobacteria in Lake Erie in September 2013 created toxins that caused a 2-day loss of drinking water for city of Toledo (Landers 2014). In Africa, algal blooms in Lake Victoria caused the collapse of indigenous fish stocks (Verschuren et al. 2002). In China, eutrophication of Lake Taihu has also posed health risks (Guo 2007), such as a 5-day disruption of drinking water supply. P-driven water quality degradation has large economic costs. For example, the average economic damage caused by eutrophication in the United States in 2008 was estimated to be $2.2 billion (Dodds et al. 2008). The total economic loss incurred from Lake Taihu's eutrophication in 1998 was estimated at $6.5 billion (Le et al. 2010).

Eutrophication in lakes is caused by the accumulation of P from agricultural runoff and wastewater discharge (Guo 2007; Macintosh et al. 2018; Paerl et al. 2019). For example, P runoff from farms can be severe when excess P fertilizer is lost via leaching or erosion or where manure is over-applied (Kleinman et al. 2015). Phosphorus also enters lakes through direct sewage discharge or leakage from septic tanks (Guo 2007). Phosphorus pollution from agriculture usually is a non-point source, while P from sewage can be a point source from urban sewage or a non-point source from septic tanks and landscape runoff. Thus, the second P-sustainability concern involves P pollution from agriculture and cities. My focus, particularly in Chapters 2 and 3 of this thesis, is on cities.

How Phosphorus Flows Through Cities

Cities always have substantial P flows because they demand food to support their high population density. Thus, flows of food and of human waste usually dominate the P budget in urban areas (Baker 2011; Chowdhury et al., 2014; Fissore et al. 2011; Lin et al. 2014; Metson et al. 2012). However, the intensity and pathway of P flows through food and human wastes can vary widely based on each city's unique socio-economic situation (Newcombe et al. 1978; Li et al. 2011; Warren-Rhodes and Koenig 2001; Burstrom et al. 2003; Færge et al. 2001; Metson et al., 2012; Egle et al. 2014; Han et al. 2011; Metson and Bennett 2014; Meinzinger et al. 2009; Zoboli et al. 2016). For example, Neset et al. (2008) studied the P flows in Linköping, Sweden from 1870-2000. Assuming that the city's consumed food products were produced and processed in the surrounding region, Neset et al. (2008) found that an increase of population drove the rise in the total amount of P imported through food. More importantly, they found that shifting to a more meat-based diet increased P inputs through fodder and fertilizers needed to produce that fodder. In another study, Li et al. (2011) reported P flows in urban China from 1985-2006. They found that higher per capita income in cities led to greater total P flows through food consumption.

Emerging Approaches for Phosphorus Recapture and Reuse

Capturing and reusing P is easier in cities than on farmlands, since most P in city waste is concentrated at wastewater treatment facilities, incineration plants, and landfills (Lin et al. 2014). For example, about 52% of total P in output fluxes goes through wastewater treatment plants (WWTP) in the Minneapolis-Saint Paul metropolitan area (Fissore et al. 2011). The P entering wastewater treatment can be captured and reused in various outputs, such as struvite or biosolids (Morse et al. 1998; Rittmann et al. 2011; Abdel-Raouf et al. 2012).

Adding magnesium in the presence of ammonium forms the mineral struvite (magnesium ammonium phosphate hexahydrate, $MgNH_4PO_4{\cdot}6H_2O$), an increasingly common practice used in WWTP to reduce pipe clogging and, as a secondary benefit, to recover P. Although forming struvite is still quite expensive (Hao et al. 2013), studies show that struvite can be used as a slow-release fertilizer. It can produce the same crop yield as monocalcium phosphate and dicalcium phosphate fertilizers and be as effective as conventional P fertilizers, such as diammonium phosphate and superphosphate (Johnston and Richards 2004).

Biosolids produced during sewage treatment present another means to recover P. Being mostly comprised of microbial biomass, they contain nutrients that can be used by crops if introduced to soil (EPA 2011). In the United States, the three types of biosolids are identified: Exceptional Quality, Class A, and Class B. Exceptional Quality has the lowest content of pollutants such as heavy metals and pathogens, while Class B has the highest pathogen content (EPA 1994). Approximately 60% of biosolids are used for land application and approximately 0.1% of agricultural land is treated with biosolids (National Research Council (NRC) 2002). However, some agricultural areas rely more heavily on biosolids. For example, in the state of Arizona (USA), biosolids have been used for land application since the 1960s (Artiola 2011) and 22% of P applied to soil in the Phoenix area is in the form of biosolids (Metson et al. 2012).

Another biological means to capture P from wastewater is via using the growth of photosynthetic microorganisms, or microalgae. Sutherland and Graggs (2017) described how microalgae are used to capture nutrients from effluents from secondary wastewater treatment, dairy farms, aquaculture, stormwater, and agricultural runoff. The microalgal biomass can be harvested and used directly as a fertilizer or it can used after lipids have been extracted for biofuel production (Mulbry et al. 2004).

Building a Smarter Plant to Reduce Phosphorus Demand

One important step in achieving improved global P sustainability is to reduce fertilizer use by developing crops that can access soil P more effectively, since P deficiency strongly impairs plant development (Brady and Weil 2012). Domestication of crop plants subject to intensive fertilization has resulted in loss of some valuable traits associated with efficient P acquisition and efficient internal use of P: e.g., large root systems and improved root architecture (root morphology, topology, distribution patterns); high root/shoot ratio, extensive root branching, root elongation, and root hairs; and formation of specialized roots (Shen et al. 2011), all of which enhance a plant's P foraging in topsoil. Furthermore, root-induced chemical and biological changes (exudation of phosphatases and organic acids, release of protons (Hinsinger 2001) and low plant-P content (Lambers et al. 2011; Sulpice et al. 2014) are other traits of plants with high P-use efficiency (PUE). High-PUE traits in domestic crops may be regained through conventional breeding, genetic engineering, or a combination of these approaches (Veneklaas et al. 2012).

Researchers have been targeting several PUE-related genes to engineer improved crops. For example, the *PHT1* gene family is responsible for production of high-affinity Pi/H^+ symporters (Shin et al. 2004). Genes in this family include *PHO84* from the yeast *Saccharomyces cerevisiae* (Bun-Ya et al. 1991), *PHO5* from the filamentous fungus *Neurospora crassa* (Versaw 1995), and *GvPT* from the arbuscular mycorrhizal fungus *Glomus versiforme* (Harrison and van Buuren 1995). Transporter gene *Pht1:1* was over-expressed in tobacco cell cultures and showed increased P uptake under P-limited conditions (Mitsukawa et al. 1997). Another gene, *OsPSTOL1*, *PHOSPHORUS STARVATION TOLERANCE 1*, was introduced to rice and resulted in enhanced root growth under both high- and low-P conditions (Gamuyao et al. 2012).

Another promising gene for improving P efficiency is the *AVP1* gene. "*AVP1*" stands for type I *Arabidopsis* Vacuolar Pyrophosphatase, and it is responsible for type I H^+-pyrophosphatase (H^+-PPase). *AVP1* was first isolated by Rea et al. (1992) from vacuoles of *Arabidopsis*. Yang et al. (2007) reported that increased expression of the *AVP1* gene results in enhanced root growth and more efficient scavenging of phosphate from P-poor soil. Type I H^+-PPase depends on cytosolic K^+ for its activity, and it is hypothesized that this PPase participates in pyrophosphate-dependent sucrose metabolism and auxin transport in the root system (Gaxiola et al. 2012; Li et al. 2005). The up-regulation of Type I H^+-PPase increases of root growth and root density.

Based on these findings, the *AVP1* gene has been genetically engineered and expressed in cotton, creeping bentgrass, alfalfa, tomato, rice, and romaine lettuce (Gaxiola et al. 2011; Paez-Valencia et al. 2013; Yang et al. 2007). These studies show that over-expressing the *AVP1* gene results in higher PUE in alkaline soils. However, these altered varieties have not yet been extensively tested for a wide range of soil conditions nor for P recovered in biosolids.

While no “silver bullet” will solve P sustainability issues, every step taken to alleviate P scarcity and eutrophication problems counts. As shown in **Figure 1.1**, Cordell et al. (2011) proposed a pathway of interventions to transition from our currently unsustainable “business as usual” trajectory towards a future with enhanced P sustainability that uses less P to produce our food and reduces our reliance on finite phosphate-rock reserves. These measures to enhance P sustainability include shifting away from animal-based diets, increasing agricultural efficiency, and reducing food waste. P recovery and reuse include reapplying crop residues in the field or as compost, recycling of food waste to be composted or as feedstock for fertilizer supplements, and recycling of manure and human excreta as fertilizers. These scenarios show that transforming current approaches into sustainable P practices will require changes in cities and in agriculture.

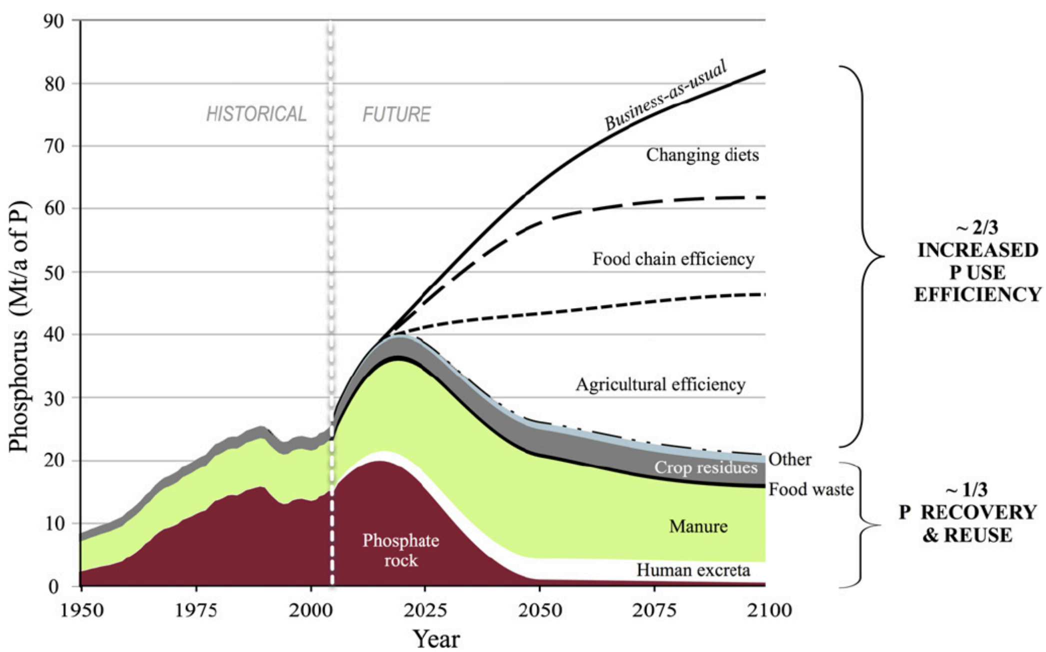

Figure 1.1. Different future scenarios achieved by adopting different P sustainability management through PUE and recovery. Figure adapted from Cordell et al. (2011).

To increase food-chain efficiency and P recovery and reuse in a city, I need to know the city's P flows and how socio-economic factors affect P flows. Such information can help us make reliable recommendations about when, where, and how to change the current system into a more sustainable P configuration. In **Chapter 2**, I use the Macau Special Administrative Region as a case study to understand P flows in a rapidly growing urban ecosystem that is dominated by tourism. I want to know what the major P flows are and the factors affecting them. For example, P imported through food usually is a large flow. How does tourism affect this P flow and other P flows? By examining the P budget of Macau, I seek to identify opportunities to implement P recycling and reuse.

It is important to appreciate that each P-sustainability strategy (e.g., those is **Fig. 1.1**) not only should be feasible for society, but also compatible with each other strategies. For example, PUE-improved crops should be able to use recycled nutrients, such as in biosolids. My thesis takes a first step to evaluate components of such a future system. In **Chapter 3 and 4**, I compared the yield of *AVP1*-transformed lettuce *Lactuca sativa* cv. Conquistador (a PUE-improved crop) to its wild-type counterpart in a series of experiments using two distinct types of "alternative" fertilizers that involve recycled P in biosolids from wastewater treatment and in microalgae. I explored how well the PUE-improved crop performed when P-recycled fertilizers were applied, and I compared the performance to commercial inorganic fertilizers.

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CHAPTER 2

THE PHOSPHORUS BUDGET OF MACAU, A RAPIDLY GROWING TOURISM CITY (1998 – 2016)

Abstract

Because cities have high phosphorus (P) demand and produce concentrated-P waste streams, they are sinks and sources for anthropogenic P. P flows in a particular city is affected by the city's particular socio-economic situation. Exploiting available data, I completed a P-substance-flow analysis from 1998-2016 for Macau, a city with a unique economy that shifted to reliance on tourism during this period. From 1998 to 2016, Macau increased P imports of many commodities but food and detergent showed especially large increases that mirrored increases in the number of tourists. Over the study period, phosphorus in imported food increased from 220 to 500 megagrams (Mg) per year, which was larger than the increase in the number of visitors, likely due to a shift to a more animal-based diet. Input of detergent P increased from 190 to 440 Mg per year, making detergents a major contributor to Macau's P budget by 2016. Macau's land reclamation also imported large amounts of P with river sand. The major outflow of P was wastewater discharge (100 Mg in 2016) but most of the P input to Macau accumulated P as incinerated ashes buried in landfills (739 Mg in 2016). Based on this P-flow analysis, Macau has considerable potential to reduce P waste by properly managing food waste and by restricting the P content of laundry detergent. Furthermore, Macau could recycle P by expanding wastewater treatment facilities to capture and reuse P, which would recover P that is currently sent to landfill as ash or discharged to the ocean.

Introduction

Phosphorus (P) is essential to all life. Human society mainly uses P to grow food to sustain its population, and P is one of the key elements in fertilizer. Since the start of the Green Revolution in the 1940s, global food production has increased three-fold, and the increase of food production was enabled by large increases of fertilizer use (Childers et al., 2011). Global food availability will need to double by 2050 to establish food security for 9 billion people, and fertilizer demand is expected to increase as a consequence (Godfray et al., 2010; Tilman, Balzer, Hill, and Befort, 2011).

The increasing use of P for food production to feed an expanding human population has come with costs. On the one hand, phosphate rock is a finite resource (Smil, 2000), and only a few countries have large extractable deposits (Jasinski, 2019). Increasing demand for P may raise prices and heighten risks of geopolitical instability, which may exacerbate uncertainty about future supplies. On the other hand, human activities have accelerated global P throughput about four times compared to pre-industrial fluxes (Falkowski et al., 2000; Lavelle et al., 2005; Liu et al., 2008). This four-fold increase of P flux, coupled to improper management of P from field to fork to feces, has led to large losses of P from ecosystems, which result in eutrophication and impaired water quality (Smith and Schindler, 2009). Notable examples are the freshwater toxic algal blooms in Lake Taihu, China (Qin et al, 2007) and the Toledo drinking water crisis caused by P derived from surrounding farms and feedlots (Landers, 2014). These nutrient losses also have impacts in coastal oceans, where over 400 hypoxic "dead zones" and toxic algal blooms are harming fisheries and contaminating seafood (Hallegraeff, 1993).

Cities contribute to P pollution but also suffer from it. Of particular note, red tides have been more persistent and covered a larger area around Macau (the subject of this paper) in recent years (Stone, 2009; Williams, 2014). Understanding P flows in urban ecosystems can improve P management for cities by identifying major sources of pollution and targets for P recycling. In urban settings, flows of food and waste usually dominate the P budget (Baker, 2011; Chowdhury et al., 2014; Fissore et al., 2011; Lin et al., 2014; Metson et al., 2012) but urban P flows can vary widely based on each city's unique socio-economic situation (Li et al., 2011; Warren-Rhodes and Koenig, 2001; Burstrom et al., 2003; Færge et al., 2001; Metson et al., 2012; Egle et al., 2014; Han et al., 2011; Metson and Bennett, 2014; Meinzinger et al., 2009; Zoboli et al., 2016).

This study focuses on the city of Macau, a rapidly transforming city in a dynamic region of east Asia. Macau has a population of 645,000, of which 95% are Chinese, similar to Hong Kong (Augustin-Fean, 2002). However, the population density in Macau is three times that of Hong Kong and Macau had the world's third highest per capita gross domestic product (GDP) in 2016 (World Bank, 2016). Macau's urban area has also been expanding by land reclamation, i.e., building new land in previously marine habitat. In 1998, Macau encompassed 23.6 km^2 of area but grew to 30.5 km^2 in 2016 through land reclamation using imported river sand from China. In 2016, Macau's land surface had 14% roads, 23% green space, 0.7% commercial, 2.3% industrial, and 9.5% residential land uses, with the rest of the city's boundaries being the ocean (Report on the State of the Environment of Macao, 2017; DSCC, 2016; Mu and Zhang, 2008). The percentages of different land uses will change in the future due to new areas from land reclamation being put into use.

Macau is renowned in the gambling world and has been called "Monte Carlo of the East" (Lai and To, 2011). Macau's gambling revenues were $45 billion in 2013, seven times greater than those for Las Vegas (Sheng et al., 2017). Since Macau became part of China in 1999, tourism in Macau has been rising, especially from mainland China. For example, after Macau was returned to China in 1999, tourism increased by 20 million from 1999 to 2007, until the global recession in 2007. After 2009, the increase of tourism resumed and continued until 2014, when it stabilized at 42 M tourists per year. Macau's tourists are mostly short-term visitors, with those staying for only one day about three-fold more numerous than tourists visiting more than one day. Tourism from 1998 to 2016 increased five-fold, a much greater increase than the 50% growth of Macau's resident population in the same period (Yearbook of Statistics, 2013-2016). Macau's dense population and tourist-oriented economy make it a unique case study to evaluate the P budget of a tourism-intensive city.

While Macau's P flows have not been studied, the rise of tourism likely has increased its P flows. For example, the increase of tourism boosted the hotel industry in Macau: the number of hotels increased from 25 in 2001 to 121 in 2016, while the number of guest rooms in hotels and guesthouses increased from 9431 in 1999 to 36278 in 2016 (DSEC, 2018). Phosphorus in detergent has long been recognized as an important potential contributor to eutrophication in water bodies (Knud-Hansen, 1994), and the large increase in hotel rooms means more detergent is being used for hotel cleaning, laundry, and personal care. In recent years, many countries have banned used or sale of detergents that contain P or have significantly lowered the P concentration allowed (European Commission, 2015; Chu et al., 2014; van Puijenbroek et al., 2018). However, Macau does not regulate detergent P.

