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A combination of temperature and urea sanitization of blackwater – optimization of a full scale system in Hölö, Sweden

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Abstract

There are an abundance of environmental problems in the world today, some of which can be traced back to a lack of proper sewage treatment. Human toilet waste, also called blackwater, is discharged untreated into the environment where it can contaminate water bodies, including drinking water sources. However, blackwater is an excellent source of plant available nutrients, and can be used as a fertilizer after proper treatment. The risks involved in the reuse of blackwater include pathogenic microorganisms, heavy metals, and pharmaceutical substances. This study however focuses only on the inactivation of pathogenic microorganisms during blackwater treatment. The aim of this study was to optimize a current blackwater treatment method at a plant in Hölö, Sweden, and confirm that the treated blackwater reached the required microbial reductions based on SPCR 178 (SP, 2012). The current method treats 30 m^3 of blackwater within 2 weeks through an increase of the materials temperature to 40°C and then the addition of 0.5% urea, based on the wet weight. The method in this study treats 160 m^3 with a similar treatment procedure, but rather than heating all of the treated blackwater only one third (60 m^3) was heated to 40°C and then mixed with the other two thirds (100 m^3) which remain at ambient temperature. After the blackwater was mixed, 1% urea (based on the wet weight) was stirred in to ensure an even distribution, after which the treatment lasted 21 days. The study was performed twice (set 1 and set 2), with samples being taken six times per set. Samples were analyzed for physiochemical properties (temperature, pH, total nitrogen and ammonia concentrations) and microorganisms (*Ascaris suum* eggs, *Enterococcus* spp., *Salmonella* spp., thermo tolerant coliform bacteria/*E.coli*, f-RNA bacteriophages, and somatic coliphages). The temperature, after the heated blackwater was mixed in with the blackwater at ambient temperature, started at approximately 17°C and then decreased to around 11°C by day 21. After the addition of 1% urea, the total-Nitrogen concentration rapidly increased and then remained constant. The pH increased during the first week and then reached a steady state. The total ammonia Nitrogen (TAN) also increased over the 21 days, appearing to reach a somewhat steady state in the last week. *Salmonella* spp. reached the required inactivation (absent in 25 g of sample based on the wet weight) by days 4 and 2 in sets 1 and 2 respectively. TtC/*E.coli* reached below the detection limit (1 CFU ml^{-1}) by day 7 in both sets, which is below the <1000 CFU g^{-1} (TS) required for the certification. The only microorganism which didn't reach its required end-product concentration (<1000 CFU g^{-1} [TS]) by day 21 was *Enterococcus* spp. Further studies using a combination of different amounts of heated and unheated blackwater in addition to different urea doses are needed to find the ideal treatment of the blackwater, where the required sanitization goals are met using the most cost efficient parameters.

Keywords: ammonia, *Ascaris*, blackwater, Enterococci, f-RNA bacteriophage, low temperature, *Salmonella*, somatic coliphage, thermo tolerant coliform bacteria/*E.coli*, urea

Popular science summary

It was estimated that in 2010 around 1.5 billion people still discharged their untreated blackwater, which is exclusively wastewater that's flushed down the toilet (human excreta), into the environment (Baum *et al.*, 2013), where it can cause a major health risk. Blackwater can spread pathogenic viruses (e.g., norovirus and enteroviruses), bacteria (e.g., *Vibrio cholera*, *Salmonella* spp.) and parasites (e.g. *Ascaris* spp.) into water bodies and drinking water sources, from where they can be ingested by people and animals and cause individuals to become sick and the potential for diseases to spread. To avoid infection and the spreading of diseases, blackwater needs to be properly treated before it is released into the environment. Blackwater is an excellent source of plant available nutrients, which means that if it is properly sanitized it can be used as a fertilizer in agricultural production. Simple treatment methods include using urea, which is already used as a fertilizer and would increase the value of the blackwater as a fertilizer, and heat.

At the blackwater treatment plant in Hölö, Sweden, where this study was performed, the current method for treating blackwater is to heat it up to 40°C for one week, then add 0.5% urea (which degrades into the disinfectant ammonia), and let it sit and sanitize for an additional week. After treatment the sanitized blackwater is stored until it is needed as a fertilizer in agricultural productions. The goal of this study was to optimize the current treatment method of the blackwater. Rather than heating all of the blackwater up to 40°C only about one third was heated while the other two thirds remained at ambient temperature. The heated and unheated blackwater were then mixed, received a 1% dose of urea, and then stored for 21 days during which sanitization took place. To ascertain that the treatment was working properly, samples were taken on 6 of the 21 days. These samples were analyzed for temperature, pH, total Nitrogen concentration, and ammonia concentration, in addition to pathogenic and indicator microorganisms which need to reach specified limits based on the current plant certification criteria (SP, 2012).

The results showed that over the 21 day period the temperature decreased by 6°C to settle at around 11°C, while the pH and total Nitrogen concentration increased until they reached a steady state after the first week. The ammonia concentration also increased over the 21 days, appearing to reach a steady state in the last week. Under the current plant certification criteria (SPCR 178), only one of the microorganisms did not reach its required reduction limit; by day 21 the concentration of *Enterococcus* spp. was still far above the <1000 per gram of dry material. *Salmonella* spp. and *E.coli* on the other hand had reached their reduction limits by the end of the first week (day 7). Further studies will need to be performed on the use of different doses and mixtures of heated and unheated blackwater, to ensure that the necessary *Enterococcus* spp. reduction will also be met.

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Abbreviations

µm	micrometer
ABP	Animal by-products
ANOVA	analysis of variance
BAB	blood agar base
CFU	colony forming unit
FAO	Food and Agricultural Organization of the United Nations
g	gram
kg	kilogram
kWh	kilowatt hour
L	liter
m	meter
m^3	cubic meters
MDG	Millennium Development Goals
mg	milligram
mL	milliliter
mm	millimeter
mpn	most probable number
MSRV	Modified Semisolid Rappaport Vassiliadis
NB	nutrient broth
N-tot	Nitrogen-total
PFU	plaque forming unit
SEK	Swedish Krona (currency)
SlaBa	Slanetzy Bartley
spp.	sub species
TAN	total ammonia Nitrogen
TS	total solids
TSI	triple sugar iron
TtC	total thermotolerant coliform bacteria
UDT	urine diverting toilet
USEPA	United States Environmental Protection Agency
VS	volatile solids
WHO	World Health Organization
ww	wet weight
XLD	Xylose lysine deoxycholate

1 Introduction

Worldwide there are many environmental and social inequality problems which have been addressed through proposals like the Millennium Development Goals (MDG) by the United Nations. A point that has been addressed by MDG, to ensure environmental sustainability, is an increase in the availability of safe drinking water and basic sanitation (UN, 2013). In 2010 it was estimated that around 1.5 billion people still discharged their sewage into the environment untreated (Baum *et al.*, 2013). The absence of a sanitation infrastructure with which to treat waste and sewage leads to water pollution, creating an inadequate drinking water source and a health concern for local populations due in part to transmissible pathogens (Langergraber & Muellegger, 2005).

In more developed countries, wastewater, as well as fertilizers and animal manure, cause more problems related to environmental nitrogen and phosphorus pollution, rather than as sources of microbial health hazard (USEPA). However, the effluent wastewater that's released into the environment does not meet drinking water standards; it still has the potential to cause pathogenic outbreaks (Naidoo & Olaniran, 2014). When treated wastewater enters waterways ecosystems can be disturbed or damaged due to excess algae growth from eutrophication (Vinnerås, 2002; USEPA). If large algal blooms occur, in addition to many aquatic animals dying, elevated toxin and bacterial growths in the water can make humans sick via contact with or consumption of the water or contaminated aquatic animals (USEPA).

Wastewater, and especially human excreta, are considered exactly that – waste – and not as the potential agricultural resource they could be after proper sanitization (Langergraber & Muellegger, 2005). Fecal sludge, for example, has all macro- and micro-nutrients needed by plants to grow, reducing or possibly eliminating the use of commercial fertilizers (WHO, 2006; Jönsson *et al.*, 2005). Additionally, it is also an excellent soil conditioner due to its organic matter (WHO, 2006). Part of the reason for the negative association with wastewater and other sewage derived products are the potential risks involved; transmissible diseases to humans and animals through food and water (Mankad & Tapsuwan, 2011; Massoud *et al.*, 2009). Wastewater, since it includes industrial waste, may also contain high levels of heavy metals and chemical contaminants even after treatment, making it less desirable as a fertilizer (Oghenerobor Benjamin Akpor, 2014; Templeton *et al.*, 2009).

Using blackwater would be a way to avoid any potential industrial contamination, while still reaping the benefits of human excreta. Blackwater is defined by the United States Environmental Protection Agency (USEPA) as "liquid and solid human body waste and the carriage water generated through toilet usage" (USEPA). While blackwater only makes up approximately 1-2% of all domestic wastewater, it contains the majority of nitrogen (more than 95%) and phosphorus (about 90%) that is found in domestic wastewater, in addition to other plant nutrients (Alp, 2010). Similar to wastewater, when in excess, these nutrients can however cause environmental problems.

In Sweden excess nitrogen and phosphorus has led to high deposits in the Baltic Sea, and even though Sweden has already reduced its output, further reductions are still needed to allow the Baltic Sea to recover (SwedishEPA, 2009). The suggested reduction goals originate from 15 environmental quality objectives defined by Sweden in 2000 in a report titled "The future environment – our common responsibility." In an effort to reduce the phosphorus and nitrogen concentrations in the environment, excluding agricultural land, and to create a closed system, reuse goals have been established. The report initially proposed that by 2010, 75% of the phosphorus from waste and wastewater should be recycled to arable or productive land without harming human health or the environment (Hultman *et al.*, 2003). However, this 75% was not reached, so the proposal was changed to recycling 60% of the phosphorus in wastewater by 2015 (Jönsson, pers). Again, this goal needed to be adjusted, with the latest proposal now suggesting that by 2018, 40% of the phosphorus and 10% of the nitrogen in wastewater should be recovered to arable land (Jönsson, pers). In Sweden, companies such as the blackwater treatment plant in Södertälje (Hölö) are striving to help meet this goal by using properly treated blackwater as a fertilizer.

2 Aim

The aim of this thesis was to test, on a full scale, an alternative combination of heating and ammonia treatment as a complement to the current blackwater ammonia treatment, to optimize the capacity of a wet compost plant in Hölö, Sweden. The sanitizing efficiency of the alternative treatment was evaluated by analyzing pathogenic and indicator organisms, specifically *Salmonella* spp., total thermotolerant coliforms (TtC) and *E.coli*, *Enterococcus* spp., f-RNA bacteriophages and somatic coliphages, and *Ascaris suum* eggs. The physiochemical factors, TAN, N-tot, temperature, TS, and pH, which were assumed to cause the reduction and inactivation of the microorganisms were also examined over the 21 day study period. The hypotheses tested were:

1. The addition of 1% urea will cause an increase in the total-Nitrogen, TAN, and un-ionized ammonia concentrations, resulting in a reduction of the studied microorganisms.

2. The mixture of heated and ambient temperature blackwater will result in a temperature high enough to, in combination with ammonia, cause significant inactivation of the studied microorganisms.

3. The end concentration of the studied microorganisms after the 21 day study period will be in accordance with the SPCR 178 certification criteria.

3 Background

Potential pathogens

Blackwater consists of human excreta which is flushed down the toilet, i.e. urine, feces, toilet paper, and varying amounts of flushwater. Pathogenic microorganisms, bacteria, viruses, and parasites, found in human excreta and thus in blackwater, pose a threat of transmissible diseases to animals and humans if released into the environment (Fidjeland *et al.*, 2015b; Fidjeland *et al.*, 2013b; Emmoth *et al.*, 2011). Infection can occur through many different routes, some of which are illustrated in Figure 1.

