EVALUATION OF VIRAL FATE DURING ANAMMOX TREATMENT OF MUNICIPAL WASTEWATERS

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Abstract

Conventional secondary treatment of municipal wastewater designed to achieve biological nutrient removal typically utilize methods of nitrification-denitrification to convert ammonia to nitrogen gas to reduce the environmental impact of human wastewater streams. However, this process requires high energy inputs while also producing greenhouse gases. An alternative nitrogen treatment process is possible that treats nitrogen through anaerobic ammonia oxidation (Anammox) which has been reported to have numerous benefits over conventional treatment. While the effect of conventional secondary treatment has been evaluated for its effects on human enteric viruses, the effect of anammox systems on these microbes is undocumented. Through a joint venture between the University of Arizona, Pima County Wastewater and the Water and Energy Sustainability Technology (WEST) center, two different anammox reactors will be established and assessed for their effects on viral fate. Three human enteric viruses (adenovirus, enterovirus, and reovirus) will be monitored as well as evaluating possible indicator organisms to monitor human enteric virus in anammox systems.

Introduction

Municipal wastewater streams contain a wide variety of chemical and biological components; that if left untreated could have severe consequences on the surrounding environment. To better protect these valuable environmental resources, wastewater treatment plants have been designed to remove harmful chemical and biological contaminants from the water source before being discharged into the environment (Arillo et al. 1981). One of the chemicals of primary concern, due to its environmental and human health impacts, is nitrogen particularly as NO_3^--N. Due to the potential risks involved with high nitrogen discharge, wastewater treatment plants must ensure that wastewater effluents contain no more than 10 mg/L

of NO_3^- - N as per federal regulations (Cohen & Sonosky 1962). This requires effective nitrogen removal systems to not only meet the required standard, but for the continued protection of valuable water resources.

Biological nitrogen removal processes can be used to reduce nitrogen from domestic wastewater. These processes relies on the biologically driven nitrification-denitrification cycles to convert ammonia (NH_3) into nitrogen gas (N_2) through a series of intermediates and multiple reactors (Khin & Annachhatre 2004). The many systems utilize alternating aerobic and anaerobic chambers designed to promote nitrification and subsequently denitrification. During the first nitrification step, performed by the genera *Nitrosomonas* and *Nitrobacter* for example, ammonia is converted to nitrite (NO_2^-) and ultimately nitrate (NO_3^-) under aerobic conditions (Shrestha et al. 2001). Nitrate in solution is then subjected to anaerobic conditions for subsequent denitrification by bacteria such as *Pseudomonas denitrificans* (Parvanova-Mancheva & Beschkov 2009). This anaerobic process converts nitrate into inert nitrogen gas effectively removing nitrogen from the water.

While this process is effective for biological nutrient removal, there are three main disadvantages related to these processes. First, the nitrification step requires an oxygen content of 4.2 g to convert 1.0 g of ammonia nitrified (Khin & Annachhatre 2004). This leads to high energy costs to aerate the nitrification tank. Second, there is a large amount of greenhouse gases generated throughout the process. Aerobic treatment of wastewater results in approximately 2.4 kg CO_2/kg COD removed ,partially due to the cost of aeration (Keller & Hartley 2003). Likewise, the denitrification step produces two additional greenhouse gases, nitric oxide (NO) and nitrous oxide (N_2O). With up to 14.6% of the nitrogen being treated being converted to the nitrous oxide in full scale studies (Kampschreur et al. 2009). Finally, this process can only convert ammonia into nitrogen gas with a 75-80% efficiency (Ahn & Choi 2006).

A novel biological pathway has recently been discovered, termed anammox, for anaerobic oxidation of ammonia. This reaction is drastically different than traditional nitrification-denitrification reactions described previously in that it is a single step reaction that converts ammonium (NH_4^+) directly to nitrogen gas under anaerobic conditions (Strous & Jetten 1997). This eliminates many of the inefficiencies and environmental impacts that occur during conventional treatment. Additionally, the unique lifecycle of *Planctomycetes*, the primary bacteria involved in the reaction enables many innovative reactor types to be used to promote this reaction (Ahn 2006). Due to the novelty of this pathway, many questions remain unanswered regarding its optimization, effects on biological and other entities including viruses, and the overall practicality of this system for municipal wastewater streams (Lackner et al. 2014).

Anammox

The anammox reaction is carried out by a family of bacteria, the *Planctomycetes* which have a unique set of characteristics. These bacteria live in many marine environments but have also been isolated from many soils worldwide (Buckley et al. 2006). Marine *Planctomycetes* can be found in environments ranging from wastewater treatment plants (Sun et al. 2011) to the Black Sea and greatly contribute to nitrogen cycling in these environments (Kuypers et al. 2003). Factors that make these bacteria unusual are the reproductive, structural, and metabolic strategies that they employ for their survival.