Similar to the demand for detergent, the large increase in tourists demands a greater flow of food, and its P, into Macau. Since China has been growing economically, the increased wealth has resulted in shifts in food choices (Veeck and Veeck, 2000), including dietary changes to more meat-based meals (Hansen and Gale, 2014). Indeed, China and Macau's overall and per capita meat consumption increased 35% from 1981 to 2005 (FAOSTAT, 1998-2013) (Masuda and Goldsmith, 2010). Since meat generally contains more P than vegetables (Metson et al., 2012; Metson et al., 2014), increased meat in Chinese tourist diets could affect the P budget of Macau beyond that expected just from the increased numbers of residents and tourists.

Macau's P budget also is affected by extensive engineering projects during recent decades. Since 1999, a substantial portion of Macau's land was created by land reclamation using sand transported from the Pearl River estuary (China) (Zhang et al., 2010). Phosphorus in the sediments of aquatic ecosystems usually is stored as organic P and iron-bound P. However, the inputs of P to Macau in land-reclamation sand have not been studied.

While assessments of Macau's energy dynamics have shown that the city depends on external imports to support energy demand (Lei et al., 2006; Lei et al., 2014; Lei et al., 2016; Lei et al., 2018; Lei et al., 2008; Lei et al., 2011; Lei et al., 2010; Lei and Zhou, 2012; Song et al., 2016) P research for Macau is lacking. Nevertheless, a P budget for Macau is especially important today, since Macau has been experiencing water pollution in the Guangdong-Hong Kong-Macau Greater Bay Area. P is a major source of the region's water pollution, especially for reservoirs (Liu et al., 2019).

The overall goal of our study was to quantify the P budget in Macau using substance-flow analysis. The specific goal was to identify the major P fluxes into, within, and out of this unique, tourism-based, urban ecosystem. These results allow us to assess how Macau's P budget has changed due to its rapid expansion from 1998 to 2016 and to evaluate the ways in which tourism affects Macau's P budget.

Methods

I used substance-flow analysis (Brunner and Recberger, 2004; Kennedy et al., 2011) to quantify P flows in the Macau urban ecosystem from 1998 to 2016. I multiplied the mass of materials entering and leaving Macau each year by the material's P concentration (mass of P per mass of material) to calculate P mass flows. All of the P flows studied, numbered from 1 to 20, are identified in **Table 2.1**.

The P concentrations for different commodities were obtained from the United States Department of Agriculture (USDA) database for food (USDA, 2019) and published literature for other entries (**Table 2.1**). When multiple commodities comprised one category, I averaged their P concentrations. For example, I computed an average P concentration of 2.46 g P/kg for the Macau government's category (DSEC, 2018) ,"ginger, saffron, turmeric (curcuma), thyme, bay leaves, curry and other spices" using the average of P concentrations for ginger root (0.35g P/kg), saffron (2.57g P/kg), turmeric (2.68g P/kg), thyme (2g P/kg), bay leaves (1.13g P/kg), and curry leaves (6g P/kg). The quantities of materials moving in various flows in a given year were obtained directly from commodity reports by the Macau government (**Table 2.1**). Since river sand used for land reclamation was a potentially large P flow but was not "consumable" as a commodity, I separated it from other imported commodities.

Since detergent P is not regulated in Macau and, thus, data on its P concentration are not available (DSPA, 2019), I determined P concentrations in detergent by examining detergent P regulations in China, Hong Kong, and the European Union. P concentration in detergent in China was 4.25% from 1970s to 1990s (Liu et al., 1998), while currently China has detergent P standards (1.7% and 0.24% by mass for detergents that have P and do not have P, respectively) but they are not mandatory (China's Standardization Administration, 2009). The European Union (EU)'s standard for detergent P is 0.3 g/load (European Commission, 2015), where one load is about 2 oz (~57 g). Thus, the EU's standard detergent P is ~0.5% by mass. The detergent-P limit in Hong Kong is 0.5% by mass (Chu et al., 2014). A value of 4.25% detergent P (in China) likely is too high for calculating the detergent P flow but the EU standard of 0.5% is likely too low; I chose 2% P (by mass) in detergent for our calculations.

Because I could not find data on the population of cats and dogs in Macau, I used the proportion of cats and dogs from the Census and Statistics Department of Hong Kong to compute the consumption of pet food for cats and dogs in Macau; I assumed pet food was mainly imported. I chose Hong Kong's pet information because Hong Kong and Macau share a very similar culture and lifestyle.

Table 2.1. Data sources for Macau's P budget

Flow number	Flow name	Calculation	Assumptions and Specifications	Data sources
1	Imported food	Flow #2 + #3 + #5	Mass balance flows	
2	Food consumed by humans	Imported food X P concentration of food	When there are more than one food in DSEC's food import category, we averaged the P content from USDA database or literature.	supmaterial; USDA database (https://fdc.nal.usda.gov/); DSEC (https://www.dsec.gov.mo/)
3	Food waste	(P in imported food - P in exported food) X 37.1%	We averaged the food waste percentage of 2015, 2016 & 2017	Report on the State of the Environment of Macao (2016 &2017)
4	Urine and excretion	Macao population X 2.1 gP X 365	We used the P in daily excretion of a person. When the data showed that the tourists stayed for more than one day, we assumed the stay as 2 days for our calculation.	Macau Yearbook of Statistics (1998-2016); Gilmour et al., 2008
5	Exported food	Exported food X P concentration of food	same as flow #1	same as flow #1
6	Incineration	Fly ash X P concentraiton of fly ash + bottom ash X P concentraiton of bottom ash	We calculated the P in fly ash and bottom ash. When there was missing fly ash number, we subtracted the metal content from total ash/slag and then multiplied it with 1%.	Kalmykova & Fedje 2013; Astrup et al., 2016; Macao Environmental Statistics (2000-2016)
7	Imported detergent	Detergent X detergent P concentration	We could not find any regulation from Macau. We chose 1% was because UN's regulation set it as 0.5% while most of the detergent Macau imported was from China where the detergent P content was probably much higher due to lack of regulation.	DSEC 1998-2016; The European Parliament and the Council of the European Union, 2004
8	Exported detergent	Detergent X detergent P concentration	Same as flow #7	Same as flow #7
9	Detergent usage	Flow #7 - #8	Mass balance flows	
10	Wastewater treatment effluent	Wastewater X wastewater effluent P concentration	We assumed that P in the effluent is constant over time.	Macau Yearbook of Statistics (1998-2016); Hong et al., 2007
11	Imported sand	Sand X sand P concentration	We assumed that all sand came from the excavation of river sand from Pearl River in China.	Macau Yearbook of Statistics (1998-2016); Lili et al., 2013
12	Imported fertilizer	Fertilizer X 43.6 g/kg	We assumed fertilizer NPK number of 10-10-10, so P is 4.36%, which is 43.6 g/kg	Same as flow #2
13	Recycled paper	Paper X paper P concentration		Macau Yearbook of Statistics (1998-2016); Kalmykova et al., 2012
14	Incinerated waste	Flow #4 + #9 - #11	Mass balance flows	
15	Imported paper	Paper X paper P concentration		Same as flow #18
16	Imported textile	Textile X textile P concentration		Same as flow #18
17	Imported others			Same as flow #18
18	Exported paper, textile, & others	Paper X paper P concentration + textile X textile P concentration + others X others P concentration		DSEC (1998-2016); Metson et al., 2012
19	Imported pet food	Pet food X proportion of cats and dogs X cat or dog food P concentration	We assumed that pet food consisted only cats and dogs' and was consumed within that year. We used the proportion of cats and dogs from the Census and Statistics Department of Hong Kong since we could not find the number of cats and dogs in Macau. We used the proportion of 1998 for calcuation for 2005-2007, proportion of 2011 for 2008-2013, and 2019 for 2014-2016.	General Hosehold Survey (1998); Thematic Household Survey (2011, 2019); Hazewinkel et al., 1991; Kienzle et al., 1998.
20	Exported pet food	Same as flow #19	Same as flow #19	Same as flow #19

Results and Discussion

P flows ranged from as low as ≤ 1 Mg per year (other goods and recycled paper in 1998 and 2016) to 6.5×10^5 Mg per year (sand in 1998) (**Fig. 2.1**). The largest input beside sand was imported food in 2016; other large flows were associated with detergent input and use and fly ash (in 2016). The fate of this imported P was mostly wastewater treatment plants, incineration, and landfills (**Fig. 2.1**).

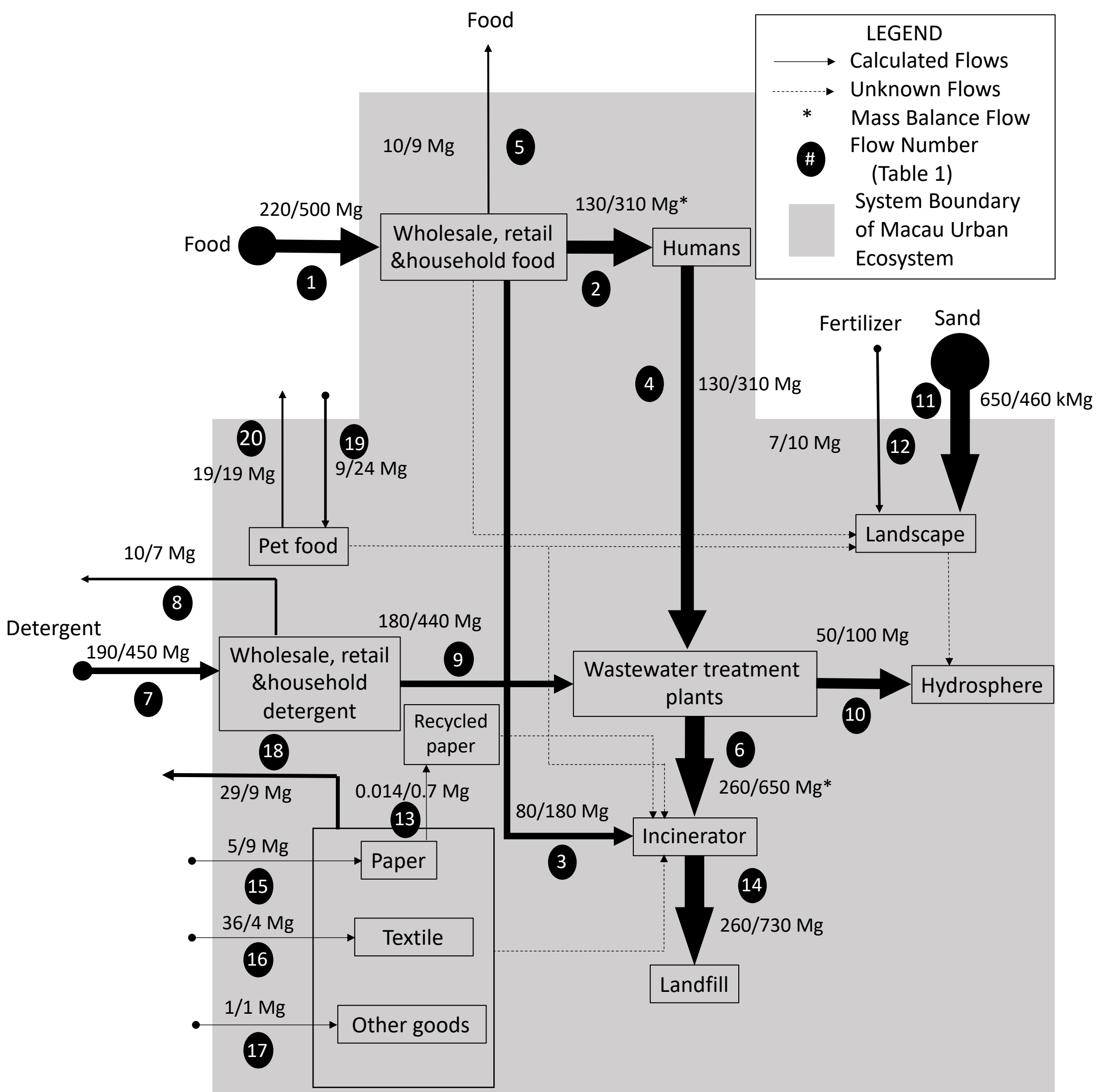

Figure 2.1. The phosphorus budget of Macau in 1998 vs 2016. Boxes represent P stocks, and arrows represent P flows (Mg/yr). The numbers on the left and right of the slash are the P flows in 1998 and 2016, respectively (except for pet food). For pet food import, the number on the left is from 2005 and on the right is 2016; pet food export P was 19 Mg in 2006 and 2009. The size of the solid arrows reflects the relative size of the flows. However, the size of the boxes is not meant to represent the size of the stocks. Dashed arrows represent unknown flows. A flow obtained by mass-balance calculation from input and output flows is indicated by *. Details of the flows and data sources used are given in **Table 2.1**.

Phosphorus in imported food was one of the biggest flows and more than doubled from 1998 to 2016 (**Figs. 2.1 and 2.2a**). Food exports hardly changed (**Fig. 2.2b**). The choice of food by tourists and Macau locals affects how much P enters Macau through food, particularly since animal- and fish-based food usually has higher P content than plant-based food (Metson et al., 2011). For example, the P concentration of seafood and meat is about 2 g P/kg but in vegetables is about 0.2-0.5 g P/kg (USDA, 2019). Data from the United Nations (UN) Food and Agricultural Organization (FAO) show that, between 1998-2013, per capita consumption of fish and pork grew strongly in Macau, with fish consumption increasing by more than 3100% (FAOSTAT 1998-2013). Although the growth of per capita animal- and fish-based food consumption in mainland China might not represent the diets of Chinese tourists, the increase of fish consumption in Macau was likely linked to the increase of Chinese tourism (**Fig. S.2.3**).

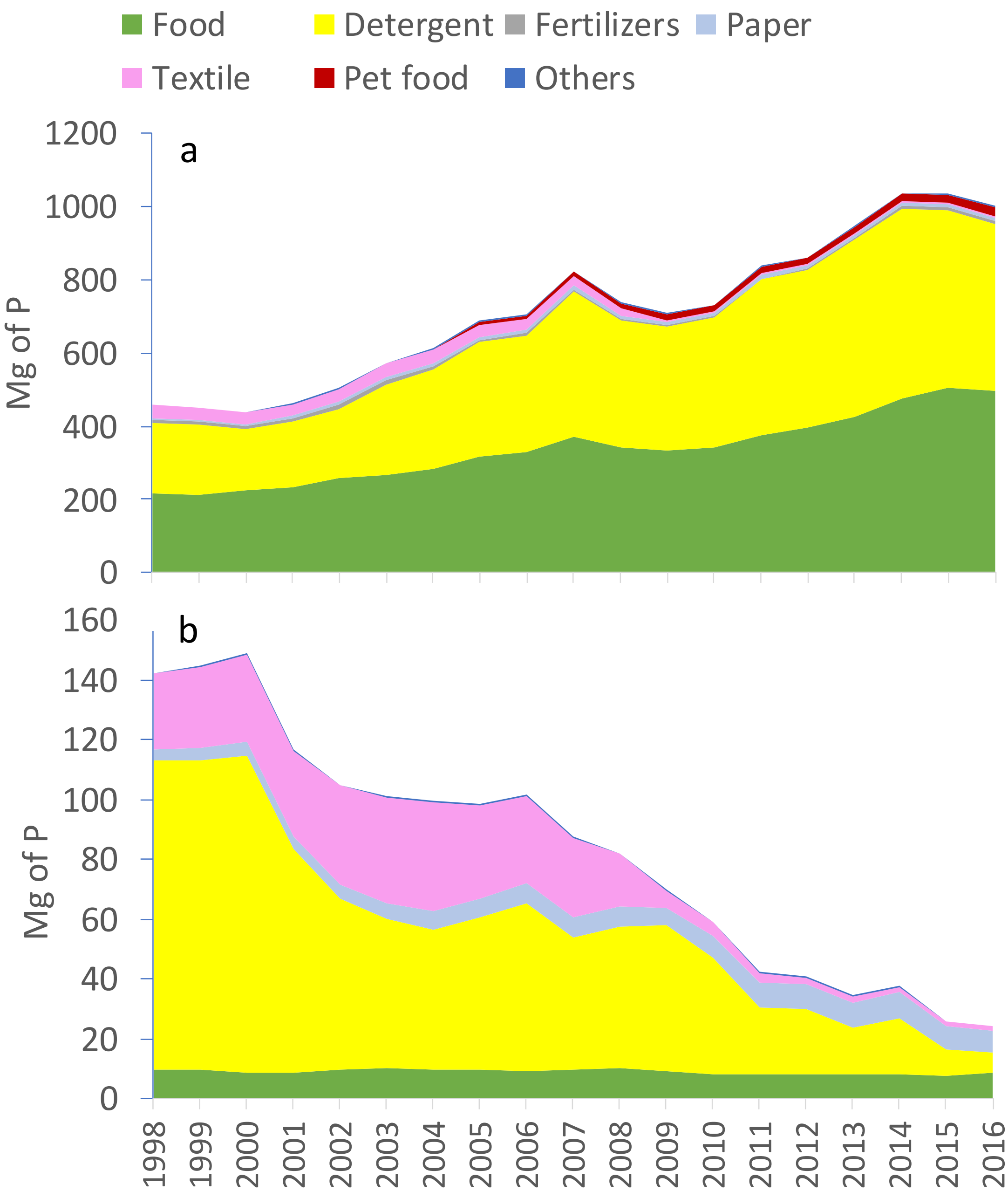

Figure 2.2. a. Flows of P in imported commodities in Macau from 1998-2016. Food imports increased while textiles and fertilizers declined. **b.** Flows of P in exported commodities in Macau from 1998-2016 Only food exports held steady while dramatic declines occurred for detergents and textiles. Note the difference in scales for P flows for the two panels.

Figure 2.3 shows the increase in P consumption per capita of Macau and Mainland China for meat-based products between 1998-2013 (FAOSTAT 1998-2013). The increase of P per capita shows a dietary shift to a meat-based diet. According to Li et al. (2011), the overall dietary shift in China from 1985 - 2006 led to an increase in per capita P consumption of meat from 0.4 g to 0.83 g P per person per day and a decrease in plant-based dietary P consumption from 1.2 g to 0.65 g P per person per day. Furthermore, Li et al. (2001) found that Chinese citizens with higher income, who are more likely to be tourists in Macau, have a much more P-rich diet than citizens with lower income.