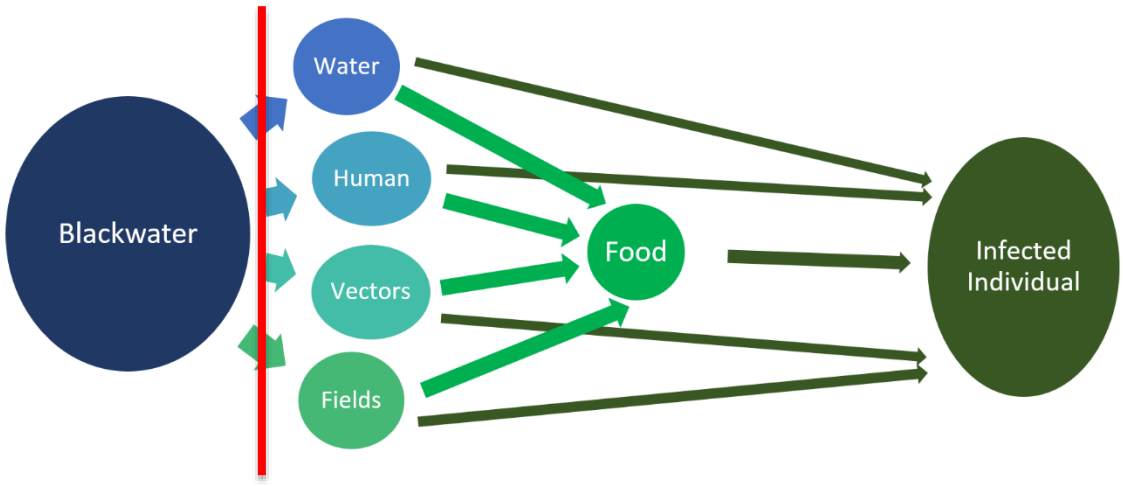

Figure 1. The possible routes of transmission for pathogenic organisms found in untreated blackwater and how sanitizing treatment creates a barrier for subsequent transmission (red line).

Microorganisms can be transmitted from improperly sanitized blackwater to water bodies, directly to humans who come in contact with it, to vector animals such as flies or pets, and to fields where it serves as a fertilizer. Treating blackwater properly before it is released into water bodies or spread on fields will cut off those routes of transmission. Additionally, the potential health risk through direct contact by humans or through vector animals is reduced due to the inactivation of pathogens during treatment. Since the initial routes of transmission to water, humans, vectors, and fields can be cut off through proper treatment performed under safe and controlled management practices, then any further routes of transmission, either directly infecting individuals or infection through the consumption of food will also be cut off.

To assure that the sanitization of the blackwater is adequate, pathogen and indicator organism inactivation must be observed. The pathogen and indicator organisms analyzed in this thesis are *Salmonella* spp., total thermotolerant coliforms (TtC) and *E.coli*, *Enterococcus* spp., f-RNA bacteriophages and somatic coliphages, and *Ascaris suum*.

Salmonella spp.

According to WHO, salmonellosis, the disease caused by strains of the bacteria salmonella, is one of the most frequent and widely distributed foodborne diseases worldwide causing tens of millions of humans to get sick each year (WHO, 2013). The two serotypes of *Salmonella enterica* that are responsible for human illness are Enteritidis and Typhimirium (WHO, 2013; PHAC, 2010b). Symptoms include those common to gastroenteritis; fever, abdominal pain, diarrhea, nausea, and vomiting, which can cause dehydration (WHO, 2013). This can be life-threatening for young and old people along with immune-compromised individuals (WHO, 2013). *Salmonella* spp. bacteria are quite resilient, they can survive for several months in water and several weeks in drier environments (WHO, 2013). Additionally, they can be transmitted by all pathways depicted in Figure 1 on page 23 above and by many vector animals, including cattle, swine, poultry, wildlife, birds, pets, and flies (WHO, 2013; PHAC, 2010b). The amount of bacteria needed to get sick, i.e. the infectious dose, varies with the different salmonella serotypes, it can range from 10^3 to 10^5 bacteria (PHAC, 2010b).

Due to its high transmissibility, regulations usually require the inactivation of *Salmonella* spp. to certain limits (Table 1). Studies on the ammonia sanitization of human excreta at a variety of different temperatures have shown positive results with regards to *Salmonella* spp. inactivation. Compared to other bacteria, such as *Enterococcus* spp., it is usually more sensitive and thus more quickly inactivated (Fidjeland *et al.*, 2013a; Fidjeland *et al.*, 2013b; Nordin *et al.*, 2009b). Depending on the temperature and urea and subsequent ammonia concentration used, the inactivation of *Salmonella* spp. can occur within a matter of days to weeks (Fidjeland *et al.*, 2013a; Fidjeland *et al.*, 2013b; Nordin *et al.*, 2009b).

Thermotolerant coliform bacteria and *E.coli*

Thermotolerant coliform bacteria (TtC) refers to members within the *Enterobacteriace* family who are able to grow at 44-45°C and contain the enzyme β-galactosidase (Hachich *et al.*, 2012; WHO, 2004). Since they are commonly found in the fecal matter of warm-blooded animals, they can be used as indicators of fecal contamination and thus the sanitary quality (Hachich *et al.*, 2012). This is commonly done to ascertain the quality of water (Hachich *et al.*, 2012). Since thermotolerant coliform bacteria can both survive and grow in water, they shouldn't be used to indicate the amount of fecal pathogens present, but rather indicate the effectiveness of treating fecal contaminated water (WHO, 2004).

A very common thermotolerant coliform bacteria, found almost exclusively in human and mammal fecal matter, is *Escherichia coli* (*E. coli*) (Hachich *et al.*, 2012; WHO, 2004). This specificity makes it an excellent indicator of contamination from feces and how well sanitization methods have worked (WHO, 2004). Many strains of *E. coli* are harmless, however, there are several strains which can cause illness in humans, examples of which are enterohemorrhagic, enteroinvasive, enteropathogenic, and enterotoxigenic *E. coli*, all of which can cause diarrhea (watery and/or bloody), fever, and abdominal cramps, in addition to symptoms individual to each type (PHAC, 2011b; PHAC, 2011c; PHAC, 2011d; WHO, 2011; PHAC, 2001). Usually the *E. coli* infection isn't life threatening; however, if symptoms gets worse, it can become deadly, especially in the elderly, young, and immune-compromised (PHAC, 2011b; PHAC, 2011c; PHAC, 2011d; WHO, 2011; PHAC, 2001). However, *E. coli* is quite sensitive to disinfection methods, more so than other thermotolerant coliforms, enteric viruses, and parasites (Fidjeland *et al.*, 2015b; WHO, 2004).

Enterococcus spp.

Enterococcus spp. is popular as an indicator of fecal contamination, with the most common strains being *Enterococcus faecalis, Enterococcus faecium, Enterococcus durans,* and *Enterococcus hirae* (Ashbolt *et al.*, 2001). Of these mainly *E. faecalis,* and less often *E. faecium*, can be found in the human intestinal tract, from where they get excreted through feces (PHAC, 2010a). These two strains of *Enterococcus* are considered to be opportunistic pathogens who mainly affect the elderly and immune-compromised people, causing urinary tract infections, wound infections, soft tissue infections, and bacteremia, especially in hospital settings (PHAC, 2010a). The route of transmission can occur from consumption of contaminated food, person-to-person, or contact with animals (zoonosis) (PHAC, 2010a).

When comparing the inactivation time of *Enterococcus* spp. by ammonia sanitization to that of other pathogens (*Salmonella* spp.) and other fecal indicator bacteria (*E. coli*), they were found to be far more persistent (Fidjeland *et al.*, 2013a; Fidjeland *et al.*, 2013b; Magri *et al.*, 2013; APHA, 2001). It has even been observed that some enterococci strains, namely *Enterococcus faecium* and *Enterococcus gallinarum*, were able to grow at significant ammonia concentrations, temperatures (up to 47.8°C), and pH ranges (up to 9.9) which would effectively inactivate salmonella and *E. coli* as well as other enterococci strains (*Enterococcus faecium* and *Enterococcus faecalis*) (Son ThiThanh *et al.*, 2011; Berghe *et al.*, 2006).

Bacteriophages

Somatic coliphages and f-RNA bacteriophages can be used as model organisms for enteric viruses as well as indicators of fecal contamination (Vinnerås *et al.*, 2008; Ibarluzea *et al.*, 2007; Rhodes & Kator, 1991). Some examples of enteric viruses are poliovirus, hepatitis A virus, reovirus, and Norwalk-type

virus, all of which can be found in water; when ingested they can cause sicknesses such as hepatitis and gastroenteritis (Ibarluzea *et al.*, 2007). Enteric viruses are often more resilient than the bacterial indicators such as *E. coli* and *Enterococcus* spp. (Ibarluzea *et al.*, 2007).

Experiments have shown ammonia sanitization is efficient at inactivating single-stranded RNA viruses, either enveloped or naked (Emmoth *et al.*, 2011; Cramer *et al.*, 1983). Additionally, somatic coliphages have been found to be more resistant than f-RNA bacteriophages (Ibarluzea *et al.*, 2007).

Ascaris suum eggs

Ascaris suum is a parasitic nematode, helminth, which is often used as a model for *Ascaris lumbricoides* (Nordin *et al.*, 2009b). *Ascaris suum* is commonly found in pigs, but can also infect humans (PHAC, 2011a). *Ascaris lumbricoides* is extremely prevalent worldwide, approximately 1.2 billion people are infected with it (PHAC, 2011a). Releasing untreated fecal material into the environment also releases the *Ascaris* eggs in it. The primary mode of transmission is through the ingestion of mature helminth eggs which can be found in fecal-contaminated food and water (PHAC, 2011a). Infections most often occur in children, while not necessarily fatal it can reduce nutrient uptake and cause developmental problems (Kawatra *et al.*, 2010).

Ascaris eggs are often treated by using a high temperature, high pH, or a mixture of the two (Nordin *et al.*, 2009b). Studies have found that ammonia can also be used to inactivate *Ascaris* eggs (Nordin *et al.*, 2009b; Pecson & Nelson, 2005). However, when comparing the inactivation of *Ascaris suum* eggs to the inactivation of other pathogens, they have usually shown themselves to be more resistant, even when treated with ammonia (Fidjeland *et al.*, 2015b; Fidjeland *et al.*, 2013b; Capizzi-Banas *et al.*, 2004).

Framework and policies

In order to regulate that the human excreta can be safely used in agricultural practices, many countries have regulations or are in the process of turning proposals into regulations, which limit certain indicator organisms, pathogens, and parasites. If these parameters are met then human excreta is considered safe for application (Table 1). While the plant is currently certified under SPCR 178, which coincides with the SEPA 2010 proposal with regards to end concentrations. A more recent proposal, SEPA 2013, has been made which will change the plant criteria for the treated end-product. The *Ascaris* and virus parameters proposed by SEPA 2013 are the similar as the European animal by-product (ABP) regulation. SEPA 2013 is aiming at being in accordance with ABP, since ABP regulates the sanitization of animal manure. However, the SEPA 2013 proposal does not include any regulations for enterococci, likely due to its stability and variability in the environment which makes it an unreliable indicator of fecal contamination. Rather, SEPA 2013 relies on *E. coli* to act as a fecal indicator. Lastly the criteria for *Salmonella*

was also adjusted to a level that complies with the European standard for the use of bio-waste in agriculture (BioAbfV, 1998).