Bacteria are often thought of as having relatively fast reproductive times due to a process called binary fission, where one cell replicates an exact double of the original. This type of replication is utilized by the common enteric bacteria typically associated with wastewater treatment such as *Escherichia coli* which has been shown to have a laboratory doubling time of around 15-20 minutes. However, *Planctomycetes* have doubling times ranging from 10-12 days

(Strous et al. 1998) and employ a reproduction strategy called budding, which is much more akin to fungal division. Budding occurs where an outgrowth of a new cell begins to form on the parent cell and continues to be attached until the daughter cell has been completely reproduced (Sagulenko et al. 2014). This makes this species one of only a few bacteria that reproduce in a manner other than binary fission.

Another defining characteristics of *Planctomycetes* is their membrane bound organelles and unique cell wall structures. Although now accepted to be a bacterial species, there was initial debate of *Planctomycetes* being of eukaryotic origin due to the membrane bound organelles, and apparent lack of peptidoglycan. However, as these issues have been reconciled and *Planctomycetes* are now correctly considered to be bacteria, but still offer an interesting insight into the evolution of life (Fuerst & Sagulenko 2012). Although this has been a source of confusion as to the eukaryotic or prokaryotic nature of the *Planctomycete*, it plays a deeper role in the metabolic capabilities of these bacteria. The anammoxosome is a membrane bound organelle within the bacteria that houses, as the name may suggest, the anammox reaction. This membrane is important in containing the anammox reaction due to the production of a highly oxidizing nitrogen species called hydrazine (Van Teeseling et al. 2013), as well as acting as an intracellular membrane in which to create a proton gradient (Van Der Star et al. 2010).

Planctomycetes obtain energy during the anammox reaction by utilizing nitrite as the primary electron acceptor under anaerobic conditions. This energy is then used to build the various structural properties of the bacteria. The anammox reaction is a redox reaction that converts the ammonia-species (NH_4^+) and nitrite (NO_2^-) into nitrogen gas (N_2) and water (H_2O). The stoichiometric equation proposed by (Jetten et al. 2001) for the anammox reaction under anaerobic conditions is:

$$NH_4^+ + NO_2^- = N_2 + 2H_2O \ (\Delta G° = -357 \text{ kj mol}^{-1})$$

This reaction is inhibited by elevated concentrations of oxygen (O_2), nitrite and nitrate and are typically outcompeted for substrate from competing microbes with faster reproduction rates therefore the proper conditions must be maintained to ensure that the *Planctomycetes* can carry out the anammox reaction optimally (Carvajal-Arroyo et al. 2013; Jin et al. 2012)

While ammonia-species (1000 mg/L) are abundant in municipal waste waters nitrite (46 μmoles/L) is significantly less abundant in the water stream (Table 3). Because of this it is necessary to generate nitrite through an aerobic partial nitrification step utilizing *Nitrosomonas* bacteria to generate nitrite (Van Dongen et al. 2001). Once both the substrate and terminal electron acceptor are present, *Planctomycetes* can reduce the substrate ammonium, using nitrite as a terminal electron acceptor. Not only does this unique pathway allow for the conversion of ammonium to nitrogen gas under anaerobic conditions, reducing the energy needed to maintain dissolved oxygen (DO) in the reactor, it also does not produce as many greenhouse gases as conventional systems (Keller & Hartley 2003).

To ensure that the anammox reaction can be carried out at an industrial scale it is necessary to optimize the reactors for two key traits; anammox reaction efficiency and *Planctomycetes* competitiveness when compared to other wastewater microbes. For the anammox reaction, this means carefully monitoring the dissolved oxygen (DO) in the reactor to prevent oxygen inhibition of the reaction. However, it also involves testing the influent water for consistently acceptable nitrite and nitrate levels. In high concentrations both nitrate and nitrite can inhibit the anammox reaction (Carvajal-Arroyo et al. 2013; Jin et al. 2012). This means that the ideal influent stream contains both low carbon and low nitrite levels, but has a high ammonium concentration (Ding et al. 2017). Phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}) also act as

inhibitors of the anammox process, and therefore must also be monitored carefully to ensure optimal efficiency (Carvajal-Arroyo et al. 2013; Jin et al. 2012).

The reactor must also provide conditions that promote *Planctomycetes* growth while suppressing other wastewater microbes. This can be achieved by utilizing a low-carbon high-nitrogen anaerobic environment (Sliekers et al. 2002). The anaerobic environment will prevent the growth of obligate aerobic microbes, whereas the low carbon environment will hinder the growth of any fast-growing heterotrophic bacteria. The limited carbon supply also helps to equalize the difference in carbon assimilation rate between fast growing aerobic bacteria, such as *Nitrosomonas* and *Nitrobacter,* with the slower *Planctomycetes*. By limiting carbon, the other wastewater microbes are unable to metabolize with a faster doubling rate, preventing the *Planctomycetes* from being outcompeted.