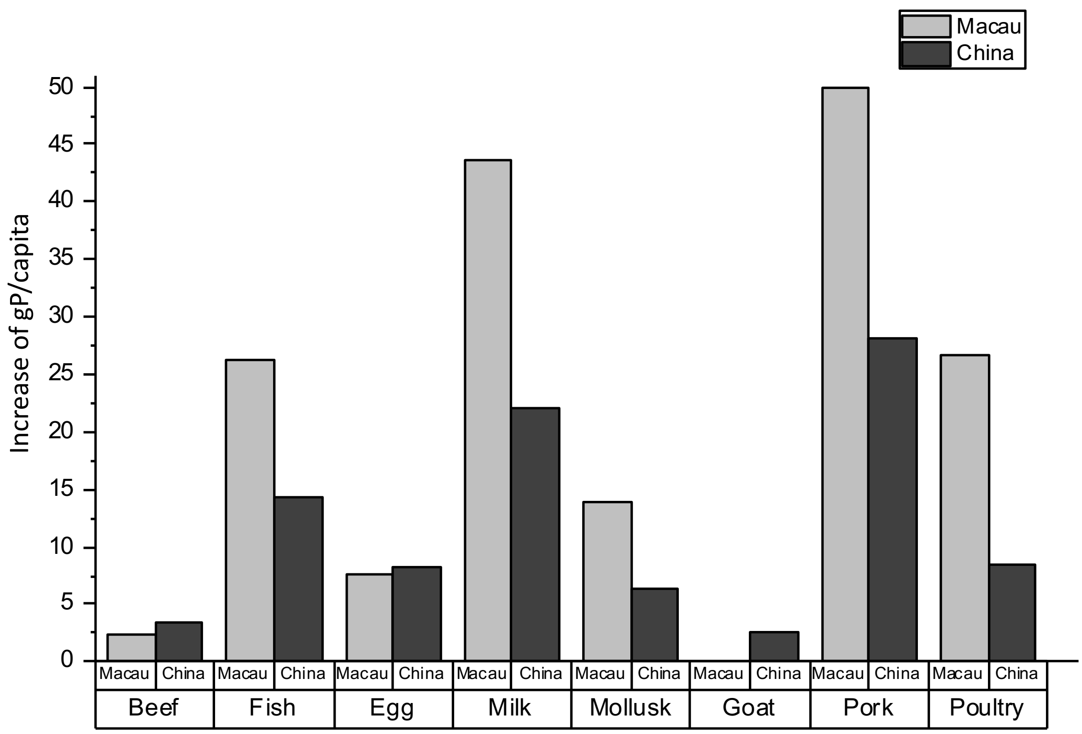

Figure 2.3. The increase of P consumption per capita in animal-based product of Macau and Mainland China from 1998-2013 (FAOSTAT 1998-2013). Note that milk and pork had the highest increase in both Macau and Mainland China.

P imported from detergent rose steadily (**Fig. 2.2a**), while the P exported from detergent decreased by 15-fold (**Fig. 2.2b**). These two counter trends indicate that the increases in tourism and in Macau's resident population created a demand that strongly outweighed locally produced detergent. Thus, detergent had to be imported. In particular, the five-fold increase of tourism (**Fig. s.2.2**) coincided with a five-fold increase of the P flow from imported detergents from 1998-2016 (flow 9 in **Figs. 2.1 and 2.2a**). Almost all P from detergent was transmitted to the wastewater treatment facilities, where it was either removed as solids going to incineration or was discharged to surrounding waterbodies as a pollutant. Over our study period, P in detergent made up about half of P going to wastewater treatment annually (flow 10 + 14) (**Fig. 2.1**).

Other imported commodities were pet food, fertilizers, paper, textiles, and other commodities. The P flows associated with these commodities contributed only a very small portion of the total P flows to the city. For example, they made up 4.9% of total P flows in 2016. Among these, only the P in textiles decreased over the years, while the others increased (**Fig. 2.1**).

Total P flows in all imported materials declined during the years of global recession (2007 and 2008) but regained momentum in 2009 before stabilizing in 2015. Total P flows in imported commodities increased 2.1-fold from 1998-2016.

P in exported commodities decreased after 2000 and stabilized in 2015, with a six-fold reduction from 1998-2016. In particular, P in textile exports dropped 20-fold from 1998-2016. This reduction was caused by four factors: 1. relocation of textile manufacturing from Macau to China due to lower labor cost and to China's joining WTO in 2001 (Trigo de Sousa, 2009, Cardoso and Cordeiro, 2019). 2. economic uncertainty given the unknown impact of the handover of Hong Kong and Macau to China at the end of the 20^{th} century. 3. the 1997 Asian financial crisis that hit southeast Asia, causing recession (Alves et al., 2015). 4. local consumption increasing due to expansion of tourism and hotel businesses.

Most of the P flux through commodities eventually ended up in incineration ash (**Fig. 2.1**, flows 3, 4, 6, 9, 13, 15, 16, and 17), which was sent to landfill (flow 14). P in landfill represents the accumulation of P in Macau urban ecosystem and this accumulation has been rising quickly (**Fig. 2.4**). Some of the net excess P was discharged through wastewater treatment effluent (**Fig. 2.1**, flow 10). P in waste increased 2.7-fold from 1998-2016, about 70% of which was waste P in bottom ash (**Fig. 2.4**). While the P flow to the hydrosphere doubled from 1998 to 2016, the flow to landfill increased almost three-fold.

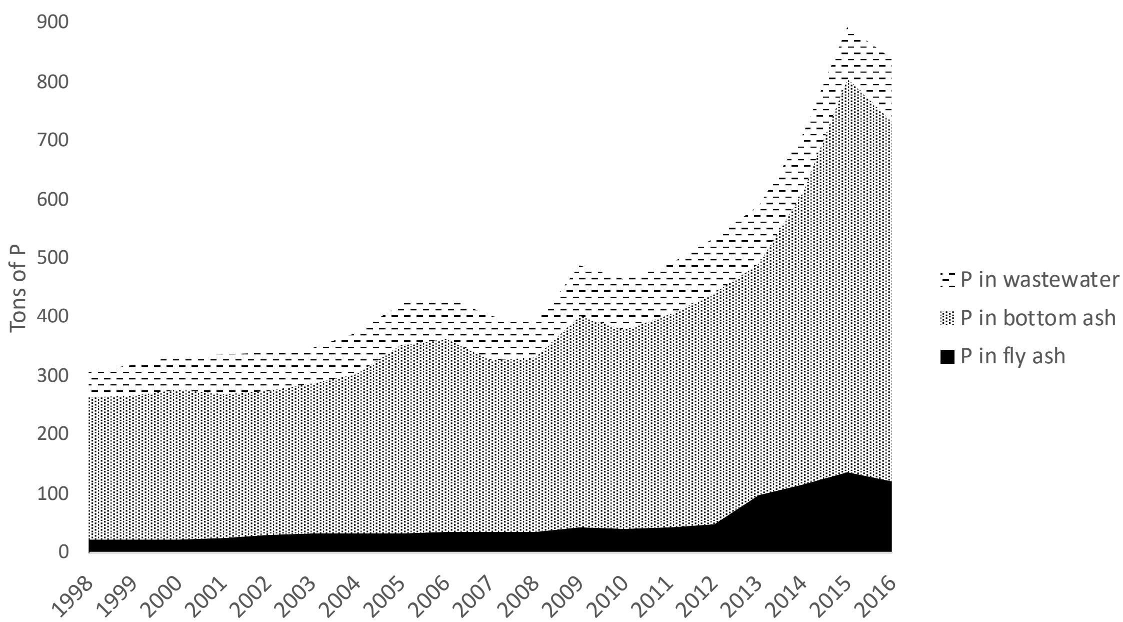

Figure 2.4. Phosphorus in incineration ashes grew substantially in Macau from 1998-2016 but P in wastewater discharge was stable.

Food waste made up ~37% of Macau's solid waste (RSEM, 2016 and 2017). P in food waste increased 225% during the study period, which almost exactly matched the relative increase of P from imported food (227%) (**Fig. 2.1**). Only a small fraction of the P in food waste was recycled via composting and then applied as fertilizer to the landscape (**Fig. 2.1**, dashed line from food waste to landscape). How much food waste per capita was contributed by tourists in Macau is unknown. Given that many factors contribute to food waste, further studies are needed to understand the differences in food waste produced by tourists versus residents. Reducing food waste in the future could reduce P flows from food imports, while composting the waste that does occur could generate a new internal P recycling flow.

The increased of tourist and resident population was one of the major contributors to P demand through food, as P in imported food increased 227% from 1998-2016. About half of the total imported P in commodities flowed through imported food (**Fig. 2.2a**). Phosphorus in food consumed by humans eventually ends up in urine and excreta, and thus that flow is strongly affected by tourism. **Figure 2.5** shows that the P discharge through urine and excreta attributable to tourists increased 450% from 1998-2016, reflecting the five-fold increase of tourism (**Fig. S.2.1b**). It is worth noting that the overall P flux in urine and excreta is dominated by Macau local residents, because most of the tourists visiting Macau only stay for one day (Yearbook of Statistics, 1998-2016). However, Macau's resident population only increased by 50% (**Fig. S.2.1a, Fig. S.2.2**).

P in wastewater-treatment sludge made up of the majority of P going to incineration, which increased from 160 Mg in 1998 to 650 Mg in 2016 (**Fig. 2.1**, Flow 6). P in ash increased rapidly after 2010 and peaked in 2015. This increase was likely driven by a large expansion in wastewater-treatment and incineration capacities in 2008 (Environmental Council 2009). P can be recovered during wastewater treatment, such as by struvite precipitation (Rahman et al., 2014; Huang et al., 2015) and enhanced biological-P removal (Rittmann and McCarty, 2020). P recovery reduces the amount of P that ends up in incinerated ash.

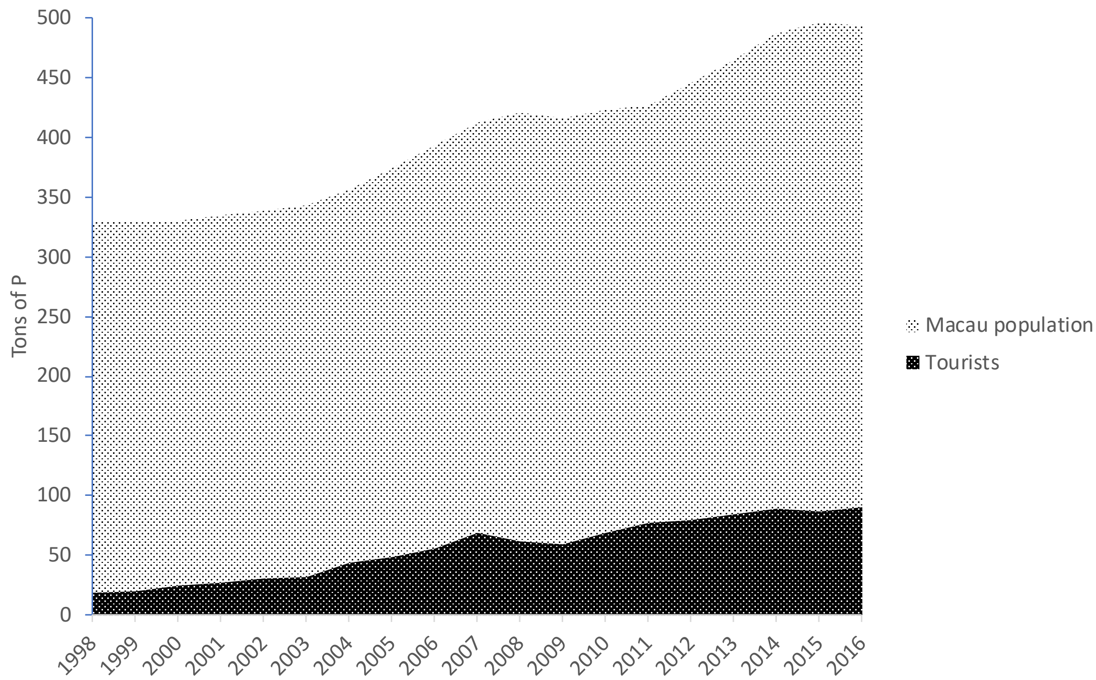

Figure 2.5. Phosphorus flows through urine and excreta from the resident Macau population and tourists from 1998-2016.

Variability in the amount of P imported in sand for land reclamation was high during 1998-2016 (**Fig. 2.6**), because the need for sand depended upon the execution of particular reclamation projects (Edmonds and Kyle, 1998; Lee, 2014). Land reclamation in Macau began in the early 20th century but it was not until the late 1980s and the 1990s that the Portuguese government invested heavily in land reclamation to develop Macau's economy in order to break away economically from Hong Kong (Trigo de Sousa, 2009; Edmonds and Yee, 1999). However, Macau's international airport, major container port and oil terminal, new ferry terminal, industrial and residential developments, and casinos were massive projects that occupied reclaimed lands after 1998 (Porter, 1993; Sheng et al., 2017). Phosphorus in the sand used for these reclamation projects was a large P flow that potentially was or is a non-point-source of pollution (**Fig. 2.1**, the dashed line connecting Landscape and Hydrosphere). However, the actual release of P from imported sand into adjacent waterbodies is unknown. Future studies are needed to assess the rates and mechanisms of P release from sources accumulated during Macau's land reclamation.

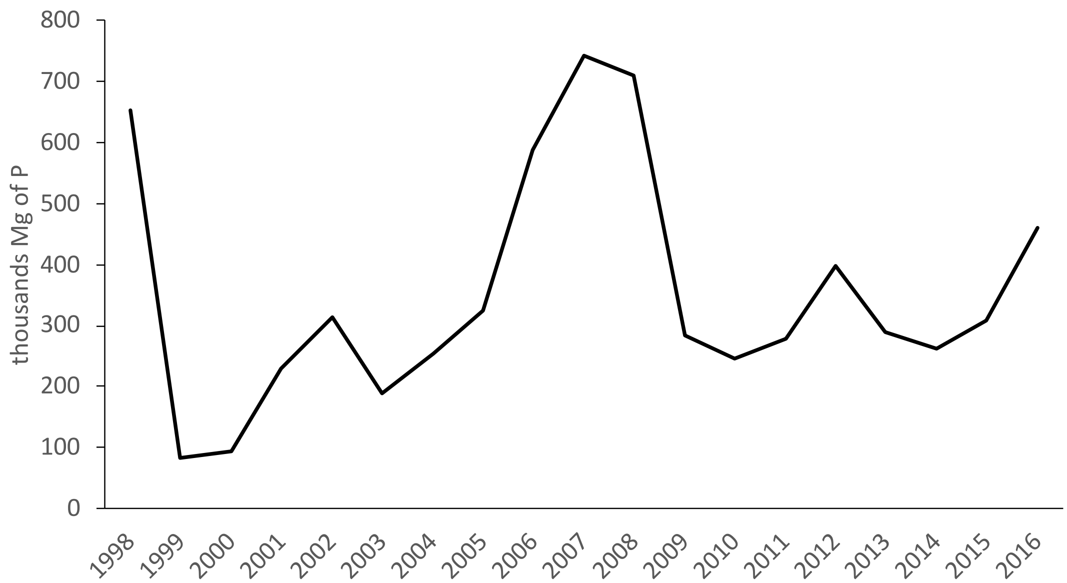

Figure 2.6. Imported sand for land reclamation in Macau from 1998 – 2016. There is no specific pattern for the P in sand because the import of sand follows the timing of sand reclamation projects in Macau.

Since Hong Kong is close to Macau and shares a similar culture, I compared our findings to the P flow studies of Hong Kong and found some similarities and distinct differences. In the late 1990s, food was 47% of total imported P in Hong Kong and Macau. However, about 40% of the P imported to Hong Kong was in animal feed and fertilizer (Warren-Rhodes and Koenig, 2001), while these were almost zero in Macau**.** This large difference reflects Macau's dwindling agriculture, which essentially disappeared in the 1980s

(Porter, 1993) while food imports continued to increase **(Fig. 2.2a**). Any imported P fertilizer was applied for landscaping.

The biggest difference between Macau and Hong Kong was in the P flows from detergent. P in detergent imported to Macau was about 50% of the total input P flow throughout the study period, while in Hong Kong, detergent P was only 14% in 1997 (Warren-Rhodes and Koenig, 2001). Detergent P was more prominent in Macau likely due to a higher relative importance of detergent use for tourism in Macau than in Hong Kong, which is a major gateway of international trade for China (Chiang, 2016).

Metson et al. (2015) reviewed P budgets for 18 cities and showed that, although the major P flows in most cities were food and wastewater and major pool was landfill, each city's own social, ecological, and technological drivers affected these fluxes and pool differently. Metson et al. (2015) listed eight drivers that govern urban P budgets: 1. biogeophysical situation, 2. infrastructure and land use, 3. market and capital availability, 4. knowledge and access to information, 5. governance and actors, 6. government and regulation, 7. cultural norms and preferences, and 8. future priorities. Our study sheds light on some of these factors (particularly 1-4) for Macau but more research will need to understand and improve our understanding of the others. Through better understanding of these drivers and by working with government and businesses, Macau could become a more P sustainable city.

Conclusion

Understanding urban P budgets is necessary to sustainably manage P because P is a finite resource and also is a key nutrient that causes algal blooms and eutrophication. Macau is a unique case study for urban P cycling because of its rapid growth after 1998 and its economic domination by tourism and gambling. From 1998 to 2016, Macau increased P imports of many commodities, but food and detergent showed especially large increases that mirrored increases in the number of tourists. Macau's land reclamation also imported large amounts of P with river sand. Most of Macau's accumulated P was in the form of incinerated ashes (buried in landfills) and wastewater effluent discharged to adjacent waterbodies. Macau has considerable potential to reduce P waste by properly managing food waste from tourists and locals and by restricting the P content of laundry detergent. Furthermore, Macau could recycle P by expanding wastewater treatment facilities to capture and reuse P, which would recover P that is currently sent to landfill as ash or discharged to the ocean.