Table 1. A brief overview of a USA and a European regulation and two proposals by the Swedish Environmental Protection Agency for the sanitation of human excreta and animal manure (ABP 2011)

	Ascaris[a]	*Enterococcus*[b]	*E. coli*/fecal coliforms[c]	Virus[d]	*Salmonella*[e]
US EPA Regulation, Class A (USEPA, 2003)	0 per 4 g TS	NA	<1000 mpn per g TS	<1 per 4 g TS	<3 mpn per 4 g TS
ABP Regulation (EU, 2011)	3 log10 red.	5 log10 red.	NA	3 log10 red.	5 log10 red.
SEPA 2010 (SwedishEPA, 2010)	NA	<1000 g TS	<1000 per g TS	NA	absent in 25 g (ww)
SEPA 2013 (SwedishEPA, 2013)	3 log10 red.	NA	5 log 10 red. or <100 per g (ww)	3 log10 red.	absent in 50g (ww)

a) For US EPA 2003 specified 'viable eggs' ; for SEPA 2013 term 'parasites' is used
b) For ABP 2011 species defined to *Enterococcus faecalis*
c) For US EPA 2003 regulation defined to fecal coliforms
d) For US EPA 2003 defined to phages; for ABP 2011 species defined to thermo resistant viruses (parvovirus)
e) For ABP 2011 species defined to *Salmonella senftenberg*

For a substantial or even total inactivation of viruses, bacteria, and parasites, WHO guidelines (2006) recommend storing fecal sludge more than one year if the ambient temperature is 20-35°C. If the ambient temperature is between 2-20°C however, a storage time of 1.5 to 2 years is recommended to eliminate bacterial pathogens and reduce viruses and parasites to below the risk level (WHO, 2006). However, simply storing material can result in variations among the final products of different treatment sets since this treatment method is so dependent on numerous uncontrolled factors. This is different to treatment methods where factors and parameters can be controlled. Based on results of studies where parameters are controlled, treatments can be repeated and predictions made about the sanitation level of the final product.

Ammonia sanitization of blackwater

There are many different treatment methods for blackwater to reach the end quality defined by the mentioned regulations, including heating, liming, anaerobic digestion, composting, and ammonia sanitization. Of these methods, this thesis focuses on a combination of temperature and ammonia sanitization to reduce pathogenic bacteria, viruses, and parasites in blackwater to levels which make it safe for use as a fertilizer.

Background chemistry

The basic idea behind treating blackwater with ammonia is that the un-ionized ammonia will allow for sanitization (Anderson *et al.*, 2015; Fidjeland *et al.*, 2015b). The ammonia treatment of blackwater can be performed through the addition of urea, which will be converted to ammonia and carbonate when hydrolyzed by the enzyme urease.

$$CH_4N_2O + H_2O \xrightarrow[urease]{} 2NH_3 + CO_2$$

Blackwater has intrinsic ammonia provided by urine, as well as the urease enzyme which is naturally occurring in feces. Temperature and pH play an important role for the presence of ammonia in its un-ionized form (NH_3), which increases with increased pH and temperature (Fidjeland *et al.*, 2015a; Emmoth *et al.*, 2011; Emerson *et al.*, 1975). The storage time needed to sanitize the blackwater depends mainly on the concentration of un-ionized ammonia and the temperature of the material (Nordin, 2010).

A theory, which is supported by studies, to explain the mechanism behind ammonia inactivation of viruses is that the viral RNA is cleaved into particles, with single-stranded RNA viruses being more easily inactivated than double-stranded ones (Decrey, 2015; Emmoth *et al.*, 2011; Burge *et al.*, 1983; Ward, 1978). In bacteria, cell alkalization, which is when ammonia enters the cell and forces the bacterium to donate protons to maintain its optimum internal pH, eventually causes cell death (Anderson *et al.*, 2015).

Process description

The current handling method of blackwater at the treatment plant in Hölö (Södertälje), Sweden (Figure 2) is as follows (Telgekoncernen, 2014). Household blackwater, currently supplied mainly by ordinary flush toilets, is collected from sealed septic tanks, which are then emptied by trucks and transported to the treatment plant. Here the blackwater is pumped into the receiving wells (2 units), which can hold 200 m^3 of blackwater each; the escaping air is sent through bio filters to reduce odors, equalize pressure, and catch any escaping ammonia (Hallgren *et al.*, 2012). From the receiving wells the blackwater is pumped into one or both of the available bio-reactors, which can hold up to 32 m^3 of blackwater each.

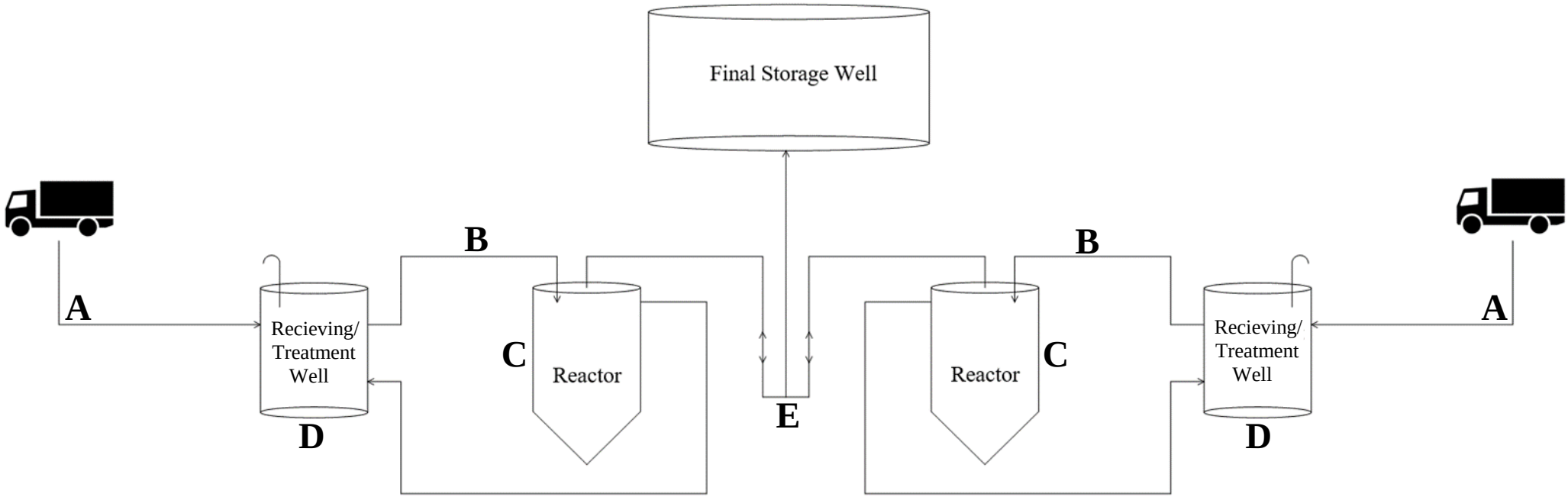

Figure 2. Depiction of the blackwater flow through the treatment plant, the water gets delivered and pumped into the treatment well (A), after which 30m^3 are pumped into each reactor (B) where the blackwater is heated to between 40-45°C (C) and then pumped back into the treatment well and mixed with 100m^3 of ambient temperature blackwater and 1% urea (ww) (D), after treatment the blackwater is pumped into the final storage well (E) (not drawn to scale)

(Illustration: Prithvi Simha; https://commons.wikimedia.org/wiki/File:Sinnbild_LKW.svg)

In the reactor an agitator is started in order to stir the blackwater, while air is pumped in through the bottom of the reactor and whipped until sufficiently sized bubbles are created. This ensures that oxygen and thus heat, due to aerobic digestion by microorganisms, are spread evenly throughout the blackwater. Any escaping air is pumped back into the receiving wells where it can exit via the bio filters. After seven days the temperature in the reactor reaches around 40°C and contagious agents (i.e. salmonella, *Escherichia coli*, enterobacteria) have been reduced by approximately 40%. To have an active flora some of the already composted blackwater is used as an inoculant for the next batch of untreated blackwater.

After the heating to 40°C, 0.5% urea, based on the wet weight (ww), is added to the blackwater in the reactor. After seven additional days, all contagious agents have been removed to below the detection limit. The blackwater is then pumped into a final storage tank which can hold up to 1500 m^3. It will remain in the tank until it is spread onto arable land, around 85-100m^3 (30-35 tons) will be used per hectare (Nordin & Calo, pers). In total, the current sanitization of the blackwater requires 14 days to treat 32 m^3.

In this study, ways to utilize another combination of heat and urea was investigated with the aim of optimizing the treatment capacity of the plant. In the studied process, blackwater will still be brought to the treatment facility and first pumped into a receiving well. However, both of the bio reactors will then be filled with 30 m^3 each, while 100 m^3 remain in the receiving well at ambient temperature. The 60 m^3 in the reactors will then be aerated and heated to between 40°C to 45°C, which takes approximately ten days. These 60 m^3 are then pumped back into the receiving well (Figure 2, D) where it is mixed with the untreated blackwater and 1% urea, based on total wet weight. The content in the receiving well is then thoroughly mixed and left alone for 21 days. The evaluation of this alternate treatment regarding its sanitizing capacity is the topic for this master thesis.

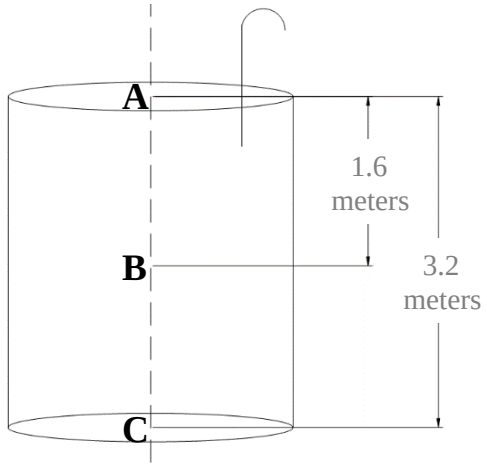

Figure 3. Close up of the well where the blackwater treatment took place and the three sampling heights; 0 meters (A), 1.6 meters (B), and 3.2 meters (C) (Illustration: Prithvi Simha)

The plant has certified its end product with the current handling according to SPCR 178, which is a voluntary certification which focuses on the end product of source separating sanitation systems (SP, 2012). The criteria set by SPCR 178, in relation to hygiene, which are listed in Table 2 shall be met. Additionally, to meet certification criteria, the plant has a control program in place to make guidelines for management. Checking the pH and temperature of the blackwater at regular intervals ensures that treatment is being performed properly and that the blackwater will have proper sanitization.

Table 2. The limitations required by SPCR 178 (SP, 2012; SwedishEPA, 2010)

Criteria	Limitation
Enterococcus spp.	<1000 per g TS
Salmonella spp.	Absent in 25 g (ww)
Escherichia coli	<1000 per g TS

Optimization and cost

Currently the plant treats about 1400 tons or 4000 m^3 of blackwater annually, which is enough to fertilize around 40 hectares of land using 30-35 tons per hectare (Nordin & Calo, pers). Using the new treatment method, the plant will then be able to treat 160 m^3 in 31 days rather than 32 m^3 (or 64 m^3 if both bio reactors are being used) in 14 to 20 days.

Additionally, approximately only one third of the material will have to be heated and aerated which could reduce overall production costs and treatment time. The energy consumption of the plant was 73119.6 kWh in 2015 using the old blackwater treatment method (Nordin & Calo, pers). Assuming the same amount of blackwater will be handled using the new method, this energy consumption could be reduced by around 62%, since only about one third of the blackwater is heated.

However, the amount of urea used would be doubled to 1% (ww). The urea currently used by the plant costs approximately 5700 SEK per ton (Nordin *et al.*, pers). When adding 0.5% urea (ww), the plant will annually use 7 tons of urea and spend almost 40,000 SEK. With the treatment method examined in this study, the amount used will be doubled to 14 tons and the price to almost 80,000 SEK.