While utilizing wastewater streams that already provide these conditions is ideal, it is not always possible to have an optimal influent stream. This can be resolved by using a high ammonia low carbon influent, such as side stream wastes resulting from the dewatering of anaerobic biosolids, and then promoting the generation of ammonium. The conversion of ammonia to ammonium is dependent of the alkalinity of the water, promoting the speciation of one or the other. To promote nitrite, it is possible to use a partial nitrification to convert the abundant ammonia into nitrite using *Nitrosomonas*. Using *Nitrosomonas* to control the nitrite level is ideal because it limits the nitrite levels while using a high ammonium influent that will not inhibit the anammox reaction. Because nitrification is an aerobic process it requires low levels of oxygen to be present to oxidize ammonia into nitrite however, anammox requires anaerobic conditions due to the inhibitory effects of oxygen on the anammox process. This difference in metabolic requirements can be alleviated using a novel biofilm strategy between *Nitrosomonas* and *Planctomycetes*.

The Consortium

This biofilm strategy can be employed to create a microbial consortium between *Nitrosomonas* and *Planctomycetes* that will allow sufficient oxygen to be present for the conversion of ammonia to nitrite while preventing the inhibition of the anammox reaction. By allowing a biofilm of *Nitrosomonas* over the *Planctomycetes,* a redox gradient occurs where there is a microaerobic environment interacting with *Nitrosomonas* and an anaerobic environment around the inner *Planctomycetes* [Fig 1] (Sliekers et al. 2002). This strategy provides *Nitrosomonas* the oxygen substrate needed for the partial nitrification of ammonia to nitrite. The resulting nitrite can then be utilized by the *Planctomycetes* in conjunction with the ammonium to carry out the anammox reaction. Currently, no other wastewater nitrogen removal process utilizes this novel strategy of biofilm consortium in a redox gradient, and it is currently unknown how this process may affect the survival of other biological contaminants, including virus, in these water streams.

Reactor

The utilization of biofilm based anammox reactors has stark advantages over many conventional biological nutrient removal processes systems, biological nitrogen removal processes typically utilizes multiple reactors in a series with each tank being optimized for the growth of a specific bacteria and a specific metabolic process (Whitacre et al. 2008). This creates a large physical footprint for these reactors to house the different bacterial species for nitrogen removal. Additionally, the aerobic reactors system require high energy inputs to maintain an oxygen rich environment for the nitrification step (Khin and Annachhatre 2004). Both the footprint requirements and energy inputs are exacerbated for each additional chamber needed for nitrogen removal in the treatment train.

Anammox systems can bypass both issues by utilizing the biofilm consortium in a singular reactor. The biofilm creates a redox gradient that enables the *Planctomycetes* to survive in an aerobic environment. The anammox reactors can then be designed to utilize a micro-aerobic environment to encourage the partial denitrification by *Nitrosomonas* while enabling the anammox reaction to occur (Van Dongen et al. 2001; Sliekers et al. 2002). By doing so only a single reactor is needed to house the reaction and a significantly lower amount of energy is needed to aerate the reactor (Jetten et al. 2002).

Multiple types of anammox reactors can be employed using this biofilm strategy such as the expanded granular sludge bed reactor (EGSB) and moving the bed biofilm reactor (MBBR). The EGSB allows the *Planctomycetes* to be covered in *Nitrosomonas* to form a granular sludge (Chen et al. 2011). This sludge is then mixed through an influent up -flow allowing the sludge to grow into the headspace of the reactor while maintain minimal washout due to the unique design [Fig 2]. The EGSB method has been shown to be effective at growing large amounts of *Planctomycete* biomass in a granular form (Chen et al. 2011). A second type of reactor, MBBR, is a continuously stirred reactor that utilizes K-5 disks to encourage biofilm growth on the carrier substrate [Fig 3]. Constant mixing encourages high nutrient flow and potentially greater nitrogen removal (Kouba et al. 2016).

Virus

Municipal wastewaters contain a multitude of biological contaminants including human associated virus (Haramoto et al. 2018; Kitajima et al. 2014). Commonly studied viruses in wastewater streams are adenovirus, enterovirus, reovirus (genotype I, II, III) pepper mild mottle virus (PMMoV) and bacteriophages such as MS2.These viruses are studied due to their relevance in public health or as a potential indicator organism. Each of these viruses has different qualities

which may make them susceptible to various disinfection or removal methods [Table 1]. By understanding what methods of disinfection are effective will help researchers and regulators determine the best method of water treatment in addition to expanding basic scientific knowledge. The collaboration between the University of Arizona and Pima County Wastewater enables the evaluation of different types of anammox reactors.

Determining viral fate through an anammox EGSB and MBBR using the same wastewater stream enables the opportunity to directly compare both the reactor type and the process effects on viruses. Traditional secondary treatment methods utilize high biomass methods of treatment, and it has been found that a large portion of the viral particles are sequestered in the sludge (Kuo et al. 2010) .However, the limited biomass found in the continuous MBBR may play a role in viral fate, but it is also possible that the unique redox gradient between the *Nitrosomonas* and *Planctomycetes* will also play a role. Additionally, research is currently lacking that describes the effects of high concentrations of ammonia intermediates on viral fate in wastewater.