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Supplementary Material

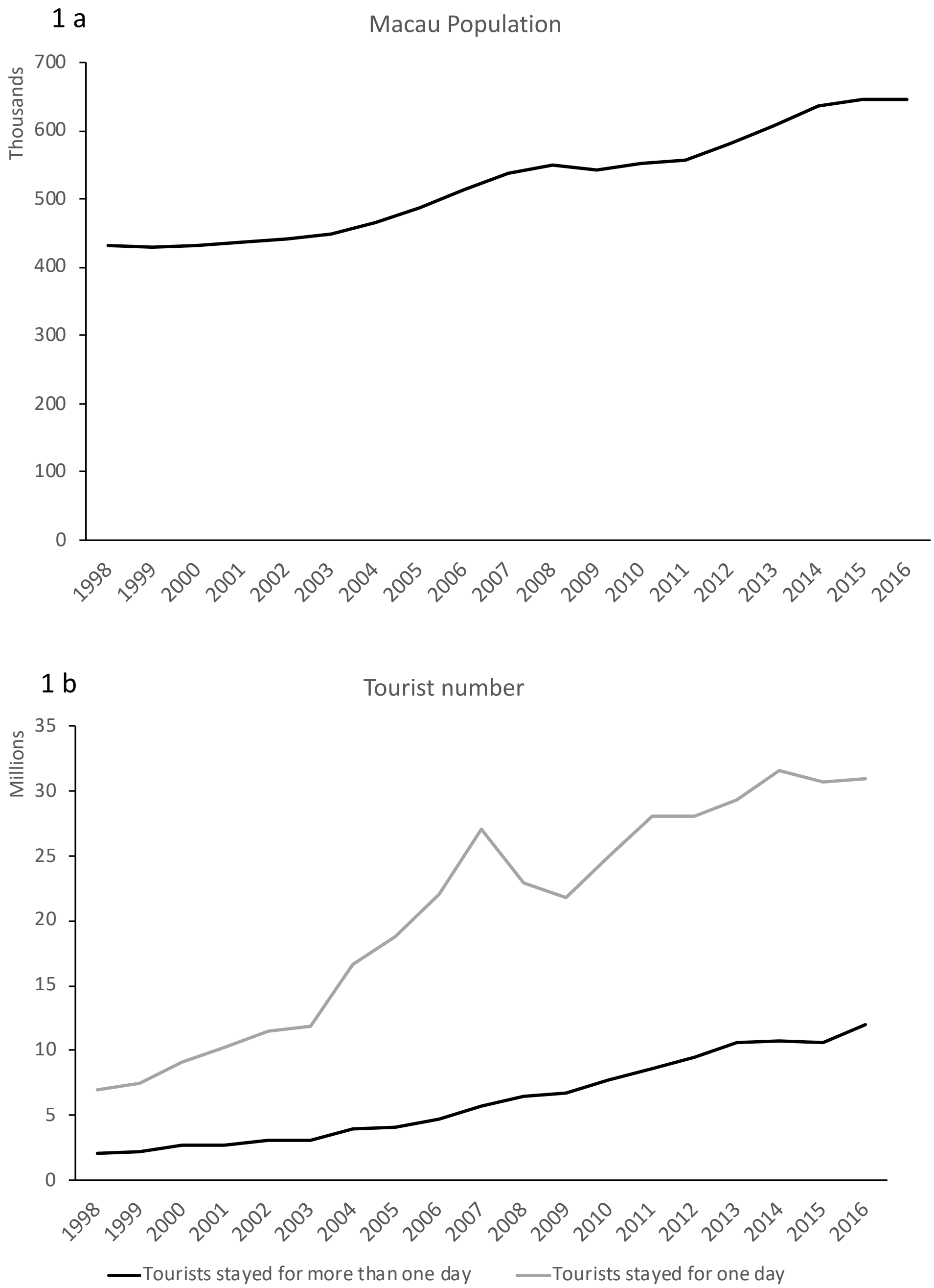

Fig. S.2.1. a) The Macau population grew steadily from 1998-2016 (Yearbook of Statistics, 1998-2016). **b)** The number of tourists visited Macau grew 5-fold from 1998-2016, and the tourists staying for one day were about 3-times greater than those staying two or more days. (Yearbook of Statistics, 1998-2016).

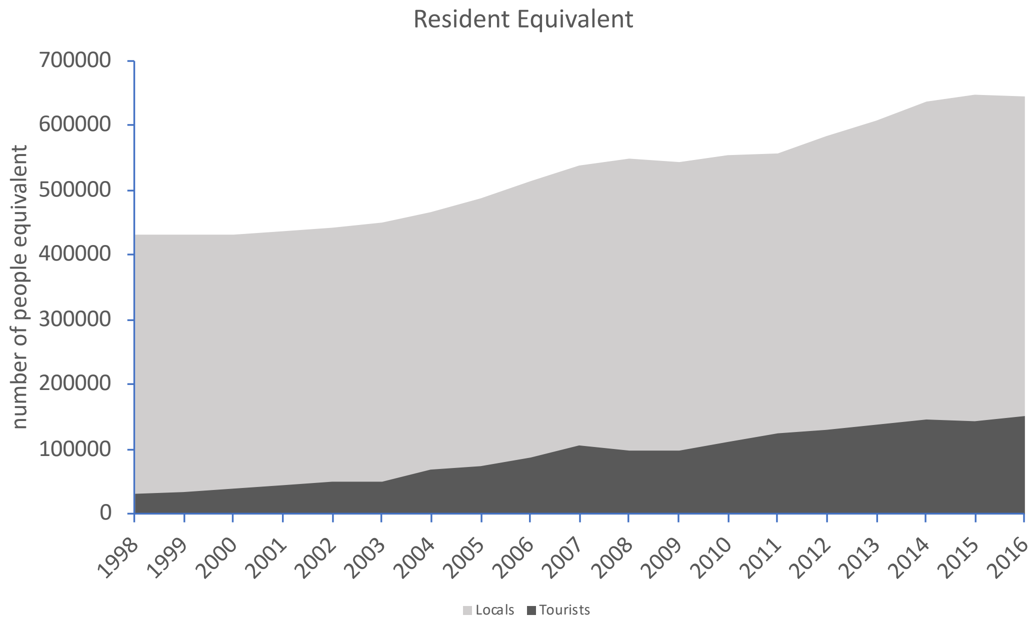

Fig. S.2.2. Macau population versus tourist population normalized by the days they stayed in Macau.

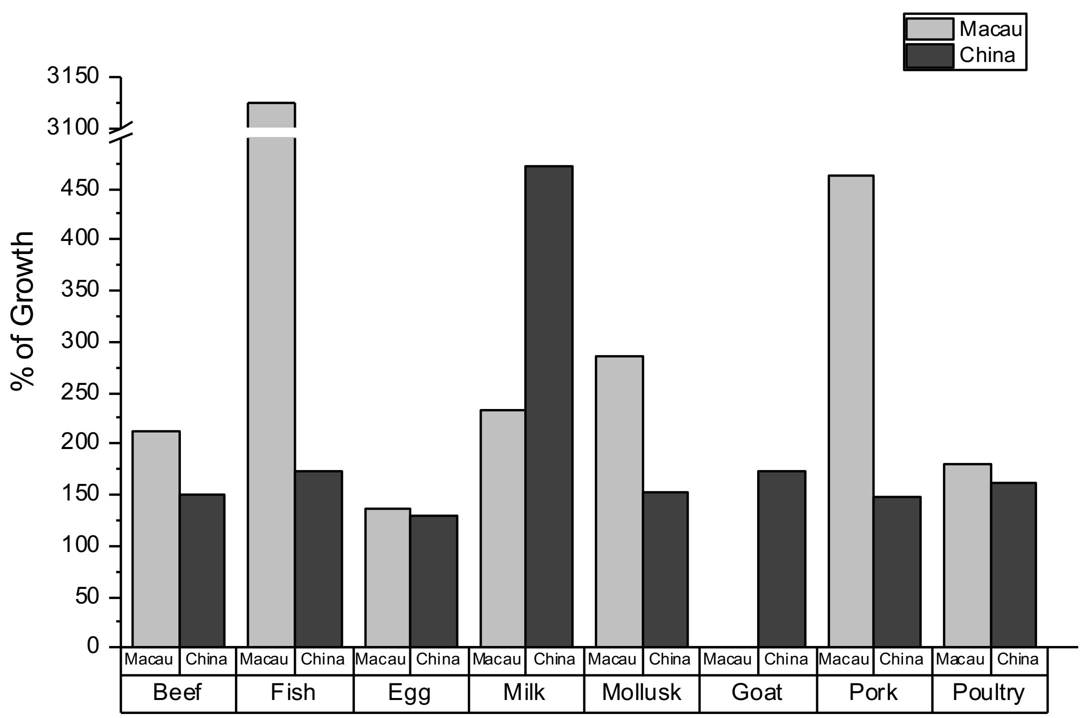

Fig. S.2.3. The percentage of growth of per capita animal-based product consumption of Macau and mainland China from 1998-2013 (FAOSTAT 1998-2013). Note that fish and pork have the highest growth of consumption in Macau, while milk had the highest growth of consumption in mainland China.

CHAPTER 3

PERFORMANCE OF BIOSOLIDS IN COMPARISON TO COMMERCIAL FERTILIZER FOR P-USE EFFICIENT GENETICALLY TRANSFORMED LETTUCE

Abstract

Measures for improving the management of phosphorus (P) include P recycling and enhancement of a crop's P-use efficiency (PUE, defined as biomass generated by per unit of P taken up). In this study, I compared plant yield and the PUE of lettuce growing with soil amendments of biosolids from three wastewater treatment plants in comparison to yield and PUE with commercial fertilizer. Furthermore, I simultaneously compared performance of *AVP1*-transformed lettuce (*Lactuca sativa* cv. Conquistador), which is genetically improved to enhance its PUE, to non-transformed (wildtype, WT) lettuce in greenhouse conditions with both types of fertilizers. Both lettuce varieties produced higher yields with commercial fertilizer than biosolids. However, *AVP1* lettuce produced similar yield as WT in biosolid treatments, although the *AVP1* lettuce had a higher PUE than WT lettuce. Although metals contents in the soil and the plants did not provide evidence of metal toxicity, the results of soil P fractionation support that the main cause for low yields with biosolids was having most of the P in plant unavailable forms, and this is consistent with the higher PUE.

Introduction

Phosphorus (P), an essential element for all life, is particularly important for food production due to its essential role in fertilizer. By 2050, global food production will need to double to meet increasing demands from increasing population and growing affluence (Godfray et al., 2010; Tilman et al., 2011), but this will put a strain on finite geological P supplies. Increased food production also will affect environmental quality, because P pollution is a major driver of water quality degradation in lakes, rivers, and coastal oceans (Metson et al., 2012; Tilman et al., 2002). Accelerating global P usage (Falkowski et al., 2000), coupled to inefficient management of P from field to fork to feces, results in surface-water pollution that drives eutrophication, hypoxia, and toxic microalgal blooms (Smith and Schindler, 2009; Landers, 2014).

To help slow the depletion of low-cost P reserves and to mitigate damage from P-derived water pollution, P-recovery technologies and improved P-management practices need to be developed and implemented (Kleinman et al., 2015; Li et al., 2015; Macintosh, et al., 2018; Rittmann et al., 2011). P recovered from livestock manure and human sewage can serve as an alternative to fertilizer based on mined P (Hao et al., 2013), while recovering P from these sources also intercepts the P before it becomes a water pollutant.

An important example of P recovery and reuse is the use of biosolids. The US Environmental Protection Agency (EPA) defines biosolids as “the nutrient-rich organic materials resulting from the treatment of sewage sludge” (Lu et al., 2012, EPA 2011).

Biosolids typically contain about 3% P by dry mass (Rittmann and McCarty, 2020; Artiola, 2011). In the USA, approximately 60% of biosolids are used for land application, and approximately 0.1% of agricultural land in the USA is treated with biosolids (National Research Council (NRC), 2002). However, in some regions, biosolids use is extensive. For example, in the state of Arizona (USA), biosolids have been used for land application since the 1960s (Artiola, 2011), and 22% of P applied to soil systems is in the form of biosolids in the Phoenix (Arizona) area (Metson et al., 2012).

Besides providing a source of P, application of biosolids also can improve soil physical properties, such as the soil's water retention capacity, organic matter, aggregation, bulk density, and total porosity (Aggelides and Landra, 2000; Beniston and Lal, 2012; Alvarez-Campos and Evanylo, 2019). Reflecting these impacts, biosolids have been shown to increase vegetable production in urban agriculture (Alvarez-Campos and Evanylo, 2019). Composted biosolids can also reduce the bioavailability of lead (Pb) in urban soils by increasing the strength of adsorption of Pb to soil particles. Reduction of Pb in contaminated soil in urban area is important since Pb is a common pollutant in urban soils (Farfel et al., 2005). However, applying biosolids also raises concerns about contamination by heavy metals and endocrine disruptors, which are common contaminants of urban wastewater (Shober and Sims, 2002; Elliott and O'Connor, 2007; Pepper et al., 2008).

Another strategy to improve the overall sustainability of P use is to enhance the ability of crop plants to access soil P and convert it to useful yield. To increase P-use efficiency (PUE), farmers and scientists have been working together to improve PUE in crop varieties via selective breeding or transgenic approaches. Among the genes that are related to P uptake, recycling, and transport in plants (Liang et al., 2014), an especially promising gene for improving PUE is *AVP1*, the gene that codes for type I *Arabidopsis* vacuolar pyrophosphatase. This gene, first isolated from *Arabidopsis* by Rea et al. (1992), is responsible for producing type I H^+-pyrophosphatase (H^+-PPase), which is a proton pump that produces chemical energy by forming pyrophosphate via a proton gradient (Gaxiola, et al., 2015). Increasing the expression of the *AVP1* gene leads to enhanced root growth (Yang et al. 2007), increased pyrophosphate-dependent sucrose metabolism and auxin transport in the root system, and more efficient scavenging of P in P-poor soil due to release of organic acids that reduce soil pH (Li et al., 2005; Pizzio et al., 2015; Gaxiola et al., 2015). Past studies have found that the *AVP1* gene increased crops' PUE by 13 – 82% (Yang et al., 2007; Paez-Valencia et al., 2013). However, no studies have yet examined the performance of an *AVP1*-transformed crop when fertilized with biosolids.

Here, I report the results of a study examining a combination of two strategies to improve P-sustainability—P recycling via biosolids and PUE improvement—to understand how such approaches might work in joint application. In greenhouse experiments, I applied biosolids or commercial fertilizer to *AVP1* genetically transformed lettuce and its wildtype (WT) counterpart using a factorial design. I measured plant yield, plant P and heavy metal concentrations, PUE, and soil P fractionation over 8 weeks. The results identify risks and benefits from using biosolids in conjunction with *AVP1*-transformed lettuce and, thus, illuminate potential complications when combining these two P-sustainability strategies.

Methods

I used biosolids from three wastewater treatment facilities in the Phoenix (Arizona) metropolitan area: the Northwest Water Reclamation Plant (NWWTP) and Greenfields Water Reclamation Plant (GWWTP) in the city of Mesa and the Tolleson Wastewater Treatment Facility in the city of Casa Grande (CGWTP). The biosolids from the two Mesa facilities came from anaerobic digestors, while the biosolid from Casa Grande came from an aerobic digestor. I obtained biosolid heavy metal concentrations from the records of the three WTPs for the month I sampled the biosolids for our experiment.

The soil used in the growth experiments was Mohall sandy loam collected from the University of Arizona Agricultural Center in Maricopa (Arizona). Mohall sandy loam is an aridisol with bulk density of 1.65 g/cc, pH 8.1, and low P content. Its water-holding capacity is 130 mm m^{-1}, and its composition is 16.4% clay, 72.5% sand, and 11.0% silt

(Lesch et al., 2005; Alexander et al., 1988). Romaine lettuce *Lactuca sativa* cv. Conquistador -- WT and *AVP1*-transformed -- was the test crop (seeds provided by R. Gaxiola, Arizona State University).

The experiment involved a factorial randomized block design. The factors were type of lettuce (WT and *AVP1*), type of fertilizer (commercial fertilizer and biosolids), and level of fertilizer addition (high and low). All factorial combinations of these treatments were blocked within the three biosolid sources, which necessarily differed in N:P ratios (among other factors) that were matched in formulating the commercial fertilizer within each block. The full factorial design involving two lettuce types, two fertilizer treatments, two nutrient levels, and three blocks based on the biosolids source. This gave 24 treatment combinations in total. Each combination was replicated eight times. I analyzed the results using 3-way Analysis of Variance (ANOVA) using IBM SPSS Statistics software (Mac version 26.0.0.1, 64-bit edition; IBM Corp., Armonk, N.Y., USA).

I oven-dried the biosolids within one month of receiving them from the wastewater treatment plants (WTPs) as preparation to measure their P content. I quantified P content (P as a percentage of dry weight) of the dried soils using concentrated $HCl+HNO_3$ (1:1) to extract all P (EPA, 1996; Church et al., 2017), followed by colorimetric analysis of P (APHA 1998). I selected 0.2 g P/cup as the high fertilizer level (HP) and 0.1 g P/cup as the low fertilizer level (LP), based on Nagata et al. (1992) and Paez-Valencia et al. (2013). I applied commercial fertilizers of 10-10-5 and 0-10-6 (the numbers represent the ratio of urea N-P_2O_5-K_2O) to match the N and P contents of each biosolid in the block.

The 10-10-5 fertilizer was Liquinox^{TM} All Purpose Liquid Fertilizer, and the 0-10-6 was Future Harvest^{TM} Ton O Bud Fertilizer. **Table 3.1** summarizes the N and P added in the form of commercial fertilizer or of the corresponding biosolids. The different treatments using biosolids are designated as: NWWTP LP or HP, GFWTP LP or HP, and CGWTP LP and HP; the corresponding treatments with commercial fertilizer are designated using the same format with Com added as: e.g., NWWTP Com LP and GFWTP Com HP.

I put 800 g of soil in a 10-cm diameter plastic cup with holes at the bottom for drainage. Biosolids were evenly mixed into the initial soil mixture. Six lettuce seeds were planted per cup. The first germinating seedling was selected, and others were removed. Growth experiments were conducted from October 2nd to November 23rd, 2017 in a temperature-controlled greenhouse (20^{o}C) located in Tempe, Arizona.

Lettuce was harvested at the 8-leaf stage. Fresh above-ground biomass in each cup was weighed immediately after the harvest, after which fresh below-ground biomass was cleaned, weighed, and dried. Above- and below-ground PUE were calculated as the above- and below-ground biomass divided by the total P in biomass (kg fresh biomass/g P in the biomass), respectively (Dobermann, 2007; Fixen et al., 2014).

After harvest, one soil sample was randomly selected from each treatment for P-fractionation analysis using the Hedley P-fractionation method (Hedley et al. 1982). I used Dowex 21K XLT anion-exchange resin for the dissolved inorganic P extraction (Gifford 2012). I measured metals in above-ground lettuce biomass using ICP-MS in the

W. M. Keck Foundation Laboratory for Environmental Biogeochemistry at Arizona State University. The heavy metals that I measured were copper (Cu), nickel (Ni), lead (Pb), chromium (Cr), cadmium (Cd), zinc (Zn), silver (Ag), arsenic (As), molybdenum (Mo), and selenium (Se).