Blackwater reuse

Similar to excreta from animals such as cattle, pigs, and chickens, human waste also contains essential plant nutrients, nitrogen, phosphorus, and potassium, in plant available forms (WHO, 2006; Vinnerås, 2002). This means that human excreta has the potential to be used as a fertilizer in crop production. The

concentration of nutrients in blackwater is dependent on the country of origin since it is directly affected by both the amount and quality of the food consumed by the local people; an example of which can be seen in Table 3 (WHO, 2006; Jönsson & Vinnerås, 2004). The majority of the nutrient concentrations in Table 3 are found in the urine (WHO, 2006; Vinnerås, 2002).

Table 3. Concentration of nutrients found in blackwater from different countries (Jönsson & Vinnerås, 2004)

	Excretion in kg per person per year		
Country	Nitrogen	Phosphorus	Potassium
Sweden	4.6	0.5	1.4
China	4.0	0.6	1.8
India	2.7	0.4	1.5
Uganda	2.5	0.4	1.4

The high nutrient values in urine make it comparable, in its nutrient composition, to ammonium-based and urea-based chemical fertilizers; it is similarly inefficient however when the soil is in a poor state, for example when it has low organic matter (WHO, 2006). Therefore blackwater, which incorporates fecal matter and urine, has the added benefit of both the additional nutrients in the feces as well as organic matter, which can improve soil structure (WHO, 2006). According to WHO (2006), 130 million tons of fertilizer are sold worldwide each year, 73 million tons of which are nitrogen and 13.7 million tons of which are phosphorus, of which 63% is used by developing countries. If agricultural practices began using human excreta as a fertilizer one third of the chemical nitrogen fertilizer and almost one fourth of the chemical phosphorus fertilizer could be replaced by it (WHO, 2006).

While being an excellent source of plant macro- and micronutrients, blackwater also has certain risks associated with it besides pathogenic microorganisms. Heavy metals, the concentrations of which vary based on the diet of the local population, is one of these risks (Jönsson *et al.*, 2004). The majority of heavy metals excreted can be found in the feces, they include some which are essential micronutrients needed for plant growth, copper and zinc, and non-essential metals, chromium, nickel, lead, cadmium, and mercury (Jönsson *et al.*, 2004; Vinnerås, 2002). The concentrations of heavy metals are generally lower than those in chemical fertilizers and farmyard manure (WHO, 2006). Therefore, if it is not properly treated these contaminants can be spread into and within the environment where they can cause detrimental effects.

Additionally, blackwater contains hormones and pharmaceutical substances, which are excreted mainly through urine (Jönsson *et al.*, 2004). These substances, especially estradiol, have caused problems in aquatic environments with regards to the sexual development of fish and reptiles (Jönsson *et al.*, 2004). However, studies have shown that when applied and mixed intro arable land, the hormones and pharmaceutical substances found in blackwater are degraded by soil microbes, showing that it is better to apply them on land rather than empty them directly into water systems (Jönsson *et al.*, 2004). It is theorized that the degradation of hormones and pharmaceuticals by soil microbes happens because hormones, which are naturally produced in the human body, and pharmaceuticals, which may be synthetically produced but still based on substances found in nature, have been excreted into the terrestrial environment during the course of evolution, allowing soil microbes to adapt and degrade them (Jönsson *et al.*, 2004). Additionally, manure produced from domestic animals contains far greater concentrations of hormones than human excreta (Jönsson *et al.*, 2004). However, microbial adaptation and eventual resistance to certain pharmaceutical substances which may be more resistant to microbial degradation still requires further monitoring (Thiele-Bruhn, 2003).

Social and environmental impact

The intended use of the blackwater, whether for private or communal use, in addition to available water resources impacts what toilets and the accompanying infrastructure is needed. The concentration of plant available nutrients and organic matter in the blackwater depends on the amount of flushwater used, which differs among toilet types; greater volumes of flushwater dilute the nutrients and organic matter available per unit of blackwater.

There are several different types of toilets available, however, the toilet waste would have to be separated from the rest of the household water waste. The easiest way to do this would be to have a storage tank connected directly to the toilet. This tank could be used either for treatment domestically, or be emptied by a collection truck and brought to a treatment facility. The type of toilet used will determine the dry matter content of the blackwater, which changes based on the amount of flushwater used. Classic flush toilets or cistern toilets (Figure 4) use quite large quantities of water when compared to other types of toilets; older toilet designs can use up to 20 L of water, while more modern designs use between 6 to 9 L (Tilley et al., 2014). Due to the high flushwater volume, cistern toilets both greatly dilute the nutrient and organic matter content of the blackwater in addition to not being as applicable in regions suffering from water scarcity.

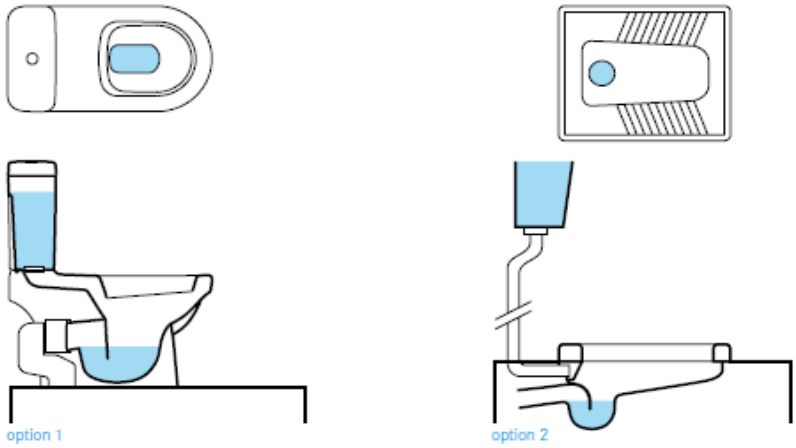

Figure 4. Depiction of a cistern toilet [Source:(Tilley et al., 2014)]

Urine diverting toilets (UDT) (Figure 5) are another option, where the urine and feces are deposited and flushed down two separate chambers; allowing for the possibility to collect the urine and feces into two separate tanks or in one tank depending on the desired use of the excreta. UDTs can be either wet or dry, meaning they do use flushwater ("wet") or they don't ("dry"). Depending on whether a "small" or "large" flush is done, these toilets use between 0.3 and 2.5 L per flush (Wostman).

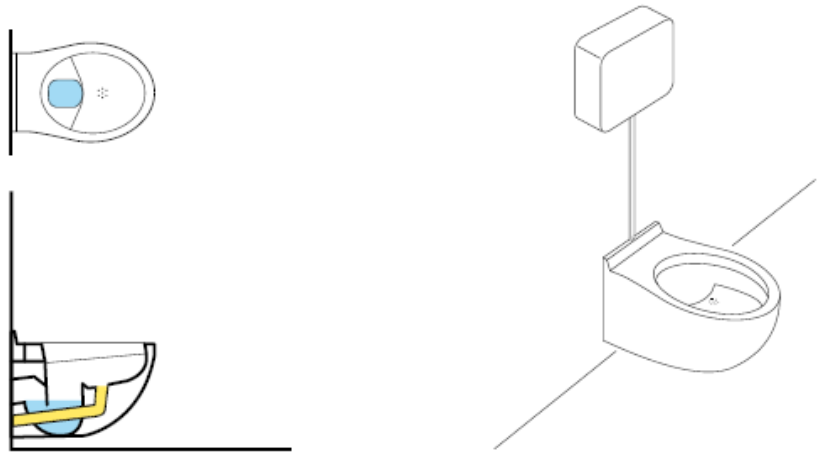

Figure 5. Depiction of a wet urine diverting toilet [Source: (Tilley et al., 2014)]

A third choice is the use of a vacuum toilet, which is similar to the cistern toilet, but uses a mixture of water and air to flush. The mixture of air and water means that only 0.6 L of water per flush are needed (Wostman). Lastly, a toilet option which may be more applicable in developing countries is the pour flush toilet (Figure 6). While in principle they are similar to cistern toilets, the main difference is that the user manually pours water to flush their excretions, usually using between 2 and 3 L of water per flush (Tilley *et al.*, 2014).

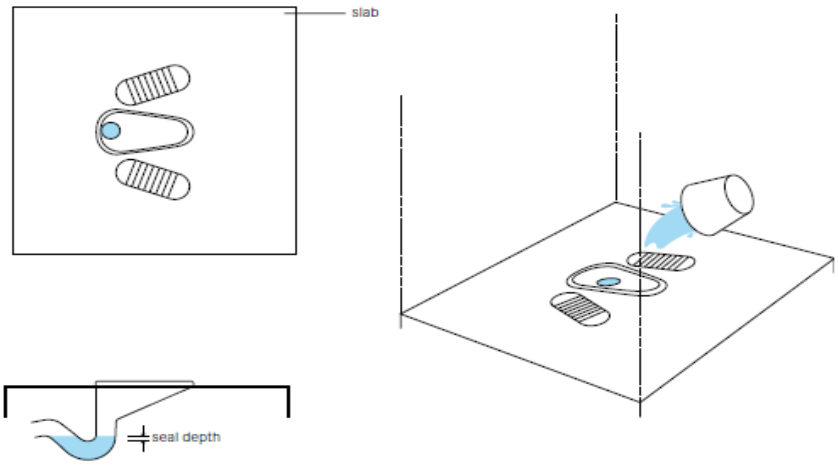

Figure 6. Depiction of a pour flush toilet [Source: (Tilley et al., 2014)]

Separating blackwater in order to treat and then reuse it may require adjustments to existing infrastructure in certain countries and complete implementation in countries where no proper sanitation systems exist. It is however, a great opportunity to recycle nutrients and water. Based on statistical data from FAO, the world population has increased by 340% between 1900-2010, while freshwater withdrawal has increased by 630% in the same time span (FAO, 2016). The majority of the freshwater, 69%, is being used for agricultural purposes, 19% is used in industry, and the remaining 12% is used for municipal purposes, including household water use (FAO, 2016). While most of the water that is withdrawn is at some point returned to the environment, it is often more contaminated than it was before it was withdrawn.

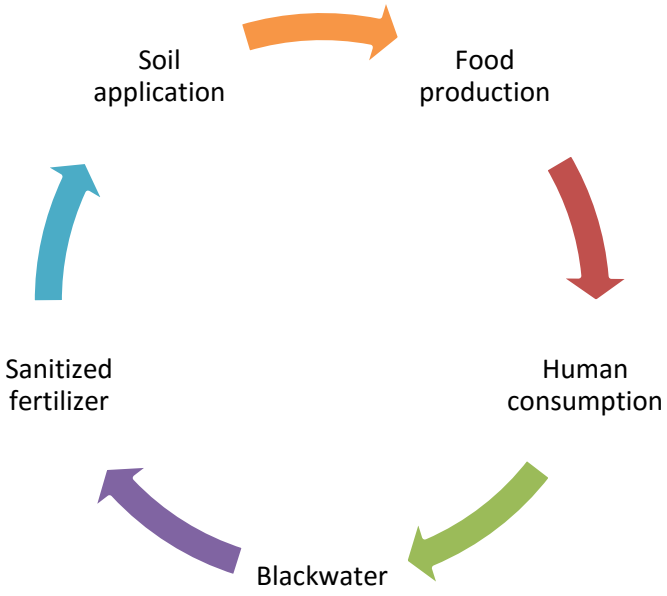

Figure 7. Closed cycle of blackwater reuse for agricultural purposes

This is creating a scarcity of freshwater sources, in some countries more than others, which impacts humans in a variety of ways, from contaminated water being used in agricultural production to a direct contamination of drinking water. By recycling and reusing blackwater some of these problems can be addressed. A closed and sustainable system as illustrated in Figure 7 could be created.