Being able and accurately monitor the amount of human associated virus in wastewater streams allows for better regulatory standards to be developed to reduce public health risks. This can be achieved through determining how different wastewater treatment strategies affect virus viability and what organisms can be used to accurately track the abundance of these viruses. Although many indicator organisms have been proposed, they currently do not correlate well with virus of human relevance in municipal wastewater (Hewitt et al. 2013; Liang et al. 2015). Some have proposed the use of PMMoV, (Rosario et al. 2009) adenovirus (Albinana-Gimenez et al. 2009), or bacteriophage (McMinn et al. 2017) to monitor these human pathogens.

Conventional treatment systems have been well studied for their effects on viral fate (Schmitz et al. 2016) but anammox systems have not, in part due to their novelty. It is unknown

as to what the effects the different anammox reactors (such as EGSB and MBBR) and the corresponding treatment parameters may have on the presence and viability of human enteric viruses. This research provides a unique opportunity to study the effects of anammox reactors on viruses using a municipal wastewater stream in a highly controlled setting. Establishing a viable indicator or human enteric viruses through the system also may enable regulators and industry experts to determine what process is optimal to treat the wastewater stream to reduce public risk.

Virus Regulations for Wastewater Treatment

Wastewater streams contain a multitude of biological contaminants that must be properly removed before the water is considered treated and allowed to be released into the environment (Arillo et al. 1981; U.S. EPA 1991). Traditionally these biological regulations have focused primarily on the abundance of bacteria in the water. States such as California and Texas are requiring additional care be taken to remove viral particles before the water can be utilized for certain processes such as the use of reclaimed water for potable reuse (California Department of Public Health 2014; U.S. EPA 1991; Virus 2016). Although this currently only applies to potable reuse waters, similar regulations may be instated in the future, expanding to recycled waters depending on the public health risk they might present.

In California and Texas there is currently a shift from non-regulation to regulation of acceptable virus levels in wastewater, and this is also true in Australia (Agriculture and Resource Management Council of Australia and New Zealand and Australian; New Zealand Council Environment and Conservation 1997). California and Texas require12-log virus removal reduction credit before that water can be used for direct potable reuse (Steinle-darling et al. 2016). Understanding what processes affect viral fate may help treatment plants meet or exceed

these regulations as new laws are implemented. Additionally, a more stringent treatment train before advanced water treatment may yield a higher effectiveness in advanced treatment.

At the state level, Arizona has stringent water quality standards but has yet to adopt treatment train specific virus removal regulations similar to those in California and Texas. However, in Arizona, reclaimed water is treated to different levels based on the intended use and its degree of potential public exposure, ranging from A+ to C. To achieve a rating of "A" or higher requires that there are "no detectable enteric virus in 4 of the last 7 monthly samples" in 40 liter samples (The Arizona Administrative Code, *Title 18.* 2016). As clean water becomes an increasingly more valuable resource, especially in the southwest, it appears likely that direct potable reuse and by extension the regulations, will become mandatory in water short locations. Addressing the issue early and ensuring that infrastructure is put into place to quickly adapt to any new regulations will be key in reducing the time required to provide quality water to residents.

The shift to a larger emphasis on removal of virus during water treatment indicates a need for the evaluation of how each component within the system affects the incidence, composition, viability, and removal of human viruses. Typically virus removal is only tracked throughout tertiary treatment where the primary purpose is biological removal, however other processes in wastewater treatment may play a role in viral abundance and survival (Sakaji 1992; Schmitz et al. 2016). Although the primary goal of secondary treatment is to remove chemical contaminants it has been shown that secondary treatment plays a role in viral fate (Katayama et al. 2008; Schmitz et al. 2016). For example, during secondary treatment, utilizing a Bardenpho system, a large portion of virus particles sorb to the activated sludge (Gerba & Smith 2005; Schmitz et al. 2016). This sludge is often dewatered and utilized for land application or otherwise repurposed. Switching from a high biomass reactor to a low biomass reactor may alter the amount of viral

sequestration in the sludge, changing the number of virus like particles (VLP) in subsequent treatment steps. Knowing how new technologies affect VLP's in the water may influence the use of different processes to be employed, depending on the regulations and desired goal of the treatment plant.