Table 3.1. Amounts of P and N applied (g/cup) with biosolids or commercial fertilizer in each treatment. LP: low P; HP: high P; Com: commercial fertilizer applied at the same P dose as for the LP or HP application of biosolids. Biosolids and their characteristics were provided by Northwest Water Reclamation Plant (NWWTP), Greenfields Water Reclamation Plant (GWWTP), and the Casa Grande Wastewater Treatment Plant (CGWTP).

	P	N
NWWTP LP	0.1	0.44
NWWTP HP	0.2	0.88
NWWTP Com LP	0.1	0.44
NWWTP Com HP	0.2	0.88
GFWTP LP	0.1	0.21
GFWTP HP	0.2	0.43
GFWTP Com LP	0.1	0.21
GFWTP Com HP	0.2	0.43
CGWTF LP	0.1	0.16
CGWTF HP	0.2	0.32
CGWTF Com LP	0.1	0.16
CGWTP Com HP	0.2	0.32

Results and Discussion

Figure 3.1 and **Table S.3.1** shows that, overall, *AVP1*-transformed lettuce produced more biomass than WT lettuce (main effect of variety: $p < 0.03$). Both lettuce varieties had seven-fold higher yield in the commercial fertilizer treatments than the biosolids treatments (main effect of fertilizer type: $p < 0.001$). This major difference in yield between commercial fertilizer and biosolid treatments could have been caused by the nutrients in biosolids not being in plant-available forms. The high fertilization rate also produced more biomass than the low rate (main effect of fertilizer level: $p < 0.001$). The yield of *AVP1*-transformed lettuce did not differ from the yield of its WT counterpart in the biosolid treatments, although *AVP1*-lettuce had higher yield than WT-lettuce with the commercial fertilizer (Variety x fertilizer interaction: $p < 0.02$). No difference in yield between the two lettuce varieties for the biosolid treatments contradicts previous studies consistently showing that *AVP1*-transformed crops had improved yield in variety of conditions over WT (Yang et al., 2007; Paez-Valencia et al., 2013). However, yields for *AVP1*-transformed and WT crops have not been compared for biosolids before.

Above-ground biomass showed statistically significant two-way interactions between block (different biosolid sources) and type of fertilizer ($p < 0.001$) and between block and fertilization level ($p < 0.02$). That is, the effect of the type of fertilizer (biosolid vs commercial) and fertilizer level (high vs low) differed depending on the source of biosolid (the blocked factor). This effect of the blocking factor was likely driven by the biosolids from Mesa Northwest Water Reclamation Plant, which had a higher N:P ratio than the other two biosolids.

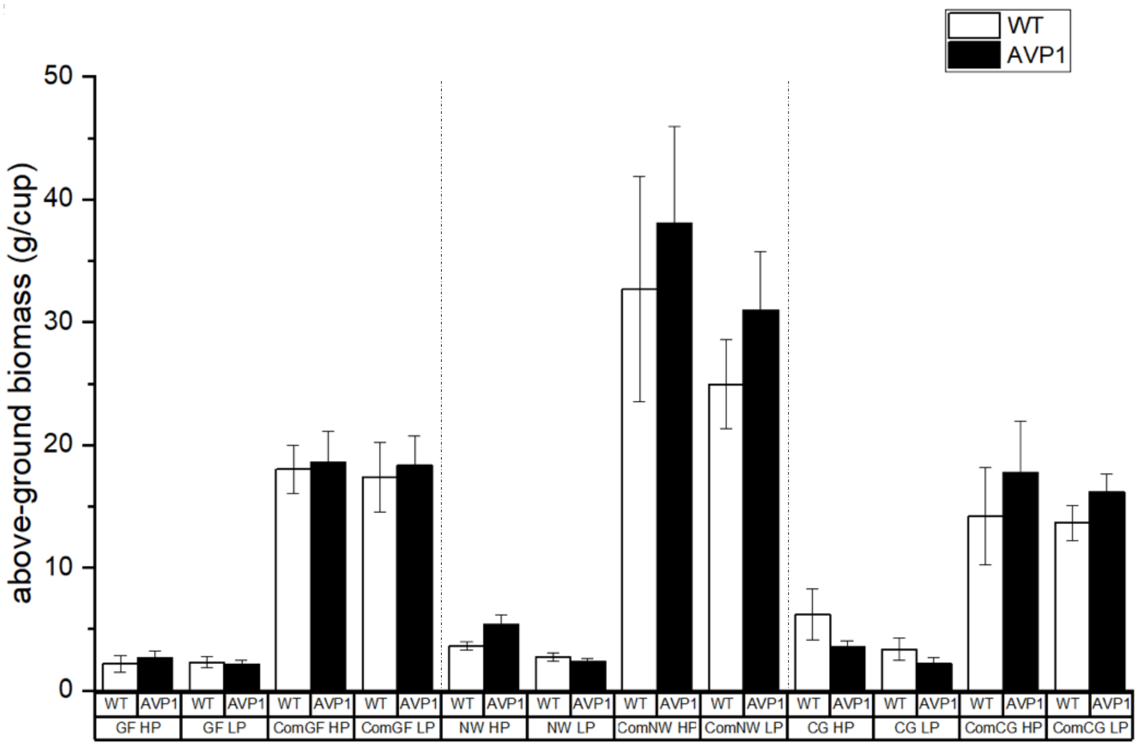

Figure 3.1. Above-ground biomass in different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. The error bars indicate 95% confidence interval. Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; and CG: Casa Grande Tolleson Wastewater Treatment Plant.

Figure 3.2 shows the PUE of the whole lettuce. PUE (kg fresh biomass/kg P in the biomass) was significantly higher in biosolid treatments than in commercial fertilizer treatments ($p < 0.001$). Higher PUE in biosolid treatments could imply that nutrients were less available for biosolids than for commercial fertilizers. Similarly, whole-lettuce PUE also was higher with a low fertilization rate than with a high fertilization rate ($p < 0.001$). The blocking factor (source of biosolids) was significant as well ($p < 0.001$):

The PUE for biosolids from the Northwest Water Reclamation Plant was different from the others.

The whole-lettuce PUE was higher in *AVP1* lettuce than in WT ($p < 0.001$); however, the whole-lettuce PUE did not differ between WT and *AVP1* lettuce in the commercial fertilizer treatment. In contrast, *AVP1*'s PUE overall was significantly higher than WT's PUE (not the case for NW) for biosolid treatments ($p < 0.001$), even though the yields of the two lettuces did not differ in the biosolid treatment (**Fig. 3.1**). Higher PUE in *AVP1*-transformed lettuce is consistent with previous studies showing that *AVP1*-transformed crops have better nutrient use efficiency (Paez-Valencia et al., 2013; Gaxiola et al., 2011). Nutrient level (high vs low) also affected PUE, with higher PUE in low nutrient treatment ($p < 0.001$).

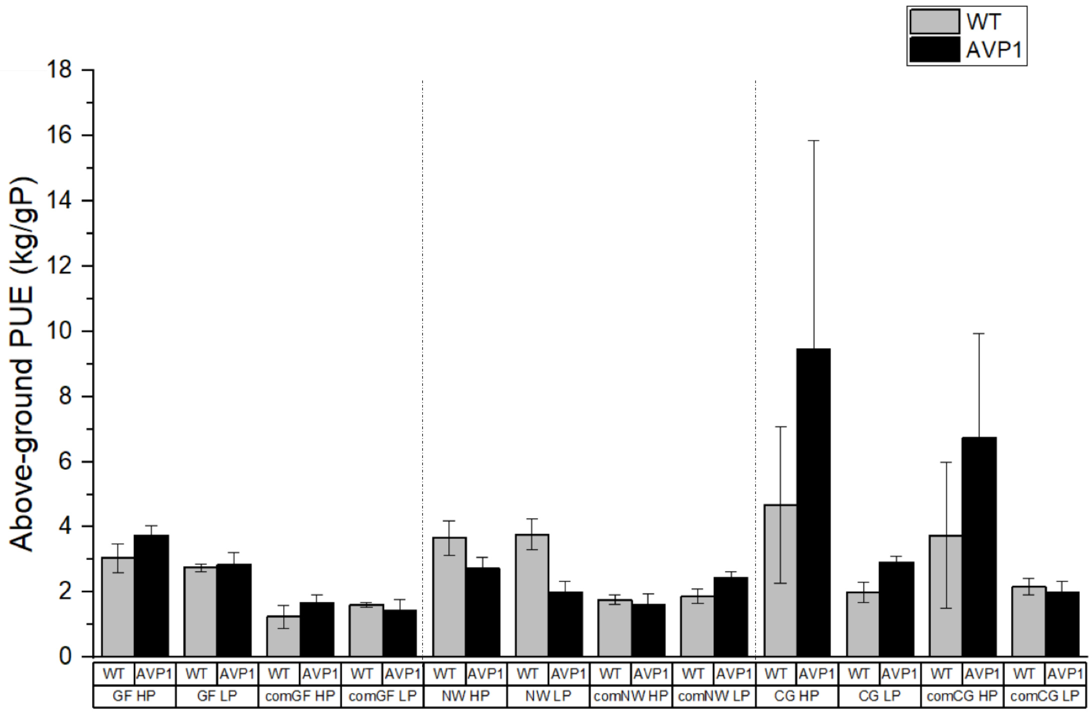

Figure 3.2. Above-ground PUE in different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. Above-ground PUE of the two lettuce types did not differ. Low fertilizer level caused higher above-ground PUE than high fertilizer level ($p < 0.001$, respectively). The two vertical dash lines separate the three sets of treatments (GF, NW, CG). The error bars indicate 95% confidence intervals. Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; CG: Casa Grande Tolleson Wastewater Treatment Plant.

The combination of biosolid use with *AVP1*-transformed lettuce produced the same yield as the combination of biosolid and WT lettuce. This lack of difference contradicts previous studies showing that *AVP1*-transformed crops improved yield in variety of conditions over WT (Yang et al., 2007; Paez-Valencia et al., 2013). It has been reported that *AVP1*-transformed crops more strongly acidify the soil in the root zone (Paez-Valencia et al., 2013; Yang et al., 2014). Root-zone soil acidification may release heavy metals bound in the biosolids; if more metals were taken up by *AVP1* lettuce, its yield may have been reduced.

Figure 3.3 shows that the concentration of resin-extractable P always was low and among the smallest fractions. This is significant because the resin-extractable pool reflects P directly available for plants, and it usually is one of the smallest P pools in soils (Hedley et al. 1982). In contrast, the largest P pool -- NaOH-extractable P – is among the least available. NaOH-extractable P is formed by chemisorption as iron phosphate and aluminum phosphate, and it usually is not directly available to plants (Hedley et al., 1982). Since Takamoto and Hashimoto (2014) reported that the NaOH-extractable P fraction using Hedley's (1982) method can be underestimated for biosolids, this highly unavailable P-pool may have been even larger for biosolids than shown in **Fig. 3.3**. This underestimation may explain why soil P was generally lower in the biosolid treatments than in commercial fertilizer treatments (**Fig. 3.3**). $NaHCO_3$ extracts the moderately labile P that is alkali-soluble in soils, and $NaHCO_3$-extractable P was roughly the same for all biosolids and commercial fertilizer.

The highest soil P levels were with commercial fertilizer, especially for ComNWWTHP treatments, which also had the highest resin-extractable inorganic P and $NaHCO_3$-extractable inorganic P. Higher soil P with commercial fertilizer appears to contradict its lower PUE, compared to biosolids treatments. This contradiction might have been caused by an underestimation of NaOH-extractable P in biosolid treatments (Takamoto and Hashimoto, 2014). It also may have been caused by more leaching of soluble P with biosolids; I could not do a P balance since I could not measure the P in leachate. I also did not digest the whole lettuce to get total P from biomass.

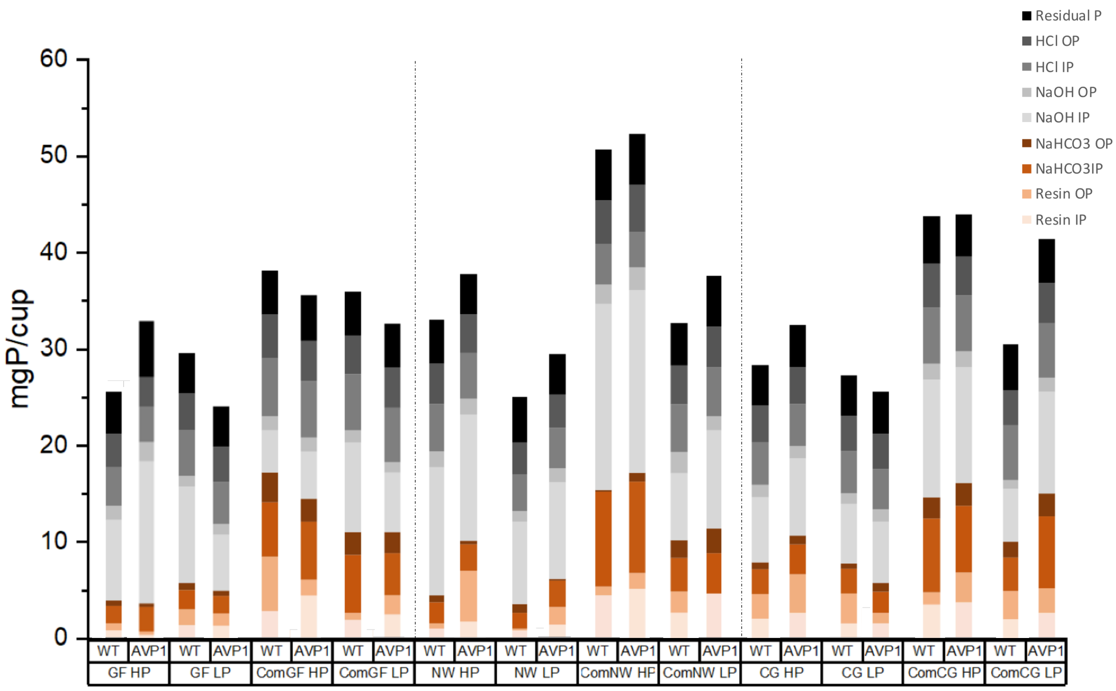

Figure 3.3. P fractionation results for different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. Different colors represent different P fractions in soil P pools; the brown and orange are the more-available pools, and with the grey/black set as the less-available pools. One replicate was randomly chosen in each treatment for the analysis. The largest two P pools were HCl- and NaOH-extractable pools. Resin- and $NaHCO_3$-extractable P pools in commercial fertilizer treatments were higher than the biosolid treatments. The two vertical dash lines separating the three sets of treatments (GF, NW, CG). Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; CG: Casa Grande Tolleson Wastewater Treatment Plant. Resin-ex IP & OP: Resin-extractable inorganic & organic P; NaHCO3-ex IP & OP: NaHCO3-extractable inorganic & organic P; NaOH-ex IP & OP: NaOH-extractable inorganic & organic P; HCl-ex IP & OP: HCl-extractable inorganic and organic P.

Table 3.2 summarizes heavy metal concentrations in the three biosolids. None of the concentrations reached ceiling concentration limits for land application (the maximum concentration limits for 10 heavy metals in biosolids when applied to land; USEPA, 1994). The lettuce's metal contents when grown on biosolids were sufficiently low that normal levels of lettuce consumption would imply metal intake levels well below the maximum permissible level for human consumption (Eissa and Negim, 2018; USEPA, 2019).

Most relevant to this study is if the metals in the soil could have inhibited plant growth. Moreno et al. (1997) showed that Cd and Zn from sewage sludge inhibited lettuce yield, since Cd and Zn have the highest bioavailability among the metals. **Table 3.2** shows that Zn and Cu had high soil concentrations with the two biosolids from anaerobic digestion, and **Table 3.3** shows that the Zn and Cu contents in lettuce leaves were the highest. However, they still were low in comparison to the inhibitory levels reported by Moreno et al. (1997): Zn 144 mg/kg and Cu 6 mg/kg. Thus, the most likely cause for lower yield in biosolid treatments than commercial fertilizer was having the nutrients in plant-unavailable forms, not the heavy metals in biosolids.

Table 3.2. Metal contents (mg/kg) in the three biosolids in comparison to ceiling concentration limits for land application (USEPA, 1994).

	Casa Grande	Greenfield	Northwest	Ceiling Concentration limits
Cu	3.4	300	430	4300
Ni	6.8	23	24	420
Pb	34	6.9	12	840
Cr	1.7	48	56	3000
Cd	1.4	2.3	2.2	85
Zn	6.8	760	730	7500
Ag	3.4	12	11	20
As	68	15	29	75
Mo	6.8	14	12	75
Se	68	23	22	100

Table 3.3. Metal concentrations in lettuce leaves (mg/kg dry weight). HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; CG: Casa Grande Tolleson Wastewater Treatment Plant.

mg/kg	GFWT HP	GFAVP HP	GFWT LP	GFAVP LP	NWWT HP	NWAVP HP	NWWT LP	NWAVP LP	CGWT HP	CGAVP HP	CGWT LP	CGAVP LP
Cu	0.45	0.35	0.50	0.51	3.3	0.72	1.00	0.72	0.28	0.29	0.66	1.12
Ni	0.043	0.012	0.021	0.064	0.263	0.079	0.085	0.066	0.021	0.026	0.079	0.086
Pb	0.014	0.01	0.01	0.019	0.164	0.006	0.055	0.017	0.005	0.097	0.015	0.061
Cr	0.028	0.019	0.03	0.049	0.392	0.026	0.142	0.042	0.021	0.025	0.048	0.122
Cd	0.096	0.085	0.154	0.103	0.048	0.082	0.057	0.142	0.055	0.055	0.13	0.087
Zn	2.1	1.5	2.0	2.9	3.51	3.01	1.65	4.15	1.75	1.7	3.2	2.53
Ag	0.0004	0.0001	0.0002	0.0003	0.006	0.0001	0.002	0.0004	0.0007	0.0001	0.0002	0.002
As	0.036	0.033	0.032	0.038	0.13	0.027	0.062	0.043	0.017	0.016	0.037	0.083
Mo	0.067	0.059	0.109	0.10	0.093	0.062	0.085	0.088	0.091	0.07	0.115	0.118
Se	0.014	0.009	0.016	0.03	0.065	0.012	0.024	0.022	0.01	0.011	0.018	0.029

Summary and Implications

This study evaluated the intersection of two potential components of a sustainable-P system: P recycling using biosolids and improved crop P use efficiency with *AVP1*-transformed lettuce. The combination of biosolid with *AVP1*-transformed lettuce produced the same yield as the combination of biosolid and WT lettuce, and all yields with biosolids were far lower than when using commercial fertilizer. However, lettuce in biosolids treatments had higher PUE than in commercial fertilizer treatments. The results of soil P fractionation support that the main cause for low yields with biosolids was having most of the P in plant unavailable forms, and this is consistent with the higher PUE.