4 Materials and Methods

Experimental setup

Two serial treatment replications were performed at a blackwater treatment plant in Hölö, which is located south of Stockholm, Sweden. For each replication, or set, the blackwater used was collected from households in the surrounding area, brought to the treatment facility, and first pumped into a receiving well. Next, both of the bio reactors were filled with 30 m^3 of blackwater each, while 100 m^3 remained in the receiving well at ambient temperature. The 60 m^3 in the reactors were then aerated and heated to between 40°C to 45°C, which took about ten days. These 60 m^3 were then pumped back into the receiving well where it was mixed with the cooler untreated blackwater and 1% urea (based on total ww). The content in the receiving well was then thoroughly mixed for 1 hour to evenly distribute the heated blackwater and urea, and then left alone for 21 days. The study took place from the middle of March 2016 until the end of May 2016.

In order to analyze if any stratification took place within the well, a structure made up of 1 L plastic bottles and string was built. The bottles were tied with the string, so that when placed in the well one bottle reached the bottom (3.2 m deep), one bottle reached the middle (1.6 m deep), and one bottle was at the top (0 m deep). A weight was attached at the bottom to make sure the bottles didn't move about and floatation devices were attached at 1.6 m and 0 m to assure that the plastic bottles remained at the desired heights. Additionally, the 1 L bottles had holes drilled into them which were large enough to allow blackwater to flow through them. On the inside they were lined with a plastic mesh to insure that anything added (the *Ascaris suum* egg bags and temperature probes) would remain within.

During each set, the temperature probes remained in the blackwater well for the entire three weeks, while the samples, listed in Table 4 below, were collected and subsequently analyzed on six days; initial day (day 0), after 24 hours (day 1), after 48 hours (day 2), after one week (day 7), after two weeks (day 14), and after three weeks (day 21).

Table 4. Overview of the samples taken to be analyzed during one set

Day	Samples
Day 0	3 samples from the reactor 3 samples from the well 3 samples of the mixture before urea addition 3 samples of the mixture after urea addition 1 *Ascaris suum* egg bag
Day 1	3 samples from the top of the well (0 m deep) 3 samples from the middle of the well (1.6 m deep) 3 samples from the bottom of the well (3.2 m deep)
Day 2	3 samples from the top of the well (0 m deep) 3 samples from the middle of the well (1.6 m deep) 3 samples from the bottom of the well (3.2 m deep)
Day 7	3 samples from the top of the well (0 m deep) + 1 *Ascaris suum* egg bag 3 samples from the middle of the well (1.6 m deep) + 1 *Ascaris suum* egg bag 3 samples from the bottom of the well (3.2 m deep) + 1 *Ascaris suum* egg bag
Day 14	3 samples from the top of the well (0 m deep) + 1 *Ascaris suum* egg bag 3 samples from the middle of the well (1.6 m deep) + 1 *Ascaris suum* egg bag 3 samples from the bottom of the well (3.2 m deep) + 1 *Ascaris suum* egg bag
Day 21	3 samples from the top of the well (0 m deep) + 1 *Ascaris suum* egg bag 3 samples from the middle of the well (1.6 m deep) + 1 *Ascaris suum* egg bag 3 samples from the bottom of the well (3.2 m deep) + 1 *Ascaris suum* egg bag 3 samples after the blackwater has been thoroughly mixed

Each sample consisted of 100 ml of blackwater and was analyzed for total nitrogen (N-tot), total ammonia nitrogen (TAN), pH, *Salmonella* spp., total thermotolerant coliform bacteria (TtC)/*Escherichia coli*, *Enterococcus* spp., f-RNA bacteriophages, and somatic coliphages as can be seen in Figure 8. Extra samples were sent on day 0 of the mixture after the addition of urea and day 21 of the blackwater after mixing for dry matter/total solids (TS) analysis.

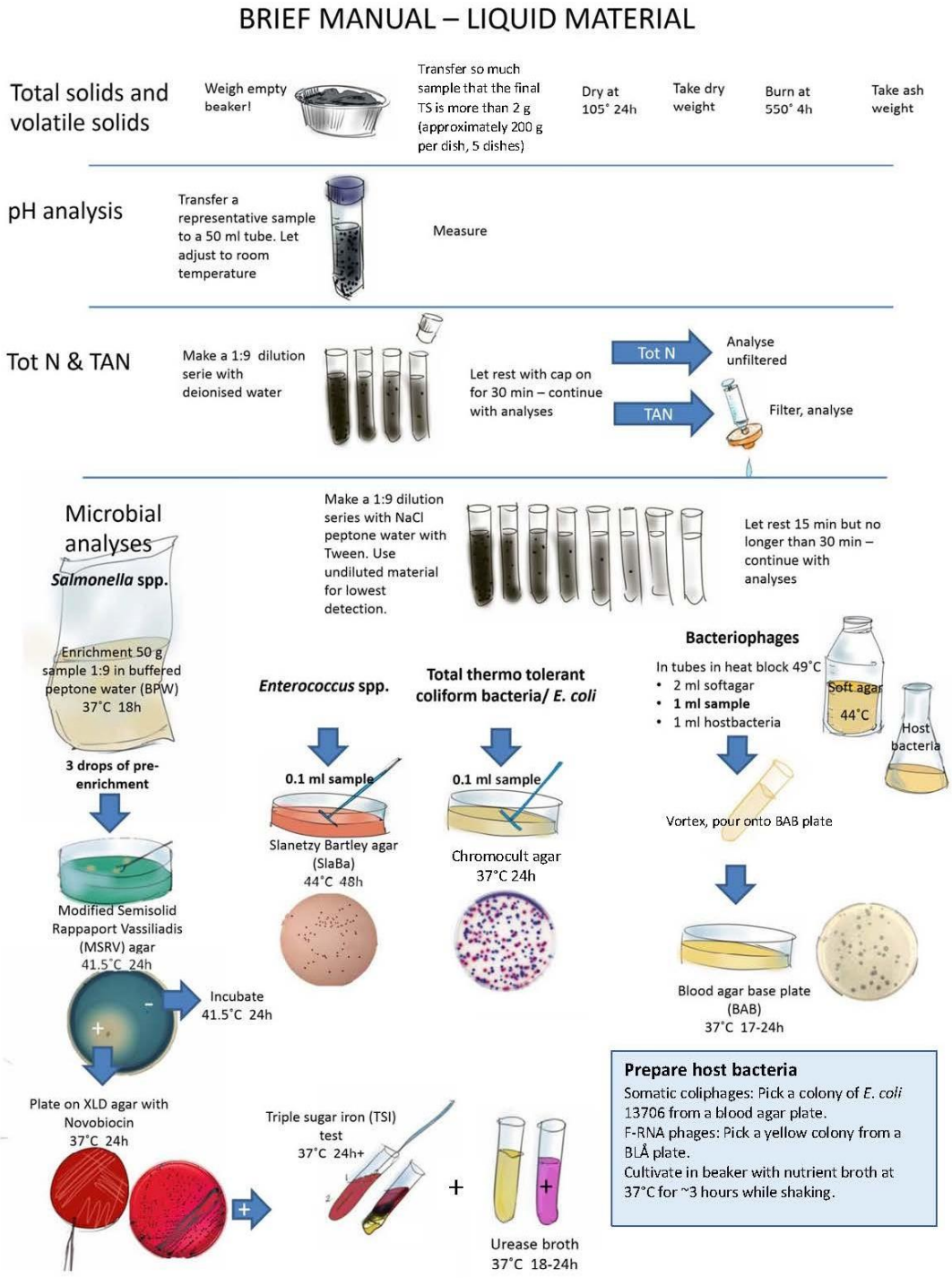

Figure 8. A brief overview of the analyses performed on the blackwater samples (Illustration: Annika Nordin)

Physiochemical analysis

The sections below provide a more in depth description of the physiochemical analysis methods performed on the blackwater and *Ascaris suum* egg samples, which were briefly described in Figure 8 above.

Temperature

Temperature probes, Tiny tag Aquatic 2 (Intab, Sweden), were placed in the same 1 L bottle that contained the *Ascaris suum* egg bags in order to measure temperature at the different depths throughout the 21 days of each experimental set.

N-total and TAN

Before any analysis on the samples was performed, the spectrophotometer, which was used to measure the concentration of both N-tot and TAN in the samples, was tested for accuracy. It did not require making a calibration curve, since the test kits used contained the necessary calibration equipment (Figure 9). The test kits, Nitrate Test (Spectroquant® 109713; Merck, Germany) and Ammonium Test (Spectroquant® 100683; Merck, Germany) were used on samples that contained known amounts of nitrate and ammonium, respectively. The samples were then pipetted into empty test tube cells and measured using the Spectroquant® NOVA 60 (Merck, Germany) spectrophotometer, which showed readings close to that of the known concentration.

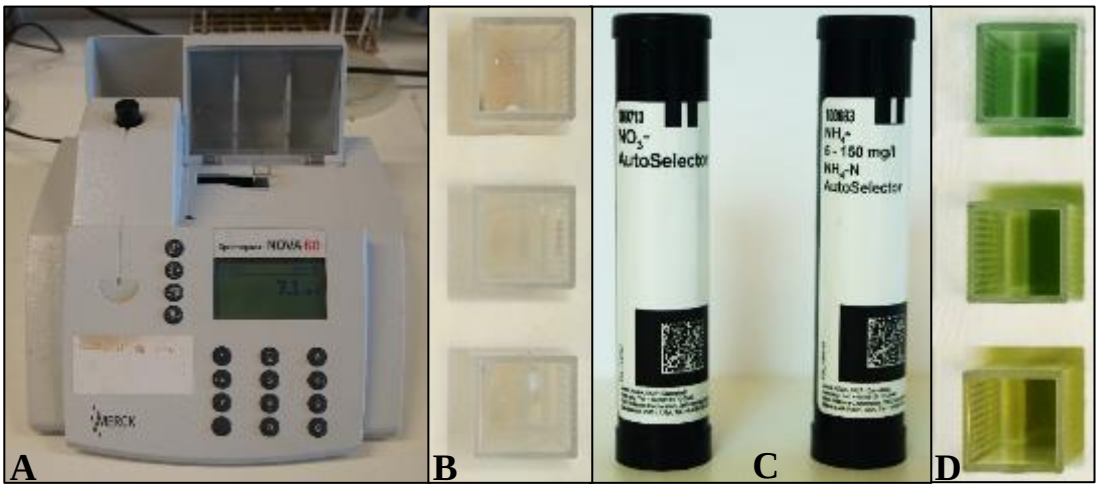

Figure 9. Depicts the spectrophotometer (A) for reading the test cells of the Nitrate test (B) and the Ammonium test (D) using provided calibration tools (C) (Photos: Astrid Max)

In order to assure that any ammonium losses were kept at a minimum, 2 ml of sample were removed each for N-tot and TAN analyses before any other analyses were performed. After shaking the sample containers to ensure homogeneity, a syringe was used to remove a sample of blackwater. For the TAN analysis, the blackwater was then pressed through a 0.45 µm filter (Sarstedt, Germany), while for the N-tot the sample remained unfiltered, to include particulate matter in the total nitrogen analysis. The samples were then kept refrigerated in sealed containers at 4°C until they were analyzed.

The analysis for N-tot began by first diluting the sample with deionized water 100 times for samples without the added urea and to 1000 times for samples after the addition of the 1% urea in order to be within the analysis range of the test kit, these dilutions were based on prior knowledge of approximately how much nitrogen was to be expected. The "Crack Set 20 for the digestion of nitrogen (total)" (Spectroquant® 114963; Merck, Germany) (Appendix I) was first used in order to transform any organic and inorganic nitrogen compounds into nitrate, by heating the sample together with an oxidizing agent in a thermoreactor (TR 420 and TR 320; Merck, Germany). The nitrate (i.e. total nitrogen) concentration in the pretreated sample was then subjected to the Nitrate Test kit (Appendix II), after which the various nitrate concentrations were measured using the spectrophotometer.