Tres Rios Case

Pima County Wastewater currently has two major plants designed for municipal wastewater treatment: Agua Nueva and Tres Rios wastewater treatment plants. Tres Rios is the larger of the two treatment plants, treating up to 50 mgd, and currently utilizes a 5-stage Bardenpho treatment train for nitrogen removal. This multistage treatment train is capable of treating up to 25 mgd of influent (Pima County Wastewater Reclimation 2016). The biosolids (treated sludge) produced from this system is then transported to anaerobic digesters for additional treatment. Water in the biosolids is then removed during a thickening and dewatering process using industrial centrifuges to separate the biosolids into liquid and solid fractions. This centrifugation results in 14% dewatered sludge, which is transported for land application. The liquid fraction, termed centrate due to the centrifugation step, becomes a high ammonia side-stream waste. This centrate is still high in ammonia (1000 mg/L) but is low in its carbon content. This centrate is then returned to the head of the plant for removal of the remaining nitrogen. This results in a high energy cost to not only return the water to the headworks, but to also re-treat the water for the high ammonia content. This in turn makes the centrate an ideal waste stream to be utilized for a side-stream anammox reactor. If this technology can be optimized, it can potentially to save Tres Rios $0.5 million a year in energy costs alone.

This has created a unique opportunity for the University of Arizona to partner with Pima County Wastewater to develop multiple mid-scale anammox reactors as a preliminary step to

full-scale reactors for use in Tres Rios. Obtaining large quantities of *Planctomycetes* has been a major difficulty in the industrial scale anammox reactors. However, Dr. Fields at the University of Arizona has been able to develop bench scale anammox reactors which will be used to seed mid-scale reactors at the WEST center. This joint venture at the WEST center will provide a powerful platform to research, discover and develop anammox technologies that may eventually be implemented at a full-scale treatment plant such as Tres Rios.

Analysis of Tres Rios Centrate

Municipal wastewater streams in Tucson may be variable based on a variety of factors such as the season, flow rate, or source of the influent. These variables may make the wastewater unsuitable for use in the anammox rector due to the strict environmental control that must be taken for the reaction. To address concerns about the quality of centrate; baseline chemical and biological analysis were performed over a three-month period. Chemical and physical analysis of the centrate including ion chromatography, ammonia concentration, total dissolved solids (TDS), total organic carbon (TOC), and pH were performed over this period. Biological analysis of the centrate included heterotrophic plate counts (HPC) (*Standard Methods* 9215 A, 2005) for bacteria and quantification of bacteriophage using double agar layover plaque assay (*Standard Method* 9224 A, 2005). The analysis was performed by the WEST Center, Pima County Wastewater, and Arizona Laboratory for Emerging Contaminants (ALEC) at the University of Arizona.

Centrate was sampled directly from the Tres Rios Wastewater treatment plant's centrifuges post Bardenpho treatment. The operational conditions of the centrifuges including centrifuge speed setting, sludge feed rate, dilution water, and the amount polymer added were all recorded at the time of sampling (Table 2). All samples were collected between July and October

between 12 pm-4 pm every Tuesday for the duration of the sampling period. The samples were then transported on ice and stored at 4C for no longer than 72 hours after collection. Samples that were to be sent ALEC for TOC and ion chromatography were processed within 4 hours using the protocols outlined by the laboratory. Samples that were to be sent to Pima County Wastewater for BOD, TDS and ammonia analysis were processed within 4 hours using the guidelines provided. The remaining physical and chemical analysis, turbidity and pH, were performed within 4 hours of collection at the WEST center.

Biological analysis of the centrate for HPC's and the abundance of culturable bacteriophage was performed at the WEST center. Heterotrophic plate counts of the centrate was performed using both R2A (Hardy Diagnostics. Santa Monica, CA) and Tryptic Soy Agar (TSA, Difco™ Tryptic Soy Agar. Le Pont de Claix, France) media, and incubated at 30 ^{o}C for 7-days. The abundance of bacteriophage was determined using three different host strains of *Escherichia coli* (*E. coli*). The bacteria used include a MS2 susceptible (*E. coli 15597*), F-specific susceptible (*E. coli 19853*), and phi X 174 susceptible (*E. coli 13706)* hosts. These hosts were selected based on their susceptibility to different types of bacteriophage to give a better indication of the abundance of virus in the centrate.

Results and Discussion

The water quality parameters gathered from the centrate can be split into three categories: required anammox chemicals; anammox inhibitors; and microbial composition. The analysis of the centrate showed that both the chemical and biological compositions of key parameters remained relatively constant throughout the course of the preliminary study [Table 3, 4]. This is likely in part due to the consistent and precise operational conditions utilized for centrate processing. Although operational conditions changed daily based on a variety of factors: such as

the speed of the centrifuges; amount of polymer added; and dilution water, it appears that these changes had little impact on the quality of the centrate. However, due to the complex nature of the treatment plant and the centrate processing there were occasional problems with the processes that required maintenance and drastically altered centrate processing parameters [Table 2].

The anammox system requires a high ammonia concentration, low organic carbon, and a stable slightly alkaline pH to promote *Planctomycetes* growth and anammox activity (Carvajal-Arroyo et al. 2013; Sliekers et al. 2002; Strous et al. 1999). The ammonia concentration remained stable throughout the course of sampling (n=13) with an average of concentration 1029 mg/L (s=49.1). The TOC concentration of the centrate (n=13) averaged 88,860 mg/L (s=58751), however this is skewed by the almost four-fold spike in TOC on October 3rd 2017, that could be due to a malfunctioning centrifuge [Table 2, 3]. These data indicate at a relatively stable C: N ratio. The pH of the centrate (n=13) averaged 7.7 (s=0.1) falling within required conditions for anammox reactions (Strous et al. 1999).