Although the combination of biosolids and *AVP1* lettuce did not show higher yield than the combination of biosolids and WT lettuce in our experiments, our results need to be viewed in context. I tested only one type of genetically enhanced crop (*AVP1*-transformed lettuce) in combination with biosolids that were applied to an alkaline soil from Arizona (AZ). Other *AVP1*-transformed crops besides lettuce should be tested in the future with biosolids and with other types of recycled P fertilizers, such as food-waste compost and struvite fertilizer. Likewise, other genetic transformation strategies and different soil types should be evaluated.

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Supplementary Materials

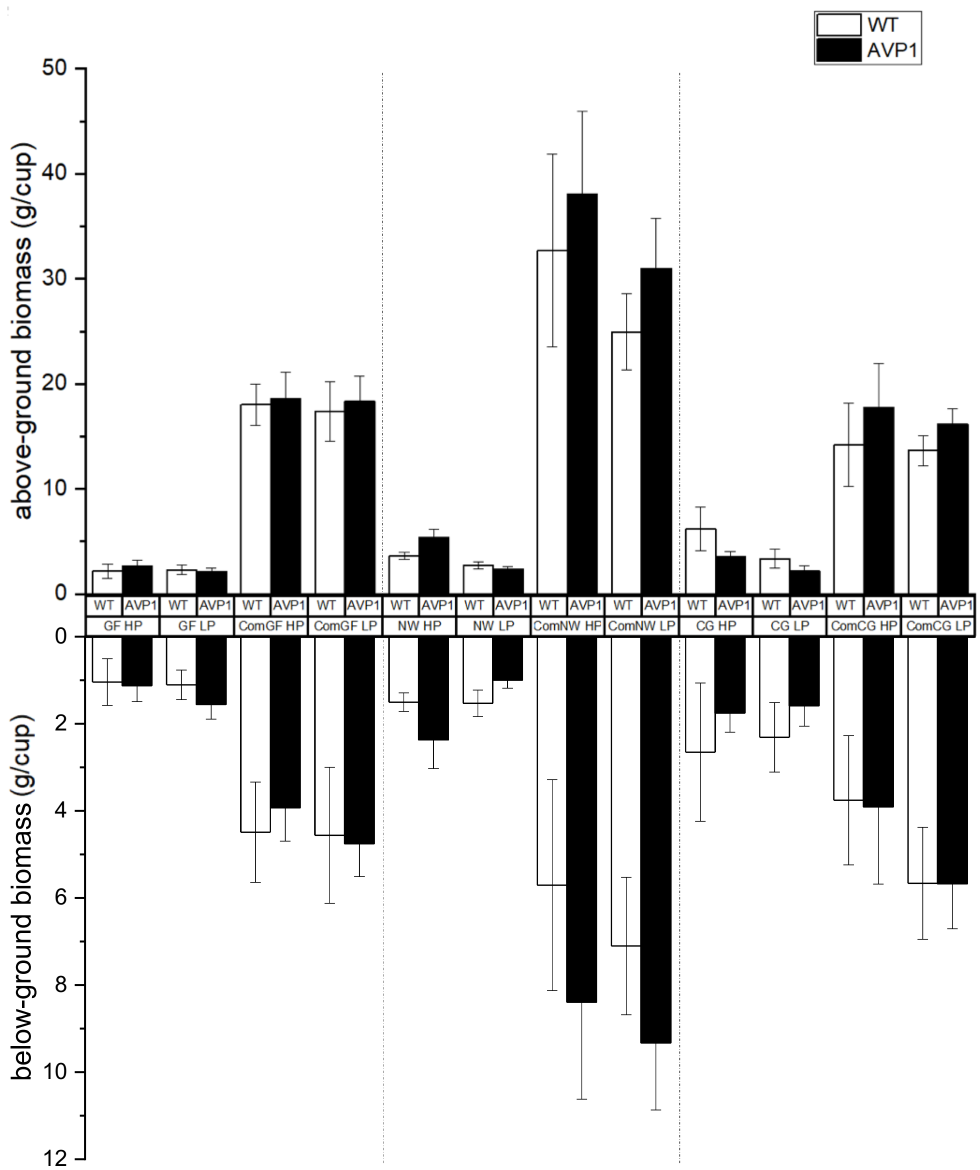

Figure S.3.1. Above-ground and below-ground biomass in different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. The error bars indicate 95% confidence intervals. The two vertical dash lines separating the three sets of treatments (GF, NW, CG). Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; and CG: Casa Grande Tolleson Wastewater Treatment Plant.

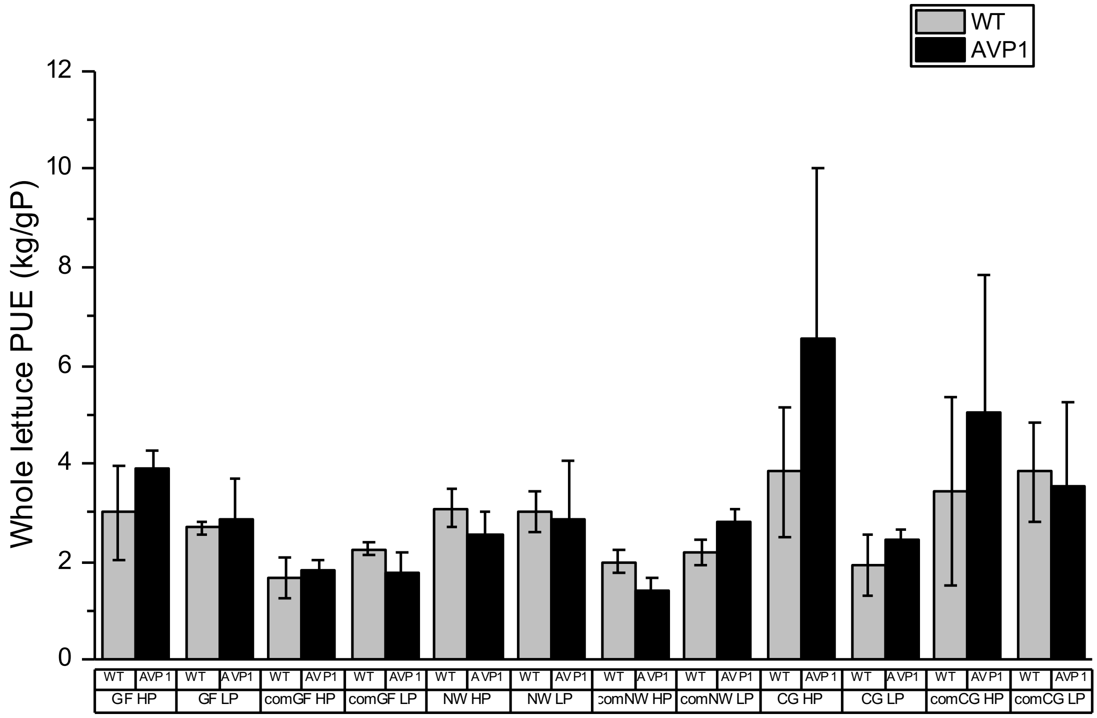

Figure S.3.2. Whole-lettuce PUE with different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. The error bars indicate 95% confidence intervals. Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; CG: Casa Grande Tolleson Wastewater Treatment Plant.

Figure S.3.2 shows the PUE of the whole lettuce. PUE was significantly higher in biosolid treatments than in commercial fertilizer treatments ($p < 0.001$). Higher PUE in biosolid treatments could imply that nutrients were less available for biosolids than for commercial fertilizers. Whole lettuce PUE was also higher under low fertilization rate than under high fertilization rate ($p < 0.001$). The blocking factor (source of biosolids) was significant as well ($p < 0.001$), in which the biosolid from Northwest Water Reclamation Plant was different from the others. Whole lettuce PUE was higher in *AVP1* lettuce than in WT ($p < 0.001$) however there was no difference in whole lettuce PUE between WT and *AVP1* lettuce in the commercial fertilizer treatment. However, under biosolid treatments, *AVP1*'s PUE was significantly higher than WT's ($p < 0.001$).

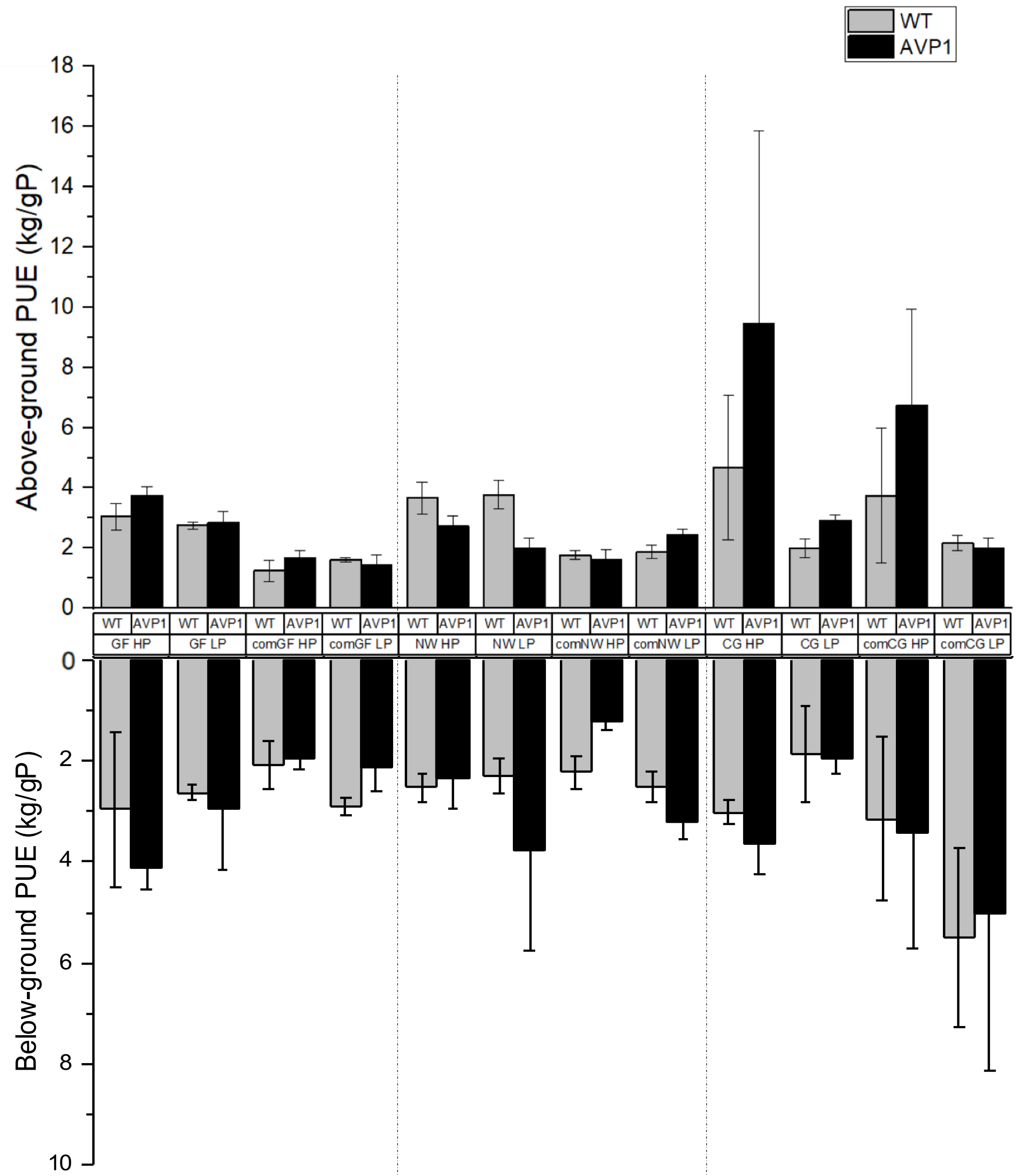

Figure S.3.3. Above- and below-ground PUE in different biosolids and commercial fertilizer treatments comparing WT and *AVP1* lettuce. Above-ground PUE of the two lettuce types did not differ, but below-ground PUE of *AVP1* was higher than WT in biosolid treatments ($p < 0.001$). Low fertilizer level caused higher above- and below-ground PUE than high fertilizer level ($p < 0.001$, respectively). The error bars indicate 95% confidence intervals. Com: commercial fertilizer; HP & LP: high and low P; GF: Mesa Greenfield Water Reclamation Plant; NW: Mesa Northwest Water Reclamation Plant; CG: Casa Grande Tolleson Wastewater Treatment Plant.

Below-ground PUE (**Fig. S.3.3**) of *AVP1* was higher than below-ground PUE of WT ($p < 0.001$). Below-ground PUE (**Fig. S.3.3**) of *AVP1* was higher than below-ground PUE of WT in biosolid treatments ($p < 0.001$) but there was no difference in commercial fertilizer treatments. Below-ground PUE in biosolid treatments was better than in commercial fertilizer treatments ($p < 0.001$), and it was also higher in low fertilization rate than high fertilization rate ($p < 0.001$). Blocking effect was significant ($p < 0.001$), in which the biosolid from Northwest Water Reclamation Plant was different from the others. In summary, these results show that lettuce above- and below-ground PUE were affected by lettuce type, fertilizer type, fertilizer level, and block, as well as by various higher-level interactions.

It is interesting that the blocking effect is different in above- and below-ground PUE. The above-ground PUE was highest with the lowest N:P (CG) block, while the below-ground PUE was highest with the highest N:P (NW) block. In biosolid treatments, the above-ground PUE had no blocking effect. The blocking effect only appeared in the commercial fertilizer treatments. While the below-ground PUE had blocking effect in both biosolid and commercial fertilizer treatments. In conclusion, low N:P commercial fertilizer increased the above-ground PUE, while high N:P in both biosolids and commercial fertilizer increased the below-ground PUE.

Table S.3.1. ANOVA table for above-ground biomass.

Source	Sum of Squares	df	Mean Square	F	Sigificance
Corrected Model	21059	23	916	48.1	0.000
Intercept	28817	1	28817	1515	0.000
Lettuce	91.7	1	91.7	4.8	0.03
Block	2432	2	1216	63.9	0.000
Fertilizer Type	15858	1	15858	834	0.000
Fertilizer Level	223	1	223	11.7	0.001
Lettuce x Block	75.5	2	37.7	2	0.141
Lettuce x Fertilizer Type	143	1	143	7.5	0.007
Lettuce x Fertilizer Level	0.507	1	0.507	0.027	0.871
Block x Fertilizer Type	2264	2	1132	59.5	0.000
Block x Fertilizer Level	157	2	78.7	4.1	0.018
Fertilizer Type x Fertilizer Level	28.6	1	28.6	1.5	0.222
Lettuce x Block x Fertilizer Type	51	2	25.4	1.3	0.266
Lettuce x Block x Fertilizer Level	1.6	2	0.822	0.043	0.958
Lettuce x Fertilizer Type x Fertilizer Level	0.579	1	0.579	0.03	0.862
Block x Fertilizer Type x Fertilizer Level	90.8	2	45.4	2.4	0.095
Lettuce x Block x Fertilizer type x Fertilizer Level	13.8	2	6.9	0.362	0.697
Error	3101	163	19		
Total	52593	187			
Corrected Model	24160	186			

Table S.3.2. ANOVA table for above-ground PUE.

Source	Sum of Squares	df	Mean Square	F	Sigificance
Corrected Model	814	23	35.4	5.2	0.000
Intercept	1667	1	1667	246	0.000
Lettuce	17.2	1	17.1	2.5	0.113
Fertilizer Type	75.3	1	75.3	11.1	0.001
Fertilizer Level	85.7	1	85.7	12.7	0.000
Block	128	2	64	9.5	0.000
Lettuce x Fertilizer Type	0.026	1	0.026	0.004	0.951
Lettuce x Fertilizer Level	22.6	1	22.6	3.3	0.069
Lettuce x Block	58.6	2	29.3	4.3	0.014
Fertilizer Type x Fertilizer Level	11	1	11	1.6	0.205
Block x Fertilizer Type	2.5	2	1.2	0.181	0.834
Block x Fertilizer Level	141	2	70.5	10.4	0.000
Lettuce x Fertilizer Type x Fertilizer Level	1.6	1	1.6	0.241	0.624
Lettuce x Block x Fertilizer Type	18.7	2	9.3	1.4	0.254
Lettuce x Block x Fertilizer Level	25.5	2	12.7	1.9	0.155
Block x Fertilizer Type x Fertilizer Level	1.3	2	0.671	0.099	0.906
Lettuce x Block x Fertilizer type x Fertilizer Level	1.3	2	0.635	0.094	0.91
Error	1414	209	6.8		
Total	4235	233			
Corrected Model	2228	232			

CHAPTER 4

SUITABILITY OF AN ALGAL BIOFUEL SPECIES, SCENEDESMUS DIMORPHUS, FOR PRODUCTION OF CONVENTIONAL AND GENETICALLY MODIFIED LETTUCE

Abstract

Nitrogen (N) and phosphorus (P) are important elements for global food production, but these nutrients cause pollution in water bodies without proper management. Furthermore, P is a finite resource with geologic reserves that are geographically restricted. Thus, sustainable use of P in agriculture has been the subject of much research over the past decade. This study examines two examples of potential sustainability measures to address nutrient challenges: improved crop varieties and use of recycled fertilizers. I tested the effectiveness of dried biomass of a freshwater alga (*Scenedesmus acutus*) as a fertilizer with conventional lettuce (*Lactuca sativa* cv. Conquistador; WT [wildtype]) and genetically modified lettuce of the same variety that was transformed for improved nutrient use efficiency (*AVP1*). In greenhouse conditions, I measured yield, soil nutrient content and losses, and soil P fractions after application of dried *S. acutus* biomass at different rates, with and without supplemental additions of conventional fertilizer. Yield was higher with commercial compared to algal fertilizer and *AVP1* lettuce consistently produced better yield than the conventional lettuce with both fertilizer types, although the effect was stronger with the algal treatments. Soil P was mostly sequestered in the pools of $NaHCO_3$-extractable organic P, NaOH-extractable organic P, and HCl-extractable P, which are

poorly available. While the algal fertilizer was ineffective in supporting short-term growth, the P was retained in the soils, which may improve soil fertility in the long term.