For the TAN analysis (Appendix III) the filtered samples were diluted with deionized water either 10 times if the sample had no addition of urea, or 100 times for samples that had the addition of the 1% urea, to make sure the concentration in the sample was within the range of the test kit, these dilutions were based on prior knowledge of approximately how much ammonium was to be expected. The Ammonium Test kit was then applied to the diluted samples, after which the spectrophotometer was used to measure the various ammonia concentrations.

Theoretical Nitrogen

The theoretical Nitrogen concentration was calculated by adding the average of the measured Nitrogen concentration (mg L^{-1}) of the blackwater mixture before the addition of the 1% urea on day 0 and the available Nitrogen in the added urea. The Nitrogen in the added 1% urea can be calculated as follows: 1 Liter of blackwater weighs approximately 1000000 mg, multiplying this by 0.01 (1%) gives 10000 mg of urea per Liter. Urea consists of 46% Nitrogen, which means the urea itself can contribute 4600 mg L^{-1} of Nitrogen.

pH

Approximately 7 ml of blackwater sample were removed from the various sampling containers and placed into 15 ml test tubes (Sarstedt, Germany). The samples were then stored at room temperature until they reached 21°C, which is the temperature at which the pH probe (pHC2011 red rod combined

pH electrode; Radiometer analytical, France) was calibrated. A 2-point calibration, from pH 4.01 (Certipur® 1.99001.0001; Merck, Germany) to 12.46 (Orion™ 910112; Thermo Fisher Scientific), was performed using buffer standards, after which the pH of the samples was measured using the MeterLab PHM210 standard pH meter (Radiometer analytical, France).

Total solids

According to the European Committee for Standardization (CEN), the total solids (TS), also called dry matter, obtained from a sample may not have a mass of less than 0.5 grams, which is why 250 grams of sample, divided between 5 drying dishes, was used (CEN, 2007). TS analysis was only performed on the mixed blackwater containing the added urea on the day of setup and then on the mixed material from the final day (day 21). This was done in order to observe any change in the TS content, as well as to ascertain the TS content of the final material which is to be used as fertilizer.

After shaking the sample containers, approximately 50 g of sample were poured into 0.5 L Toppits® aluminum foil pans and then placed into a chamber furnace oven (Model LH 30/12; Nabertherm GmbH, Germany) for drying, where they remained at 105°C for 24 hours. The samples cooled to room temperature and were then weighed. The change in weight of the sample was then used to calculate TS (Eq. 1).

$$TS\ (\%) = 100 \cdot \frac{w_d}{w_w} \qquad \text{(Equation 1)}$$

Where w_d is the dry weight of the samples, after it's time in the oven, and w_w is the wet weight of the initial blackwater sample.

After drying the samples were returned to the oven and burned at 550°C for 4 hours, allowed to cool back down to room temperature, and then weighed in order to calculate the ash content.

Microbiological analysis

The sections below provide a more in depth description of the microbiological analysis methods performed on the blackwater and *Ascaris suum* egg samples (Figure 8).

Microbial inactivation

The microbial concentration in a sample is commonly calculated as colony or plaque forming units (CFU or PFU) per gram of TS. However, since the TS was not measured on each sampling day, to be more accurate, results are expressed as CFU or PFU per milliliter. CFU or PFU ml^{-1} is based on the raw data, since it is calculated using the number of colonies counted on a plate and the dilution of the sample plated.

Additionally the decimal reduction time (D_r), or the amount of time required at set of conditions to kill 90% or $1\log_{10}$ of the measured microorganism, is calculated for each microorganism. To calculate the D_r – value, the k_D - value, which is the change in concentration over the change in time, must first be calculated (Eq. 2).

$$k_d = \frac{(log_{10}n_t - log\ _{10}n_0)}{t} \qquad \text{(Equation 2)}$$

After which the D_r – value can then be calculated (Eq. 3).

$$D_r = \frac{1}{|k_d|} \qquad \text{(Equation 3)}$$

Ascaris suum eggs

Ascaris suum egg bags, which came from sieved swine feces and had an original concentration of 100,000 eggs per ml (Excelsior Sentinel, Inc, USA), where added to the blackwater. In order to reach a number of 10^4 eggs per bag, 0.2 ml of egg solution was put into each sterile "tea bag" with a pore size of 28µm (Bigman AB, Sefar) using a 1.2 x 50 mm needle and attached syringe (Neolus® Hypodermic, Terumo Europe NV). The injection hole in the "tea bag" was then sealed using a hot glue gun.

Three "tea bags" were placed into a 1 L plastic bottle (Figure 10). There was one plastic bottle per sampling height, e.g. 0 m, 1.6 m, and 3.2 m. These 1 L bottles had holes drilled into them to assure that blackwater could flow through them, and were lined with a plastic mesh to assure that the *Ascaris suum* egg bags would remain within. A weight was attached at the bottom to make sure the bottles didn't move about and floatation devices were attached at 1.6 m and 0 m to assure that the plastic bottles remained at the desired heights.

During sampling, one *Ascaris suum* egg bag was removed on days 7, 14, and 21 for each height, placed into 0.1 N sulfuric acid, and then incubated at 28.5°C for 30 days. After the 30 days, the bags were removed and washed with NaCl peptone water containing the detergent Tween (SVA, Sweden), to release eggs stuck to the bag material. Next, 0.1 ml of eggs along with some "NaCl peptone water with Tween" were removed with a 1.2 x 50 mm needle and attached syringe, and placed on a 76 x 26 mm microscopic slide and then covered by a 26 x 60 mm slide cover (Thermo Fisher Scientific Inc.). The different stages of larval development, or lack thereof were then studied with a 20x magnification under a Zeiss AX10 microscope (Zeiss International) (Figure 10).

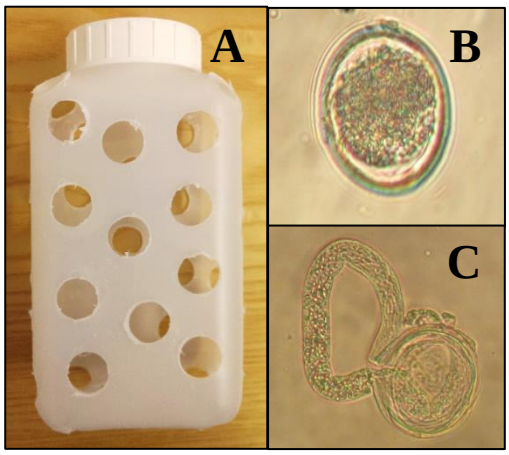

Figure 10. The plastic 1 Liter bottle without the inner plastic mesh holding the Ascaris egg filled "tea bags" during the study (A), a depiction of Ascaris suum egg (B), a depiction of Ascaris suum larvae (C)

Salmonella spp.

In each set, the detection of *Salmonella* spp. was performed on the three samples from the untreated sample in the well (day 0), in addition to one sample, chosen at random, from each sampling height (0 m, 1.6 m, 3.2 m) on the other days, until no more *Salmonella* spp. was detected. A 1:9 dilution was prepared in sterile blender bags (VWR International) with 50 grams of the blackwater sample and 450 grams of buffered peptone water (BPW) (SVA, Sweden). The samples were then incubated at 37°C for 18 hours.

The next step involved the use of Modified Semisolid Rappaport Vassiliadis (MSRV) agar (SVA, Sweden), on which 3 drops of the pre-enriched sample were placed (Koyuncu & Haggblom, 2009). Additionally a control was made using nutrient broth (NB) (SVA, Sweden) and a colony of *Salmonella typhimurium* strain WG49 (ATCC® 700730™). These plates were incubated at 41.5°C for 24 hours. If the sample plates did not appear positive after 24 hours, they were incubated for an additional 24 hours. If they were still negative after 48 hours, then the samples were considered to be *Salmonella* spp. negative.

If the sample plates appeared to be positive after 24 or 48 hours, then a Xylose lysine deoxycholate (XLD) agar plate (SVA, Sweden) was inoculated with a sample taken from the ring surrounding the inoculation droplet on the positive MSRV plate. The same was done for the positive control. The XLD plates were then incubated at 37°C for 24 hours. If black colonies grew on the XLD, which is how *Salmonella* spp. should appear, then a last step was taken to confirm *Salmonella* spp. One colony was removed from the positive XLD plate and inoculated into a triple sugar iron (TSI) slant test (SVA, Sweden), while a second colony was removed and placed into urease broth (SVA, Sweden). Both tests

were incubated at 37°C for 24 hours. The TSI test was positive for *Salmonella* spp. if the agar at the butt of the tube was yellow, the path where the inoculating needle stabbed into the agar was black, there was gas formation, and if the slant part of the tube remained red. Additionally, if the urease broth test remained negative (yellow color), then *Salmonella* spp. was confirmed.

Thermotolerant coliform bacteria and E.coli

Chromocult agar plates were prepared by mixing Chromocult Coliform agar (Merck, Germany) with boiling deionized water. Some plates also received an "*E. coli*/Coliform Selective-Supplement" (Merck, Germany), that was added to the liquid agar once it reached below 50°C. The liquid agar was then pipetted into 90 x 15 mm petri dishes and allowed to cool, after which it was stored at 4°C until it was used.

A 1:9 dilution series of the blackwater with "NaCl peptone water with Tween" was performed on each sample. Once the desired dilution was reached, 0.1 ml of sample was pipetted onto a Chromocult agar plate and then spread out. These plates were then incubated at 37°C for 24 hours.

Once the number of colonies growing on the lowest dilution became very low (1-5 colonies), to analyze how many TtC bacteria remained, 1 ml of direct sample was spread between five Chormocult plates with the selective agent, i.e. each plate contained 0.2 ml of sample. This was also done on the following sampling day to confirm that if there were any TtC left, the number was below 1 CFU per ml of sample.

Enterococcus spp.

A 1:9 dilution series of the blackwater with "NaCl peptone water with Tween" was performed on each sample. Once the desired dilution had been reached, 0.1 ml of sample was pipetted onto Slanetzy Bartley (SlaBa) agar (SVA, Sweden) and spread out. These plates were then incubated at 41.5°C for 48 hours. They were not incubated at the normal 44°C due to a lack of available incubators, and because the MSRV agar which tested for *Salmonella* spp. had an extremely small allowed temperature variation range (±0.5°C). This is different from *Enterococcus* spp., which grows well in the range of 37-44°C, however, with higher specificity at 44°C (Nordin, pers).

When the concentration of *Enterococcus* spp. was low in the lowest possible dilution (diluted 10 times), enumeration was performed by dividing 1 ml of direct sample between 5 plates (0.2 ml of sample per plate). However, no additional selective agent was added to the agar.

F-RNA bacteriophages and somatic coliphages

Salmonella typhimirium strain WG49 (ATCC® 700730™) was cultivated on BLÅ agar plates (SVA, Sweden) through incubation at 37°C for 24 hours, to serve as host bacteria for the f-RNA bacteriophages. However, only the lactose producing colonies which can be identified by their yellow color on the BLÅ agar plates can be used. The yellow color indicates that the bacteria contain a plasmid for F-pili production, which is needed by the male-specific f-RNA bacteriophages, since the F pilus which is produced is the viral receptor (Cefas). To test if the host bacteria was working properly positive controls were made using the yellow colonies, and it was discovered that the BLÅ agar plates had to almost exclusively grow yellow colonies, not just one or two colonies per plate, in order for the host bacteria and subsequent plaque formation to work properly.

E.coli 13706 (ATCC® 13706™) was cultivated on horse blood agar plates (SVA, Sweden), through incubation at 37°C for 24 hours, to serve as host bacteria for the somatic coliphages.