Ensuring a lack of inhibitory compounds within the centrate is vital to verifying its use in anammox reactors. Common inhibitory compounds included phosphate and sulfide, the latter of which can be generated from anaerobic sulfate reduction, which result in an IC_{50} (the concentration required to inhibit the reaction by 50%) at concentrations of 25.3 ± 5.9 mM and 0.03 ± 0.00 mM respectively (Carvajal-Arroyo et al. 2013). The concentration of phosphate (n=13) averaged 1595.7 μmoles/L (s=583.9) whereas the concentration of sulfate (n=13) averaged 267.1 μmoles/L (140.5).

The biological composition of the centrate was examined including the abundance of bacteria and bacteriophage. HPC's were performed using a minimal media (R2A) and an enrichment media (TSA) with (n=13). HPC's average 4.3 x 10^6 CFU/ml and 4.1 x 10^6 CFU/ml

respectively. The BOD of the centrate (n=13) averaged 55.2 mg/L (s=22.11). The (n=13) average plaque forming units found on double agar layover plaque assays using PhiX, MS2 and fr bacteriophage susceptible hosts were 246.5 PFU/ml, 137.1 PFU/ml and 114.4 PFU/ml respectively [Table 4].

Most of the key parameters including pH, ammonia, nitrite, nitrate, TOC, HPC, and bacteriophage remained relatively stable throughout the course of sampling. Large fluctuations of these parameters were rare, but these discrepancies coincided with operational issues with the centrifuges [Table 2], which may have contributed to these spikes. This indicates that although the water quality remains relatively stable long term, it may be necessary to implement a holding reservoir to mitigate the effects of sudden, and often unexpected, changes in operational conditions.

Sulfate appears to be the main inhibitory chemical of concern because the maximum concentration achieved was 690.3 μmole/L, substantially higher than the minimum IC_{50} reported due to sulfide (Carvajal-Arroyo et al. 2013). However, this would also require the reduction of sulfate into sulfide through anaerobic processes. Therefore, care should be taken to prevent or control the production of available sulfide to prevent the inhibition of the anammox reaction.

If appropriate measures are taken to address the concern of sulfide inhibition as well as create a buffer reservoir to mitigate the risk of equipment failure during centrate processing, then the centrate from Tres Rios provides an ideal influent for anammox reactors. Additionally, the presence of bacteriophage in the centrate indicates that viruses can survive the treatment processes up to and including centrifugation. This suggests that human enteric viruses may also be present in the centrate and that their fate during anammox treatment can be tracked and assessed.

Future efforts

After determining the quality of the feed waters, the reactors will be established at the WEST center through collaboration between Pima County and the University of Arizona. Two different 30-gallon reactors will be built to evaluate the anammox system, an EGSB and a MBBR. Upon completion of the reactors, the influence of the different reactor types on viral fate can be determined.

Each reactor will be fed using centrate from Tres Rios Wastewater Treatment Plant and the centrate will be analyzed for quantity and viability of five common wastewater virus pre-anammox and post-anammox treatment. These viruses include adenovirus, enterovirus, reovirus (G1, G2, G3), PMMoV, and MS2 bacteriophage. The first three viruses (adenovirus, enterovirus, and reovirus) were chosen based on their relevance in human health. The latter two viruses (PMMoV and MS2) will be used to determine their potential use as indicators of virus of human importance through anammox systems.

Quantification of these viruses will be performed using quantitative polymerase chain reaction (qPCR). The viability of any adenovirus, enterovirus and reovirus will be determined using cell culture assays followed by qPCR, to assess the infectivity of the viruses and verify the identity of the infectious viruses respectively. MS2 bacteriophage viability will be assessed using double agar layover plaque assays using MS2 susceptible strains of *E. coli*. Through these measurements of both abundance and viability of viruses in influent and effluent, over the course of multiple months, it will be possible to evaluate the effects of the two different anammox systems on viral fate in wastewater.