Introduction

Global food production will need to double by 2050 to provide food security for 9 billion people and to meet anticipated dietary changes (Godfray et al., 2010; Tilman et al., 2011). This expansion in food production will likely double the demand for fertilizers, particularly nitrogen (N) and phosphorus (P) (Odegard and van der Voet, 2014). N fertilizer is primarily produced from the energy-intensive Haber-Bosch process, while P fertilizer is now almost exclusively produced by mining phosphate rock. Fertilizer production from these processes generates large amounts of greenhouse gasses (Kool et al., 2012), the main cause of global climate change. Excessive N and P, mainly from agricultural runoff, cause eutrophication, toxic algal blooms, and oceanic dead zones (Smith and Schindler, 2009; Ferber, 2004). To reduce pollution and extend the life of finite supplies of rock phosphate, nutrient-recovery technologies (Li et al., 2015) and improved agricultural practices (Mikkelsen, 2011) are being developed. In this study, I evaluated examples of both approaches.

Microalgal biomass is a means of nutrient recovery from wastewater (Rittmann and McCarty, 2020; Brennan and Owende, 2010; Hughes et al., 2013) that also can be cultivated as so-called “third-generation” biofuels (Hughes et al., 2013), since microalgae can be induced to develop a high lipid content that can be converted to transportation fuel (Carriquiry et al., 2010). The lipophilic fraction of the microalgae also may contain valuable co-products (Brennan and Owende, 2010), such as polyunsaturated fatty acids (Hu et al., 2008) and β-carotene (García-González et al., 2005). The lipids and co-products are extracted, leaving behind a solid residue containing important elements such as N and P. In this situation, the post-extraction residue can be used as a nutrient-containing soil amendment (Brennan and Owende, 2010; Hughes et al., 2013). A good example of such a biofertilizer are N-fixing cyanobacteria, which have long been used in rice production (De, 1939; Venkataraman, 1981) and have been widely tested in other crops as well (Abdel-Raouf N, 2012; Ibraheem, 2007; Uysal et al., 2015).

The bioavailability of the nutrients in residual algal biomass is a key issue that has been considered in previous studies. Algae have been compared to urea and inorganic fertilizers for promoting growth of a variety of food crops. Some studies showed no difference from inorganic fertilizers (Dadhich et al., 1969; Mulbry et al., 2005), while others showed that inorganic fertilizers were better (Coppens et al., 2016; Wuang et al., 2016). Comparing biomass-based fertilizers, Øvsthus et al. (2015) found that algal fertilizer was the least effective because of its high C:N ratio and, consequently, lower net N-delivery potential. The physical status of the algal biomass also influences bioavailability. For example, applying 2-3 g of dry algae (*Chlorella vulgaris*) per kg of soil had a better effect on germination and growth of lettuce than did applying the same weight of fresh algae (Faheed and Fattah 2008).

Recycling nutrients via microalgal biomass might be enhanced if improved crop varieties were more efficient at obtaining nutrients from recycled sources. Sustained efforts involving conventional breeding as well as genetic modifications have been underway to improve crop nutrient-use efficiency (Veneklaas et al., 2012), the second strategy I study. An especially promising gene for improving nutrient use efficiency is the gene coding for the type I *Arabidopsis* Vacuolar Pyrophosphatase, or *AVP1*. This gene was first isolated from vacuoles in *Arabidopsis thaliana* cv. Columbia and is considered a *bona fide* vacuolar marker (Sarafian et al., 1992), although its enzyme had been isolated much earlier (Pfankuch 1936). Recent studies with gold-conjugated, H^+-PPase-specific antibodies and proteomic approaches showed that a H^+-PPase located in the plasma membrane was crucial in maintaining pyrophosphate homeostasis (Pizzio et al., 2015) and pyrophosphate-dependent sucrose metabolism and auxin transport in the root system (Gaxiola et al., 2012; J. Li et al., 2005; Pizzio et al., 2015). Over-expressing the *AVP1* gene resulted in enhanced root growth and crop yields (Yang et al., 2007), as well as higher N-use efficiency (Paez-Valencia et al., 2013). While *AVP1* crops have been tested for nutrient uptake with conventional fertilizers, their performance with algal-based fertilizers has not been assessed.

In this study, I evaluated the suitability of dried *Scenedesmus acutus* biomass as a fertilizer, in comparison to commercial fertilizer, for growing wild-type (WT) and AVP1-modified lettuce (*Lactuca sativa* cv. Conquistador). I tested the hypothesis that the yield of *AVP1* lettuce would be higher than WT lettuce with both fertilizer types because of *AVP1*'s ability to use assimilated nutrients more efficiently. I also tested the hypothesis that the lower availability of P in algal biomass, compared to commercial fertilizer, would be partially compensated by the greater ability of *AVP1* lettuce to mobilize and acquire P from soil.

Materials and Methods

Algae, Lettuce, and Soil

The green alga *Scendesmus acutus* was mass-cultured by the Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University (ASU) (http://www.azcati.com/) in standard BG-11 culture medium (Stanier et al., 1971) in 1.22 m x 14.6 m flat-panel photobioreactors. Algal biomass was harvested by centrifugation (without extracting lipids) after two weeks of cultivation, freeze-dried, and crushed by a mortar and pestle. Two strains of romaine lettuce (*Lactuca sativa* cv. Conquistador as wildtype (WT) or *AVP1*-transformed) were the test plants. Dr. Roberto Gaxiola in the School of Life Sciences at Arizona State University provided us with seeds of WT and *AVP1*-transformed lettuce. The potting soil was a mixture of Mohall sandy loam, sand, and vermiculite in 1:1:1 volume ratio with 800 g in each 4-inch (10cm) cup. The Mohall sandy loam came from University of Arizona Maricopa Agricultural Center. The soil is an Aridisol with bulk density of 1.65 g/cc, and its pH was 8.1. Its water holding capacity is 130 mm m^{-1} and soil texture is clay (16.4%), sand (72.5%), and silt (11.0%) (Lesch et al., 2005; Alexander et al., 1988).

Greenhouse Experiments

Table 4.1 summarizes the 20 treatments, which involved combinations of dried algae and commercial fertilizer based on the N addition. The treatments included a control with no additions, four algae-alone treatments (A2, A4, A6, A8, having added algal biomass equivalent to 0.2, 0.4, 0.6, or 0.8 g N per kg dry soil), four algae+P&K treatments (A2+S, A4+S, A6+S, A8+S, having the same algal N levels plus NaH_2PO_4 at 0.33 g P per cup), and one commercial-fertilizer treatment (Com, having 0.4 g N and 0.16 g P per kg dry soil). All algal treatments also were fertilized with KOH (390 mg K per cup) to match the K level in the commercial fertilizer treatment: The N-P-K nutrient proportion in the commercial fertilizer was 10-10-5 (Urea N, P_2O_5, K_2O). The algae+P&K treatments were enriched with inorganic P and K to ensure that all the lettuce would receive balanced nutrients close to or equivalent to the commercial fertilizer, as the algae biomass was high in N, with an N:P ratio of 12.8:1 by mass.

Table 4.1. The masses of dried algal biomass, NaH_2PO_4, and KOH added to each cup in the ten treatments: A2, A4, A6, A8 are algal treatments; A2+S, A4+S, A6+S, A8+S are algal treatments with augmented NaH_2PO_4; and Com: commercial fertilizer. Each cup contained 800 g of soil. All treatments (except the control) received 390 mg KOH to assure that potassium did not become limiting in the fertilized treatments.

Treatment	Algae (g)	N (mg)	P (mg)	K (mg)
Control	0	0	0	0
A2	1.56	200	15.6	390
A2+S	1.56	200	333	390
A4	3.13	400	31.3	390
A4+S	3.13	400	333	390
A6	4.69	600	46.9	390
A6+S	4.69	600	333	390
A8	6.25	800	62.5	390
A8+S	6.25	800	333	390
Com	0	400	333	390

Each treatment had 8 replicates, and I set up 160 cups: 2 (lettuce types) x 10 (soil treatments) x 8 (replicates). Growth experiments were conducted from February 4 to March 9, 2015 in a temperature-controlled greenhouse (20ºC) located in Tempe, AZ (USA).

Prior to seed planting, dried algal biomass was mixed into the soil manually using a small spade. Soil not receiving algal biomass was mixed in the same way. Then, the soil was placed in a 10-cm plastic drinking cup with holes at the bottom for drainage. Another 10-cm cup was placed under each cup to collect leachate. Six lettuce seeds were planted in each cup. I applied Nano-pure™ deionized water for irrigation. A control treatment consisting of eight cups without any lettuce was established for leachate comparison and followed for all irrigation schedules using only Nano-pure™ water. Commercial fertilizer and NaH_2PO_4 additions were split-added. Each cup received the same quantity of water. The first germinating seedling was selected, and other seeds were removed from the cup. The whole lettuce plant was harvested at the 8-leaf stage, which occurred after about five weeks. The above-ground and below-ground portions were separated, dried at 60ºC for 48 h and weighed. For the below-ground samples, I washed the roots with tap water in a mesh drainer that retained fine roots that broke off.

Leachate was collected in all treatments every 2 to 4 d throughout the experiment. Leachate from eight replicates in a single treatment was pooled together as one sample instead of analyzing each of them separately due to the limitations of time, labor, and chemical stocks.

Soil P Analyses

One soil sample was selected randomly from each treatment for P-fractionation analysis. Soil P concentrations in ten fractions were quantified before and after the experiment using the Hedley P fractionation method (Hedley et al. 1982). I used Dowex 21K XLT anion-exchange resin for dissolve inorganic P (DIP) extraction (Gifford 2012). DIP is considered to be the most biologically available P. $NaHCO_3$-extractable inorganic and organic P are forms that are sorbed onto soil surfaces and are moderately bioavailable. NaOH-extractable inorganic and organic P are held strongly by aluminum and iron components through chemisorption and are considered to be largely unavailable. NaOH-ultrasonification-extractable inorganic and organic P are the forms of P that are held at the internal surfaces of soil aggregates and are considered to be unavailable. Finally, HCl-extractable P mainly represents apatite-type minerals, which are not available. Soil ammonium-N and nitrate-N concentrations were also measured (Weaver et al., 1994), as they are soluble and mobile forms of N.

Statistical Analysis

Since our data were not normally distributed, I used Tukey's Honestly Significant Difference tests to analyze the results. I used RStudio Statistic software (Mac Version 1.2.5033) and IBM SPSS Statistics software (Mac version 26.0.0.1, 64-bit edition; IBM Corp., Armonk, N.Y., USA) for statistical analysis.

Results and Discussion

The biomass yields in **Figure 4.1** show that all treatments with dried algae gave far smaller yields than with commercial fertilizer. This trend was true for above-ground and below-ground biomass. For example, the largest above-ground yield for WT lettuce in algae treatment was 2.5 g/cup (A8), but this was only 13% of the 19 g/cup obtained for WT lettuce with commercial fertilizer. Algae treatments that received inorganic P and K amendments had significantly lower biomass than those without amendment (above-ground biomass $p < 0.0006$, below-ground $p < 0.004$).

AVP1 lettuce produced higher above-ground and below-ground biomass than WT lettuce ($p < 0.0001$, $p < 0.0001$), consistent with, but stronger than in previous studies of other plant species (Li et al., 2005; Yang et al., 2007). For example, our above-ground yield for commercial fertilizer was 27 g/cup for *AVP1*, 42% higher than for WT. The proportional effect was even larger for the algal fertilizer, especially when supplemental P was not added to the algae-fertilizer treatment. In particular for A8, the above-ground yield for *AVP1* lettuce was more than 500% higher than for WT. For comparison, Yang et al. (2007) showed that the root and shoot dry weights of *AVP1*-transformed tomato plants were 13% and 16% higher than WT tomato plants ($p < 0.01$) under unfertilized (control) conditions. Li et al. (2005) also reported that the *AVP1*-transformed *Arabidopsis* had a 50% increase in seed yield, more rosette leaves, greater leaf area, as well as more root dry weight than the WT counterpart.

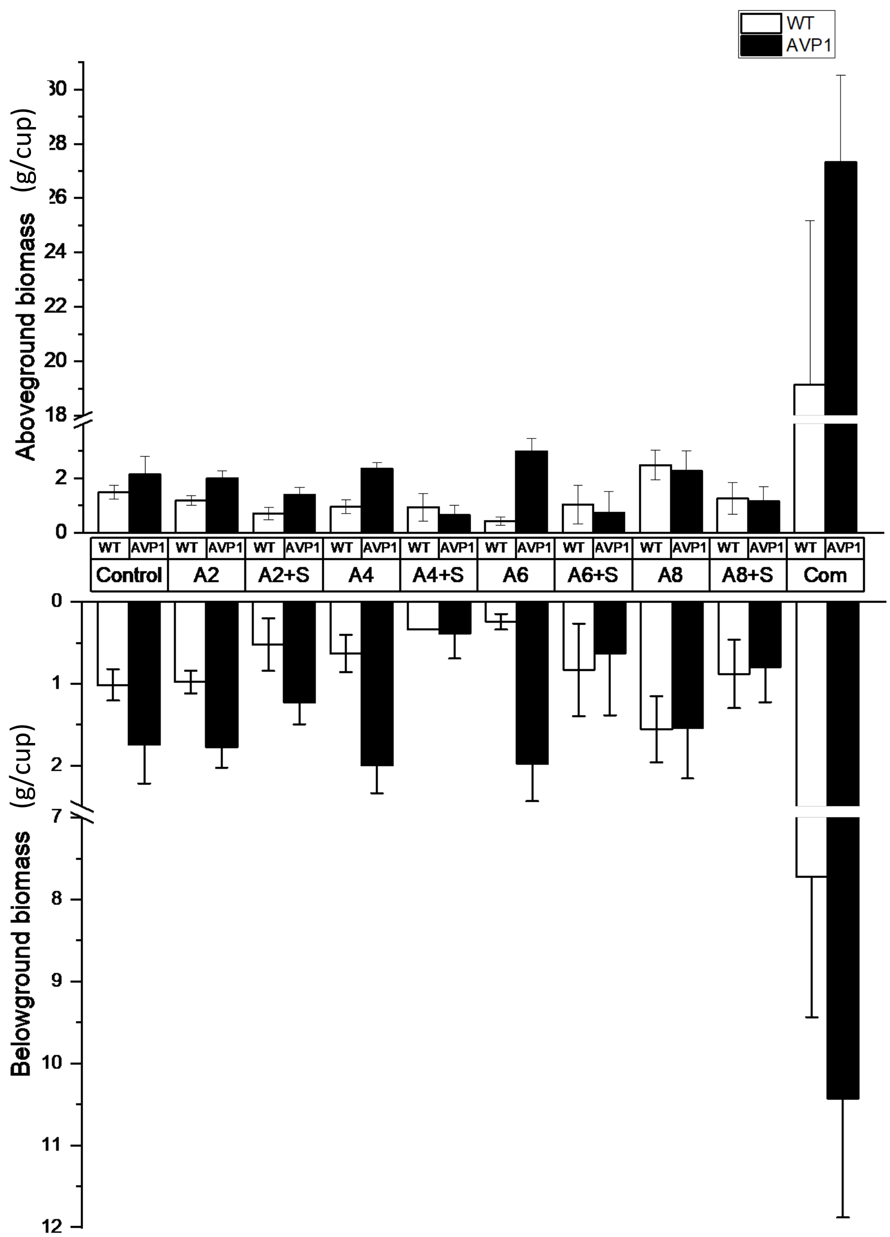

Figure 4.1. Above-ground and below-ground yields of WT and *AVP1* genetically modified *L. sativa* cv. Conquistador for all treatments. The error bars are 95% confidence intervals. See **Table 4.1** for details of the treatments.

Algae treatments with P and K amendments resulted in lower biomass yields than treatments without addition of P and K. I observed outer leaf marginal necrosis on lettuce leaves from the P and K amended algae biomass treatments. This probably was due to high nutrient and salt concentrations in the amendments. For example, added Na through NaH_2PO_4 in algae treatments were lower than 12 mg, but all the algae treatments with P and K amendments received 64 mg Na.

The low effectiveness of the algal fertilizer may have been caused by its P being unavailable for lettuce uptake. Soil P fractionation results in **Figure 4.2** show that the majority of P was in the pools of $NaHCO_3$-extractable organic P, NaOH-extractable organic P, and HCl-extractable P. These forms are generally considered to be poorly available for plants (Hedley et al., 1982; Bünemann et al., 2011; Brady and Weil, 2012). Among these three less-labile P pools, HCl-extractable P was the highest. This may have been an outcome of using KOH as the K supplement, which likely increased soil pH. Concentrations of resin-extractable P, the most available fractions, always were the lowest among all other available fractions. Total soil P was significantly higher in the algal treatments with nutrient amendments, mainly because plant growth and consequent P uptake were much lower with algae fertilizer compared to commercial fertilizer (**Fig. 4.1**).