On sampling days, a yellow colony off a BLÅ agar plate and a colony from the blood agar plate were added to flasks containing NB, and then incubated at 37°C for approximately 3 hours while being shaken. During these three hours, a 1:9 dilution series of the blackwater samples was made using "NaCl peptone water with Tween." The samples were filtered using a 0.45µm filter to reduce the potential of bacterial growth on the blood agar base (BAB) agar plates (SVA, Sweden). Next, soft BAB agar (SVA, Sweden) was heated up to 44°C in order to make it liquid, after which 2 ml were pipetted into test tubes which sat in a QBD4 heating block at 49°C (Grant, Cambridge). Once the three hours were over, 1 ml of one host bacteria and 1 ml of sample were pipetted into each BAB agar containing test tube. The mixture was then vortexed and poured onto a BAB plate. A positive and negative control were also made using 1 ml of water in the place of 1 ml of sample. Five drops of phage ϕx174 (ATCC® 13706-B1™) with a 10^8 concentration were then added to the positive somatic coliphage plate containing *E.coli* 13706, while 5 drops of f-RNA phage MS2 (ATCC® 15597-B1™) with a 10^{11} concertation were added to the positive f-RNA bacteriophage plate containing *Salmonella typhimirium* WG49. After the BAB agar had cooled and hardened, the plates were turned over and incubated at 37°C for 17-24 hours.

Statistical analysis

The statistical analysis of the raw data was performed using Excel 2013 (Microsoft, USA) and Minitab 17 (Minitab Inc., USA). Excel was used to average the raw data and perform simple calculations needed to generate the logarithmic and decimal reductions. Additionally, Excel was also used to generate the graphs used to present the analyzed data as well as run the model to predict *Ascaris suum* egg inactivation (Fidjeland *et al.*, 2015a).

The first analysis performed on all collected data in Minitab was to test for a significant difference between the three depths from which samples were taken (0 meters, 1.6 meters, and 3.2 meters). This was done to see if there would be stratification of the urea and thus nitrogen and ammonia distribution, which would in turn affect the inactivation of the tested microorganisms. A single factor analysis of variance (ANOVA) in combination with Tukey's Honestly Significant Difference (HSD) test was used to perform this analysis. To analyze the *Ascaris suum* data, a multiple comparisons for proportions test was run in Minitab, where the proportion between the number of larvae and the total number counted was taken to show if there was a significant difference between the three sampling heights per day. Lastly Minitab was also used for general regression analysis of the relationships between the chemical parameters; TAN, N-tot, un-ionized ammonia, pH. All analyses were performed with a 95% confidence interval and a p-value <0.05 representing a significant difference.

Research limitations

The research limitations of this study are as follows:

- A lack of a control to allow for the analysis of the effect of the physiochemical properties on the inactivation of the microorganisms.
- A lack of temperature data for set 2 because of the thermometers breaking.
- Unexplainable microbial growths on somatic coliphage plates in set 2, likely causing less accurate data because plaques could not be identified properly and counted.
- The inability to grow the necessary host bacteria for the f-RNA bacteriophages in set 1, resulting in no data on f-RNA bacteriophages for that set.

5 Results

Total solids

The TS of the blackwater was taken on the initial day (Day 0), after the heated blackwater ($60m^3$) and 1% urea were added to the blackwater at ambient temperature ($100m^3$) in the well and mixed for one hour. The TS was then taken again on the final day (Day 21) after the blackwater had been mixed again for one hour. These results of these samplings can be seen in Table 5.

Table 5. Results of the TS analysis of sets 1 and 2 including the standard deviations of the mean

	Total solids (%)	Ash (% of TS)[a]	Volatile solid (% of TS)
Set 1 Day 0	0.688 ± 0.147	17.5 ± 2.72	82.5 ± 2.72
Set 1 Day 21	0.351 ± 0.0698	46.8 ± 25.4	53.2 ± 22.8
Set 2 Day 0	0.375 ± 0.0403	44.9 ± 5.37	55.1 ± 5.37
Set 2 Day 21	0.262 ± 0.0367	47.6 ± 6.60	52.4 ± 6.60

a) The grams of ash weighed during each sampling was similar, the concentration on set 1 day 0 is only different due to the higher TS.

In the first set it appears that after the 21 days the TS had decreased to 0.35%, while the ash content remained similar; this is likely due to decomposition of organic matter. The decrease of VS during this time supports that decomposition is the likely cause, since it represents the amount of organic matter present. In set 2 the TS was already lower to begin with than in set 1, however there was still a slight decrease, again likely due to some decomposition.

Nitrogen and pH analysis

The concentration of total Nitrogen was measured at the three sampling depths. Single factor ANOVA showed that there was no significant difference on any of the sampling days in both sets, which is why the average measured value for the sampling day was used (Figure 11). The total Nitrogen concentration increases in the first few days due to the added urea, after which it is expected to remain constant. This was supported by a linear regression analysis, which showed that overall there were no significant trend of increase or decrease in the total Nitrogen concentration throughout the 21 days in either set 1 (p-value = 0.423) or set 2 (p-value = 0.662).

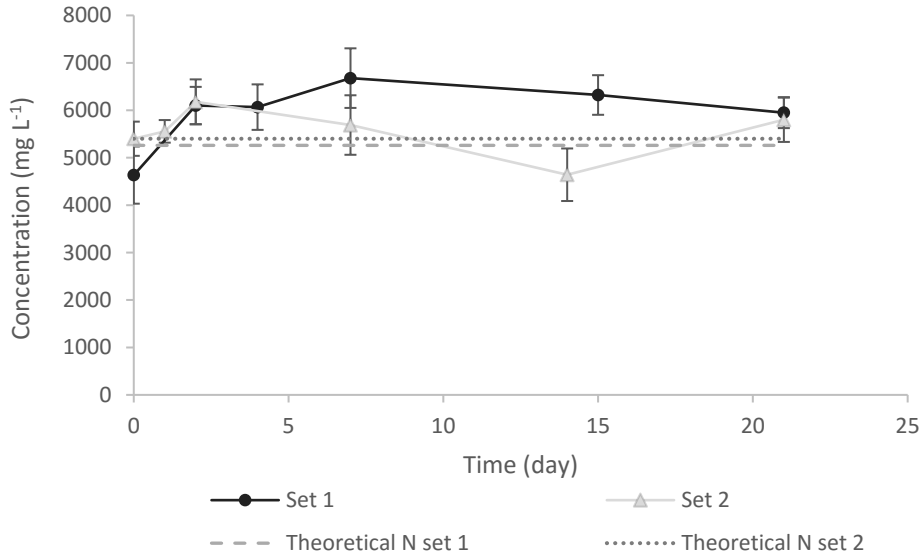

Figure 11. Total Nitrogen concentration in both sets over time including error bars to show the standard deviation

The theoretical total Nitrogen concentrations were calculated by adding the initial total Nitrogen concentration in the blackwater to the concentration of Nitrogen from the added urea (1% ww). While the theoretical Nitrogen for set 1 was 5260 mg L^{-1} and for set 2 was 5400 mg L^{-1}, it can be seen (Figure 11) that the averages are often above these values with rather large standard deviations. This could be due to variations in the organic Nitrogen concentration in the blackwater, caused by large particles. These particles may not be spread evenly throughout the mixture and are included in the results since the samples undergoing total Nitrogen analysis are not filtered.

The single factor ANOVA test for the TAN concentration between the three layers showed that there was no significant difference on the majority of the sampling days. The two days which showed a significant difference between the three layers were day 4 from set 1 and on day 14 from set 2.

On day 4 from set 1, the average values for each layer vary between the top (3500 mg L^{-1}), middle (1900 mg L^{-1}), and bottom (2133 mg L^{-1}). While statistics showed that there was not a significant difference between the middle and bottom layer, there is a significant difference between them and the top layer. This large variation between the middle and bottom to the top layer can only be explained by randomness in sampling. The concentration does not show stratification, since then the TAN concentration would be expected to be higher at the bottom rather than at the top. The trend of the TAN concentration is also not reflected in the trend of the total Nitrogen concentration, which would have to show that the concentration of total Nitrogen is highest in the top layer, and that the top layer is significantly

different from the other two layers, which is not the case. So, the average and standard deviation for day 4 in set 1 are used since the value (3500 mg L^{-1}), which caused there to be a significant difference, cannot be explained and is therefore considered to be an outlier. This allowed for the use of the daily average for set 1 (Figure 12).

On day 14 from set 2, there was no significant difference between the top (5600 mg L^{-1}) and middle layer (5500 mg L^{-1}), but that these layers were significantly different from the bottom layer (4300 mg L^{-1}). This significant difference cannot be due to stratification since the bottom layer has a lower TAN concentration than the top two layers. The TAN concentration does however reflect the variations in the total Nitrogen concentration, where the top (6033 mg L^{-1}) and middle (6067 mg L^{-1}) layer had similar concentrations and the bottom layer (4967 mg L^{-1}) had a much lower concentration. However, the single factor ANOVA test considered the total Nitrogen concentrations of the three layers to be not significantly different. Since the bottom TAN concentration likely resulted from the lower total Nitrogen concentration in the bottom layer, which did not show a significant difference, the average along with the standard deviation will also be used for day 14 from set 2 is used. This made it possible to use the daily averages for set 2 (Figure 12).

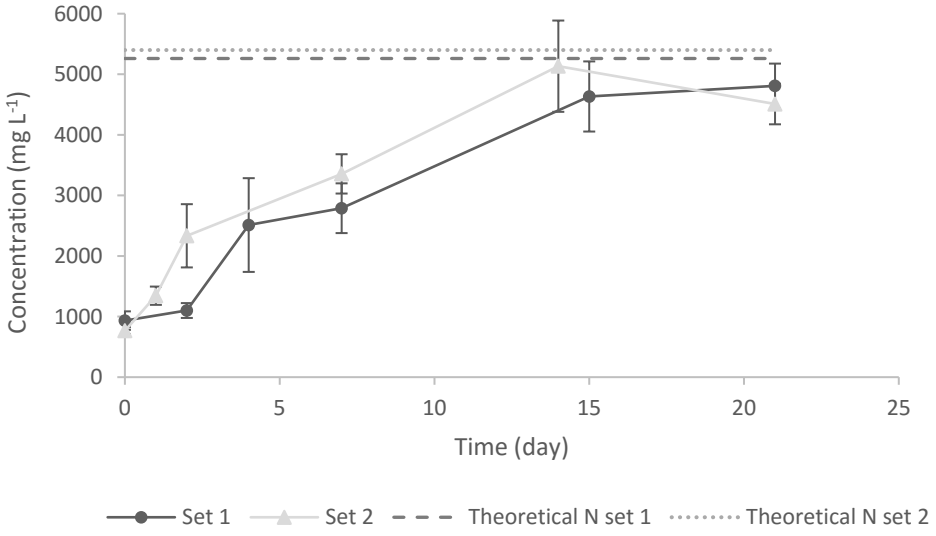

Figure 12. TAN concentration of both sets over time including error bars to show the standard deviation

While the total Nitrogen concentration should remain constant over time, the TAN concentration should be increasing, since the Nitrogen from the urea is continuously transformed into ammonia. This can be seen in Figure 12, where the trend of the TAN concentration appears to be increasing until around

day 14, after which it appears to reach a steady state, while the total Nitrogen concentration is represented by the theoretical Nitrogen concentration. A linear regression analysis of the TAN concentration during the first two weeks confirmed that the increase for both set 1 (p-value = 0.004) and set 2 (p-value = 0.005) was significant. When TAN reaches a steady state it means that the urea has been fully degraded, at which point its concentration should be close to the total Nitrogen concentration, as seen in Figure 12 around day 14.

A single factor ANOVA test for pH between the three layers shows that there was no significant difference on the majority of the sampling days. The only days which showed a significant difference between the three layers were on day 4 from set 1 and day 3 from set 2.