Figures

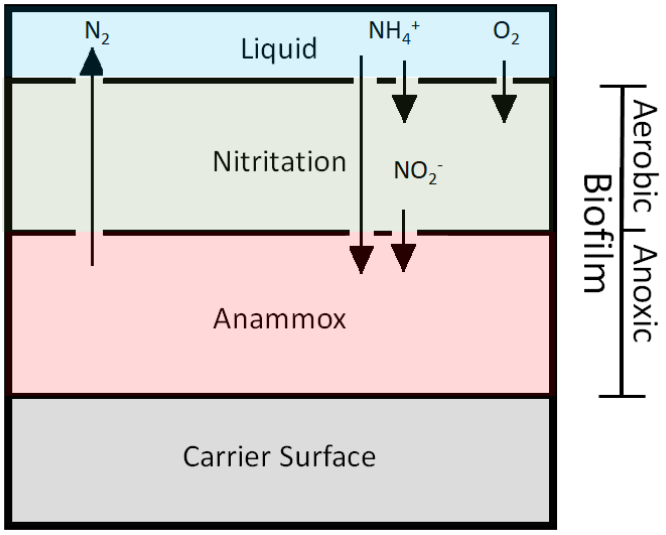

Fig.1

Biofilm strategy to enable anammox reactions in micro-aerobic environments. Two biofilms with different metabolic processes are formed, nitritation and anammox, creating aerobic and anoxic zones in the redox gradient. The liquid contains low quantities of oxygen (O_2) with a high concentration of ammonium (NH_4^+). Both the oxygen and ammonium are utilized by nitrifying bacterial biofilm, such as *Nitrosomonas*, to perform a partial nitrification resulting in nitrite (NO_2^-) production consuming the oxygen in the process. Nitrite and ammonium are then passed into the anoxic biofilm layer and utilized for the anammox reaction to generate nitrogen gas. This strategy prevents the inhibition of the anammox reaction by oxygen while simultaneously providing the required nitrite to drive the process.

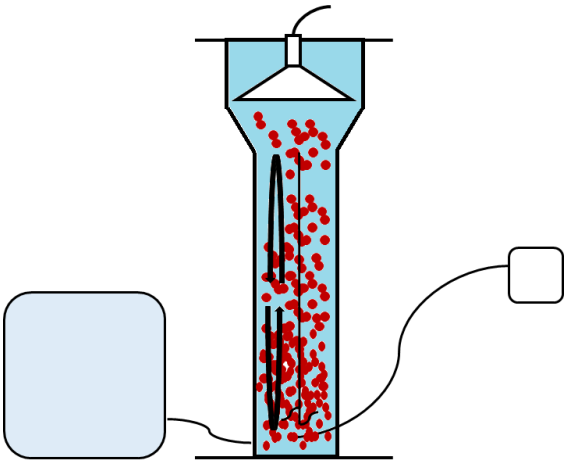

Fig 2.

EGSB Anammox Reactor. This method creates granular anammox biomass with limited culture washout. This reactor utilizes an upflow of the influent centrate to create anammox biofilm granules. The bacteria are pushed upwards in the center of the reactor until reaching the headspace and then fall to the bottom along the sides of the reactor. This upwell gives space for the generation of biomass and creates granules in the process. As the anammox reaction occurs nitrogen gas is captured and released through the gas collection space.

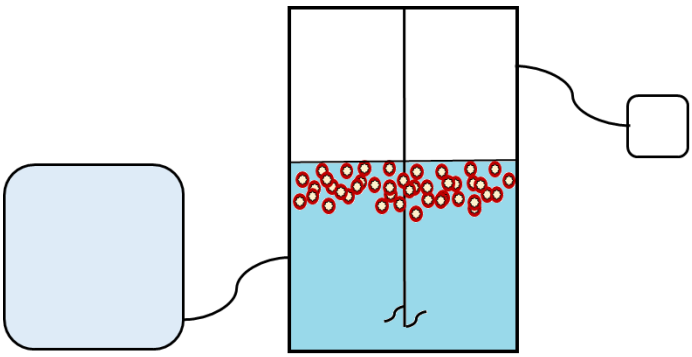

Fig 3.

Anammox MBBR. This method utilizes anammox biofilms grown on K-5 substrate disks. These disks float at the top of the reactor while the reactor is continuously mixed. This method prevents culture washout due to the size of the disks easily being contained.

Tables

Table 1. Attributes of common viruses.

Virus	Nucleic Acid	Enveloped	Shape	Host	Levels found in Raw Sewage (qPCR)	Levels in Treated Effluent (qPCR)	Reference
Adenovirus	dsDNA	No	icosahedral	Human	6.4×10^6	3.4×10^5	Katayama et al., 2008
Enterovirus	ssRNA	No	icosahedral	Human	5.5×10^5	1.3×10^4	Katayama et al., 2008
Reovirus G1	dsRNA	No	icosahedral	Human	2.0×10^7	B.D.L *	Qiu et al., 2015
PMMoV	ssRNA	No	Rod	Plant	1.4×10^7	5.6×10^6	Kitajima et al., 2014
MS2	ssRNA	No	icosahedral	Bacteria	-	-	
*B.D.L- Below Detection Limit							Table 1

Table 2. Operational conditions of the Tres Rios centrifuges.