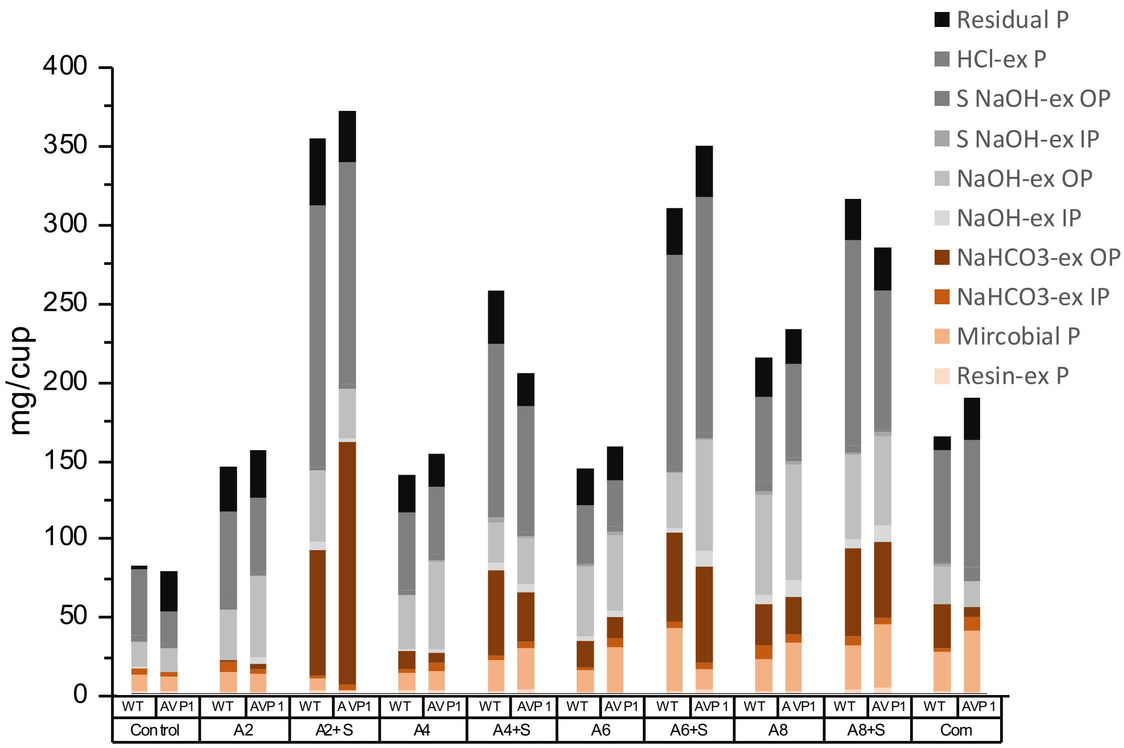

Figure 4.2. Soil P fractionation for all treatments. One sample was selected from each treatment for fractionation analysis. Different colors represent different P fractions in soil P pools; the brown and orange are the more-available pools, and with the grey/black set as the less-available pools. The Resin-ex P and S NaOH-ex Pi & Po were extremely low. Microbial P increased as the fertilization rate increased. The largest P pools were the $NaHCO_3$-ex, NaOH-ex, and HCl-ex pools. $NaHCO_3$-ex Po and HCl-ex P were higher in algae treatments with nutrient amendments than just the algae treatments. HCl-ex Pi: HCl-extractable inorganic P; S NaOH-ex Pi & Po: Sonication NaOH-extractable inorganic and organic P; NaOH-ex Pi & Po: NaOH-extractable inorganic and organic P; $NaHCO_3$-ex Pi & Po: $NaHCO_3$-extractable inorganic and organic P; Resin-ex P: Resin-extractable P.

Phosphorus that is not strongly complexed in soil leaches out over time. **Figure 4.3** shows that algal treatments with nutrient amendments leached much more P than treatments without fertilizer. Within the nutrient-amended algal treatments, soils supporting WT lettuce tended to lose more P than those supporting *AVP1* lettuce, possibly due to soil acidification by the *AVP1* lettuce that allowed *AVP1* lettuce take up more nutrients (Yang et al., 2007; Paez-Valencia et al., 2013).

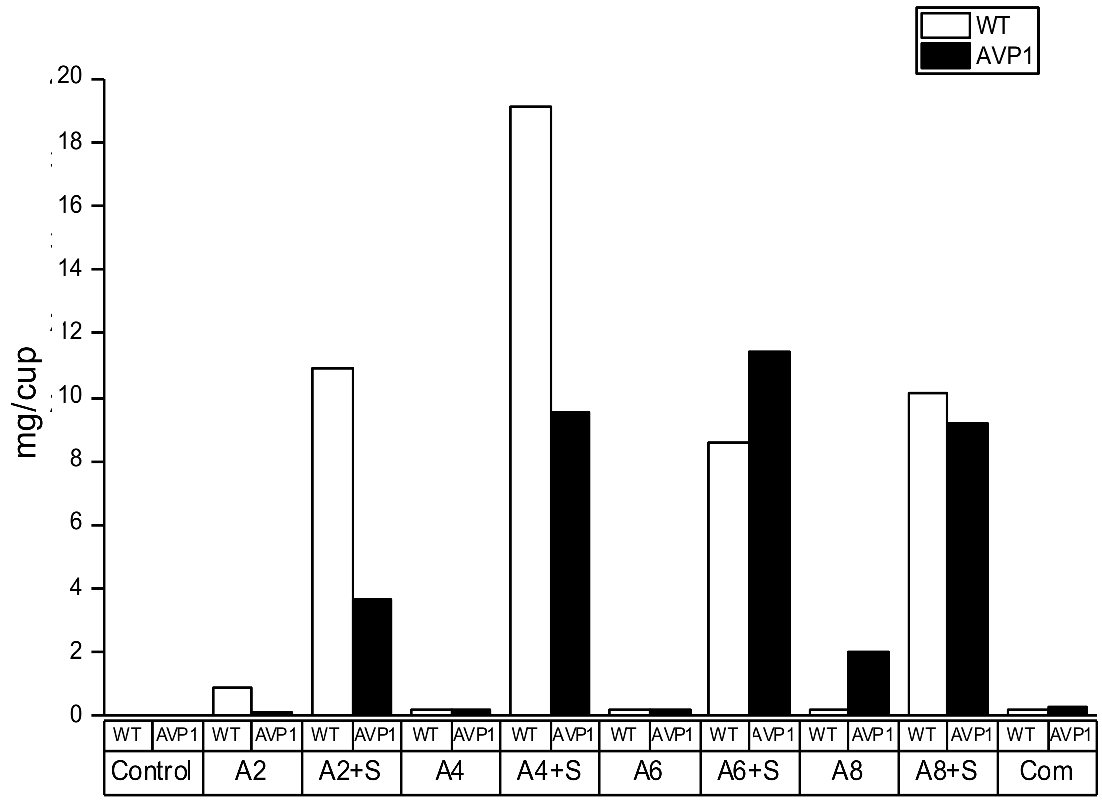

Figure 4.3. Total P in leachate over the growing season. Leachates in each treatment were pooled together and divided by the number of replicates.

Figure 4.4 shows total-P mass balance for the soil and leachate combined. Most of the P was in the soil, and leachate P was significant only for algal biomass plus added nutrients. Algal treatments with nutrient amendment also had highest P in soil and in leachate, since the lettuce did not grow well with algal biomass plus nutrients (**Fig. 4.1**).

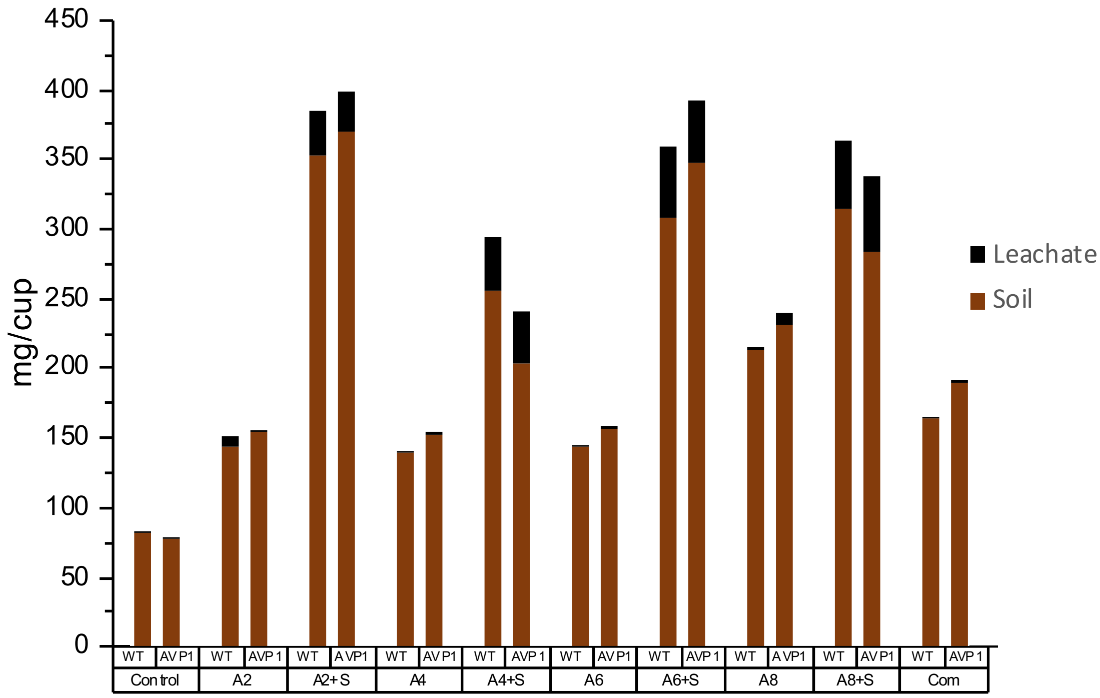

Figure 4.4. Total P in soil and leachate.

Figure 4.5, which presents the concentrations of N species for all treatments, makes it clear that all available N species were detectable in all treatments. Soil ammonium N and soil nitrate and nitrite N did not differ for *AVP1* versus WT ($P > 0.6$, $P>0.025$, respectively). Soil ammonium N increased with increased algal fertilization but remained low in the commercial fertilizer treatment. Elevated concentrations of ammonium inhibit primary root growth by causing cell death in the root meristem zone (Qin et al., 2011). The threshold of ammonium toxicity for plants is about 0.04 – 0.4 mg/L, depending on plant species (Britto and Kronzucker, 2002). The ammonium concentration in our results were much higher than this threshold. Thus, ammonium toxicity likely was a stressor that inhibited lettuce yield and its nutrient uptake for all treatments, but especially for those with algae and added nutrients (Court et al., 1964).

In contrast to ammonium, nitrate + nitrite concentrations were higher in the algae treatments than in the commercial fertilizer treatment. Soil nitrate + nitrite was particularly high in the A4 and A8 treatments and lowest in the control treatment. The commercial fertilizer treatment (Com) also had low soil nitrate + nitrite levels. These trends suggest that lettuce in commercial fertilizer treatments took up nitrogen effectively.

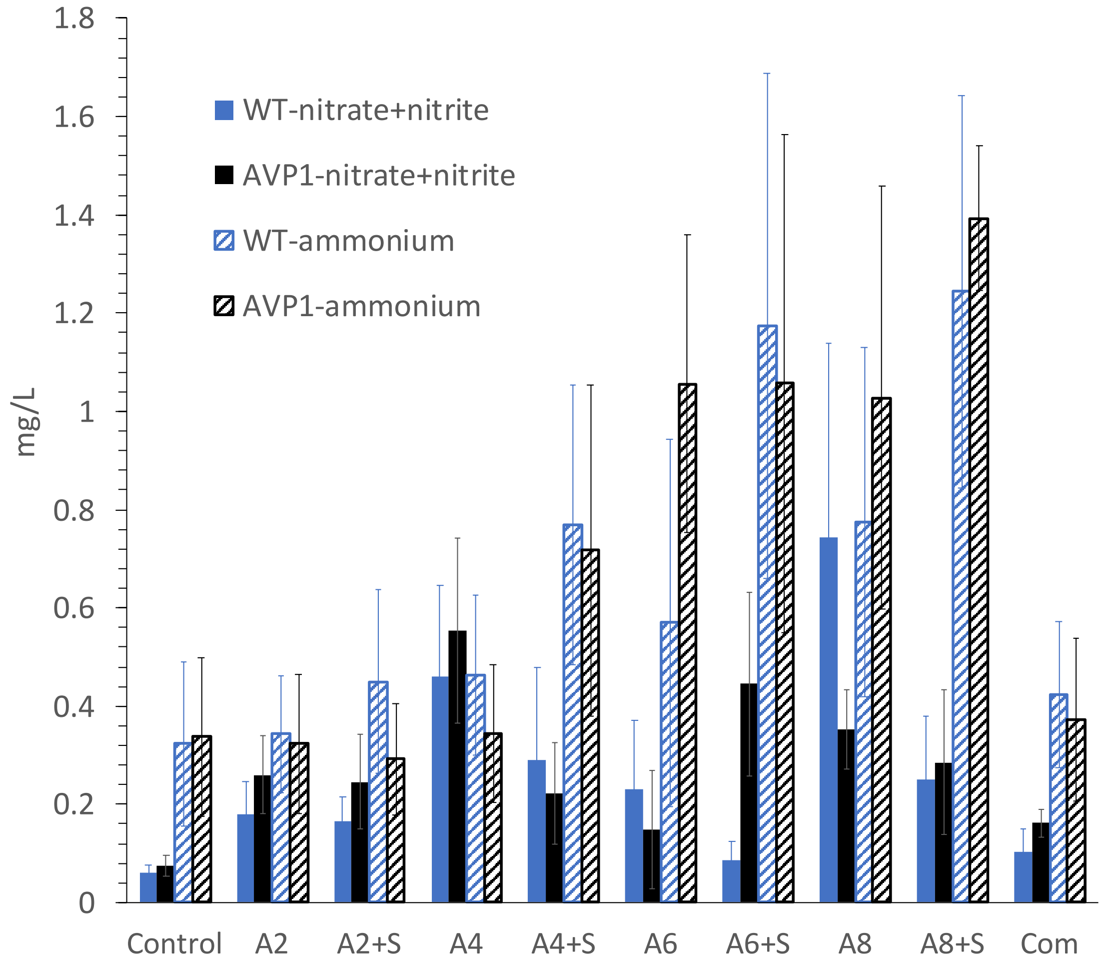

Figure 4.5. Soil concentrations of nitrate + nitrite and ammonium in various treatment combinations.

Although our results show that lettuce did not grow well with application of dried *S. acutus* in greenhouse experiments, the soils contained algae-derived nutrients that may become available to plants over the longer term, as suggested by Garcia-Gonzalez and Sommerfeld (2016). Thus, applying dried algae as fertilizer could benefit long-term soil nutrition, as well as improve soil organic content.

Conclusion

Commercial fertilizer was notably superior to dried algae in promoting yield of romaine lettuce, but *AVP1*-transformed lettuce had better yield than WT lettuce, particularly when supplemental P was not added in algae fertilizer treatments. The relatively poor results with algal treatments may have occurred because soil ammonium levels were inhibitory. While the algal fertilizer was ineffective in our short-term growth experiments, the P was retained in the soils, which may improve soil fertility in the long term. Future studies should investigate the impacts over multiple growing seasons.

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CHAPTER 5

SUMMARY AND FUTURE RESEARCH

While much P sustainability research has been focused on agriculture, research on P sustainability in cities deserves attention. My dissertation contributes new knowledge about the flows of P and the potential reuse of P flowing through cities. I first explored P flows in a unique urban ecosystem, Macau. I then tested the feasibility of P recycling via wastewater-treatment biosolids and microalgae to support the growth of a test crop (lettuce), including crop varieties engineered for greater P use efficiency (PUE). Thus, my dissertation encompasses P sustainability in cities and how it links to agriculture.

In Chapter 2, I used existing data to conduct a P-substance-flow analysis for Macau, a city with a unique economy build on tourism. The major P flows into Macau were from food, detergent, and sand. The major P flows out of Macau were through landfill burial and wastewater treatment effluent. The increase of tourism after the return of Macau to China increased certain P flows through Macau, especially through food and detergent. For example, the increase of detergent P import coincided with the expansion of hotel business and increases of hotel rooms in Macau. My P flow analysis for Macau suggests that P recovery from wastewater treatment could enhance Macau's overall P sustainability if the recovered P could be directed towards replacing mined P used to produce food. For example, P processed during wastewater treatment can be recovered with struvite and biosolids. Recovering P with struvite can offset a treatment plant's operation costs (Hao et al. 2013). Recovering P with biosolids in Macau might be sold as biofertilizer to China and Hong Kong or other countries in the region.

Among a variety of measures, the sustainable management of P includes recycling P from cities and enhancing P-use efficiency (PUE) in agriculture. In Chapter 3, I measured plant yield and PUE for lettuce grown with additions of biosolids from three wastewater treatment plants for comparison with commercial fertilizer. Furthermore, I measured their impacts on a lettuce (*Lactuca sativa* cv. Conquistador) variety that had been transformed to enhance its PUE (via manipulation of *AVP1* gene) in comparison to its wildtype (WT) counterpart. In greenhouse conditions, the yields for the two lettuce types with biosolid treatments were nearly the same for the two lettuce types and they were much lower than with commercial fertilizer. A likely explanation for the absence of the normally observed benefit for *AVP1* transformed lettuce is that *AVP1*-transformed plants acidified the root zone and mobilized heavy metals from biosolids, which reduced yield.

In Chapter 4, I tested the effectiveness of the dried biomass of a freshwater alga (*Scenedesmus dimorphus*) in stimulating the growth WT and *AVP1*-transformed lettuce. Similar to the findings in Chapter 3, yield was much higher with commercial fertilizer compared to algal solids. However, the *AVP1* lettuce consistently produced higher yield than the WT lettuce with both fertilizer types and also had lower losses of P and N by leaching. A high rate of algal fertilization may have inhibited lettuce yield due to ammonium toxicity. Overall, *AVP1* lettuce showed improved nutrient-use efficiency but the algal biomass was not an efficient fertilizer compared to commercial fertilizer.

Taken together Chapters 3 and 4 documented that commercial fertilizer was considerably superior to dried algae and wastewater biosolids in promoting the yield of lettuce. Thus, trying to combine a PUE strategy with P recycling did not work well in my experiments. However, my studies were short-term greenhouse trials. Perhaps organic fertilizers like algae and biosolids would be more beneficial as part of longer-term agricultural practices (Garcia-Gonzalez and Sommerfeld 2016; Pepper et al. 2007). This would be a good area for future research.

Future research also could focus on evaluating the benefit of pretreatment of algae and biosolids before applying them to soil. For example, algae can be grown in media that has N:P between 5:1 and 2:1 (molar), since these N:P ratios are similar to the N:P of fertilizer application in agriculture (Nagata et al. 1992). Making the algal N:P ratio fall into the usual range could also alleviate ammonium toxicity of the high N:P algae fertilizer that I used. Biosolids could be added to compost used as fertilizer, since the added organic matters could reduce the phytotoxicity of metals (Bolan et al. 2003).

Another possible area for future research is to assess the importance of the timing of application of the algae biomass fertilizer and biosolids. For example, Garcia-Gonzalez and Sommerfeld (2016) showed that applying *S. dimorphus* to the soil 22 days before planting had better results than planting seeds right after the algae application.

Future research on biosolids and *AVP1*-transformed crops also could explore how root-zone acidification mobilizes toxins from biosolids to inhibit the yield. Which toxin? Heavy metals or organic compounds? What happened to the *AVP1* crops physiologically that might be different from the WT crops?

Last but not least, future research could use life cycle assessment (Henrikke et al. 2004) to analyze the costs, benefits, and environmental impacts of a system that combines the components of P recycling and PUE-enhanced crops, the system tested in my thesis.

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