Date Sampled	Differential Speed	Polymer Speed (lbs/tonne)	Dilution Water (gallons/minute)	Feed Rate (gallons/minute)
7/25/2017	2	15	10	180
8/1/2017	2.5	15	11	240
8/8/2017 *	2.4	16	13	210
8/15/2017 **	2.3	15	11	200
8/22/2017 ***	9.9	7.8	0	250
8/29/2017	2	14	11	200
9/5/2017	4.8	14.25	10	220
9/12/2017	4.3	15	11	190
9/19/2017	5	16	7	200
9/26/2017	5.3	15	5	15
10/3/2017 ******	8.27	6.5	0	220
10/10/2017	5.1	15	5	220
10/17/2017	4.68	14	3	210
* Maintence. Centrifuges run on "speed mode".				
**Centrifuges being run on "speed mode".				
*** Maintence. Centrifuge bowl speed reduced. Solids were treated to 5% liquid content, 16% is normal.				
**** Maintence. Centrifuge run on "thickening mode".				Table 2

Table 3. Chemical analysis of Tres Rios centrate.

Date Sampled	pH	BOD (mg/L)	NH3 (mg/L)	NO2 (µmoles/L)	NO3 (µmoles/L)	PO4 (µmoles/L)	SO4 (µmoles/L)	Mean TOC (µg/L)
7/25/2017	7.3	N/S	1020	<LoD	352.1	1732	282.4	68158
8/1/2017	7.5	43.2	1050	23.19	528.8	1486	141.5	76133
8/8/2017	7.7	45.8	1050	<LoD	302.6	2618	166.2	75851
8/15/2017	7.7	40.7	914	<LoD	297.8	1572	275	90588
8/22/2017	7.6	70.5	1110	60.32	BDL	517.4	690.3	57549
8/29/2017	7.7	35.5	1010	83.32	289.8	1770	278.4	65499
9/5/2017	7.7	34.0	1030	31.24	293.9	1466	275.7	73345
9/12/2017	7.7	44.3	1030	<LoD	<LoD	1300	311.5	68848
9/19/2017	7.7	40.7	977	34.29	<LoD	1126	242.5	74012
9/26/2017	7.8	60.7	996	52.17	<LoD	1580	276.8	72144
10/3/2017	7.6	106.0	1080	36.27	<LoD	2786	123.8	282800
10/10/2017	7.8	54.9	1060	<LoD	<LoD	1395	203.8	75129
10/17/2017	7.7	85.6	1050	<LoD	<LoD	1395	203.8	75129
Average	7.7	55.2	1029	45.83	344.17	1595.65	267.05	88860.38
Max	7.8	106.0	1110	83.32	528.80	2786	690.30	282800
Min	7.3	34.0	914	23.19	289.8	517.4	123.8	57549
Range	0.5	72.0	196	60.13	239	2268.60	566.50	225251
Standard Deviation	0.1	22.1	49.08	20.84	93.29	583.94	140.47	58750.72

<LoD- Below Limit of Detection

N/S - No Sample

Table 3

Table 4. Biological analysis of Tres Rios centrate.

Date Sampled	R2A (CFU/ml)	TSA (CFU/ml)	PhiX Suceptible Host (PFU/ml)	MS2 Suceptible Host (PFU/ml)	fr Suceptible Host (PFU/ml)
7/25/2017	4.50E+06	3.30E+06	3.28E+02	TNTC	5.13E+01
8/1/2017	2.40E+06	4.45E+06	3.85E+02	3.50E+01	≤1 *
8/8/2017	2.18E+07	2.68E+07	4.73E+02	5.33E+01	8.33E+01
8/15/2017	3.20E+06	1.63E+06	7.57E+02	9.97E+02	8.93E+02
8/22/2017	3.97E+06	4.17E+06	2.33E+02	1.07E+02	7.33E+01
8/29/2017	2.87E+07	2.60E+07	1.10E+02	2.83E+02	2.93E+02
9/5/2017	2.67E+06	5.97E+06	4.27E+02	2.57E+02	3.17E+02
9/12/2017	1.13E+06	1.00E+06	3.10E+01	1.27E+02	2.07E+02
9/19/2017	3.53E+06	3.40E+06	3.33E+02	1.00E+02	1.00E+02
9/26/2017	9.33E+05	4.30E+06	4.57E+02	8.33E+01	1.07E+02
10/3/2017	1.26E+07	2.63E+06	≤1 *	2.33E+02	≤1 *
10/10/2017	3.83E+06	2.93E+06	9.00E+01	4.67E+01	3.33E+00
10/17/2017	3.80E+06	1.97E+06	2.40E+02	2.67E+02	2.30E+02
Geometric Mean	4.27E+06	4.10E+06	2.47E+02	1.37E+02	1.14E+02
Max	2.87E+07	2.68E+07	7.57E+02	9.97E+02	8.93E+02
Min	9.33E+05	1.00E+06	3.10E+01	3.50E+01	3.33E+00
Range	2.77E+07	2.58E+07	7.26E+02	9.62E+02	8.90E+02
Standard Deviation	8.63E+06	8.80E+06	2.01E+02	2.62E+02	2.47E+02
*Below Limit of Detection					Table 4

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