On day 4 from set 1, the average pH values for each layer vary from the top layer (9.3) to the middle (9.2) and bottom (9.2) layers. While statistics show there is not a significant difference between the middle and bottom layer, there is a significant difference between them and the top layer. Since TAN and pH are known to affect each other, the trend in the pH can be explained by the TAN concentration (Nordin *et al.*, 2009b). On day 4 in set 1, for both the pH and TAN concentrations, the middle and bottom layers were not significantly different from each other but were significantly different from the top layer. However, since the daily average of the TAN concentration is used and the variable causing a significant difference is considered to be an outlier, the same principle is applied to pH. Additionally, the significant difference is only 0.1 pH-unit, which holds no practical meaning. Therefore, the daily averages are used for set 1 (Figure 13).

On day 3 from set 2, the average pH values for each layer vary from the top (9.1) to the middle (9.0) to the bottom (9.1). The single factor ANOVA test stated that pH of the top and bottom layers and the middle and bottom layers are not significantly different, while the top and middle layer are significantly different. This trend in the pH can be explained by the TAN concentrations; TAN was highest in the top layer, second highest in the bottom layer, and lowest in the middle layer. However, there was no significant difference in the TAN concentration on this day. Additionally, the significant difference is only 0.1, which holds no practical meaning. This is why the daily averages are also used for set 2, with the variations visualized in the figure by error bars showing the standard deviation from the mean (Figure 13).

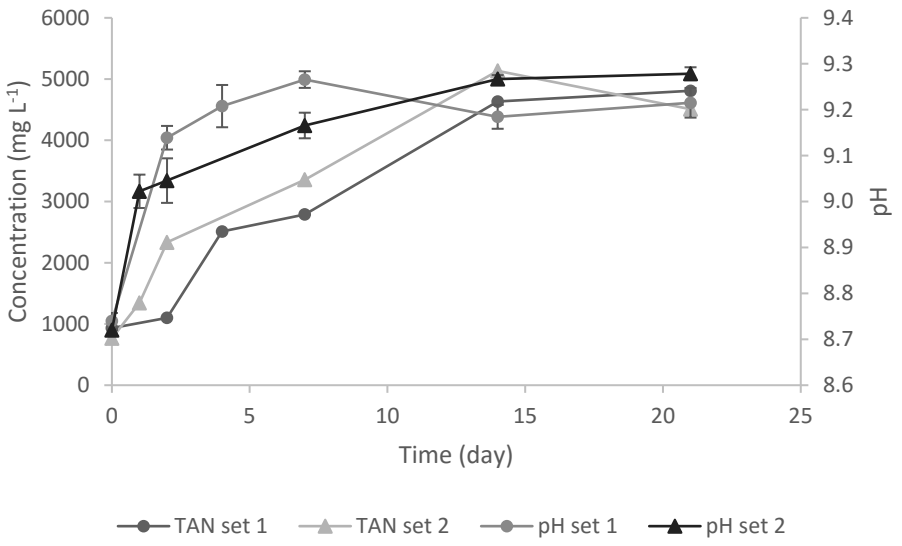

Figure 13. TAN and pH change over time including error bars to show the standard deviation of the pH

Urea itself is expected to create a more alkaline environment even before it is degraded (OECD, 2005). When urea is degraded into TAN it is expected that the pH will continue to increase until it reaches a steady state. The pH increased only at the beginning and then remains somewhat constant once it had reached a steady state (Figure 13).

Due to missing temperature data for set 2, the temperature data from set 1 was used to calculate the un-ionized ammonia concentration of set 2. Temperature measurements were taken every 10 minutes. However, the slight differences in temperature at the different depths did not affect any of the investigated parameters. So, the average daily temperature of the three layers will be used for any calculations or correlation tests involving temperature.

Using the temperature, TAN, and pH the concentration of un-ionized ammonia, which is the compound that actually causes the disinfection, can be calculated with Equations 4, 5, and 6.

$$pK_a = \frac{2728.92}{T + 273.15} + 0.090181$$ (Equation 4, (Emerson *et al.*, 1975))

$$f_{NH_3} = \frac{1}{10^{(pK_a + pH)} + 1}$$ (Equation 5)

$$[NH_3] = [NH_{tot}] \cdot f_{NH_3}$$ (Equation 6)

Figure 14 below shows the concentration of un-ionized ammonia in comparison to the TAN concentration, theoretical Nitrogen concentration, and total Nitrogen concentration. Since the un-ionized ammonia concentration is calculated using the TAN concentration it should be expected that they follow a similar trend over time. Even though the un-ionized ammonia concentration is much lower than the TAN concentration, it does increase over time like the TAN concentration.

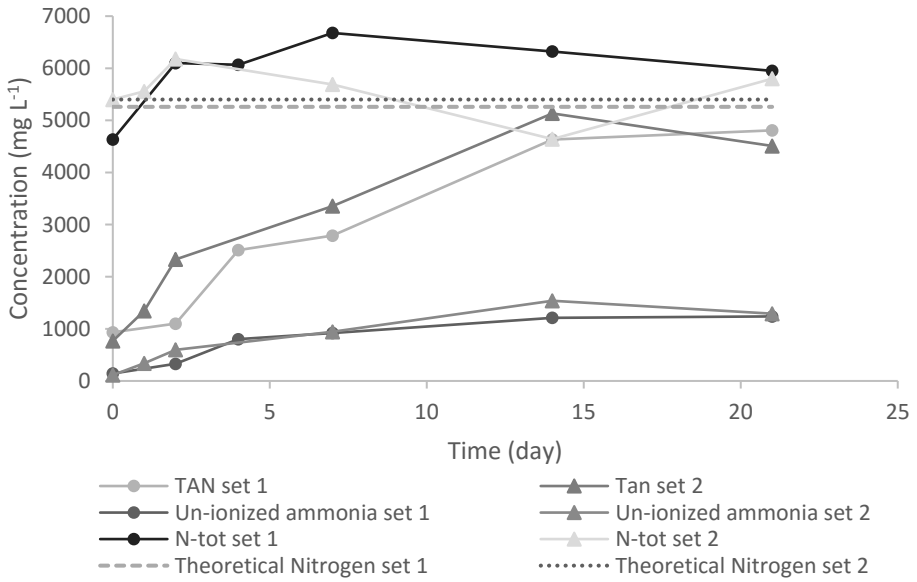

Figure 14. TAN, un-ionized ammonia, and total Nitrogen over time

A linear regression analysis shows that while lower than the TAN concentration, there is a significant and increasing trend over time in the un-ionized ammonia concentration for both set 1 (p-value = 0.016) and set 2 (p-value = 0.019).

Microbiological results

The first analysis performed on all collected data was to test for a significant difference between the three layers from which samples were taken (0 meters, 1.6 meters, and 3.2 meters) using a single factor ANOVA test. The daily averages of the layers were used if there was no significant difference between them.

Ascaris suum egg viability

The proportion of viable *Ascaris suum* eggs in the three layers was compared. The results showed that there was no significant difference on all days in set 2, and only a significant difference on day 21 in set 1.

On day 21 form set 1, the test showed that there was no significant difference between the top (78%) and bottom (81%) layer, but that these two layers were significantly different from the middle (65%) layer. The first time the number of eggs and larvae were counted for day 21 from set 1, the viability for the middle layer was approximately 58%, which appeared rather low when compared to the viability in the top and bottom layer. So, another 0.1 ml of eggs were analyzed for day 21 from set 1. The viability increased by 7% to 65%, which shows how great the discrepancy can be, most likely caused by an uneven distribution of *Ascaris* eggs and larvae on the microscopic slide during analysis. The outlier cannot be explained through stratification of total Nitrogen and TAN concentration, firstly, because the lowest viability would be expected in the bottom layer, not in the middle layer, and secondly because on day 21 in set 1 there is no significant difference in either the total Nitrogen or TAN concentrations between the layers. Since there was only one outlier (red circle in Figure 15), the daily averages, along with the standard deviation, were used to describe the *Ascaris* viability over the 21 days (Figure 15).

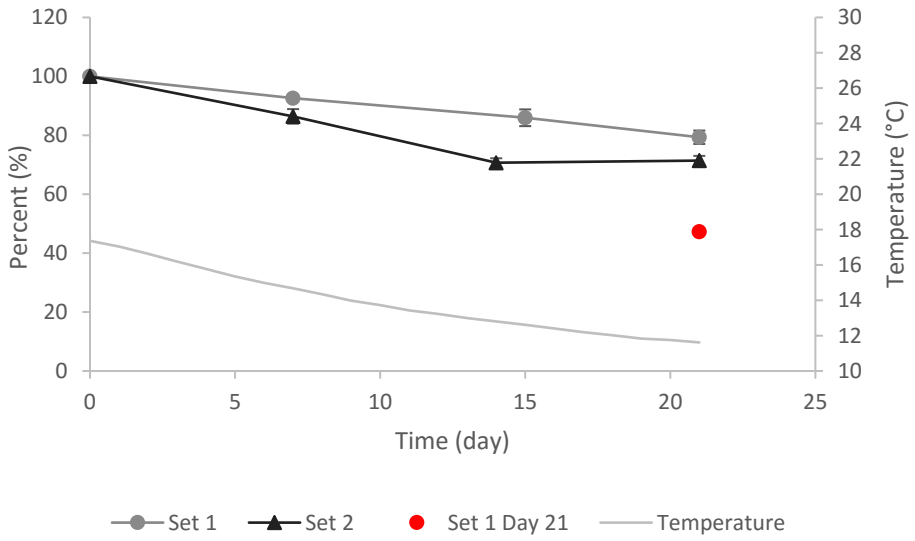

Figure 15. Viability of Ascaris suum eggs over time including the outlier from set 1 (day 21) and error bars representing the standard deviation

A linear regression analysis showed that both set 1 (p-value = 0.02) and set 2 (p-value = 0.044) had a significant decrease in the percent viability over time. However, the D_r – value for both sets showed that quite a long period of time is required to accomplish a $1\log_{10}$ reduction of *Ascaris suum*. The D_r – value varied from 163 days for set 1 to 144 days for set 2.

Salmonella spp.

The concentration of *Salmonella* spp. was qualitatively tested; analyses were done to simply test if there was any *Salmonella* spp. present in the samples per 50 g, and if so how long until no more *Salmonella* spp. could be detected (Table 6). In set 1, day 4 established the concentration of *Salmonella* spp. to be below the detection limit of 1 bacterium per 50 ml of blackwater. In set 2 this occurred on day 2.

Table 6. Salmonella spp. inactivation over time

Day	Set	Layer	Result
0	1	Top	-
		Middle	-
		Bottom	-
	2	Top	-
		Middle	+
		Bottom	+
1	2	Top	-
		Middle	+
		Bottom	-
2	1	Top	+
		Middle	-
		Bottom	-
	2	Top	-
		Middle	-
		Bottom	-
4	1	Top	-
		Middle	-
		Bottom	-

Thermotolerant coliform bacteria and *E.coli*

On the Chromocult agar used for the enumeration of the TtC, two types of colonies grew. The violet colonies were strains of *E.coli*, while the pink colonies were non-*E.coli* coliforms or TtC. The inactivation rates of the *E.coli* and TtC were very similar, *E.coli* was only slightly more sensitive to inactivation than TtC.

The single factor ANOVA test for the E.coli and TtC concentrations between the three layers shows that there was no significant difference in both sets. In set 1, the detection limit (1 CFU per ml) was reached after 7 days, however since no CFU were detected on day 4, the detection limit of the lowest concentration plated on that day (dilution factor 10) is shown (see blue marking in Figure 16). A regression analysis showed that there was a significant reduction in set 1 (p-value = 0.001). In set 2 on day 0 no CFU were detected on any of the plates, which is why it is represented by the detection limit of the lowest concentration (dilution factor 1000) plated on that day (see red marking in Figure 16). A linear regression analysis showed that in set 2 there was also a significant reduction (p-value = 0.002).

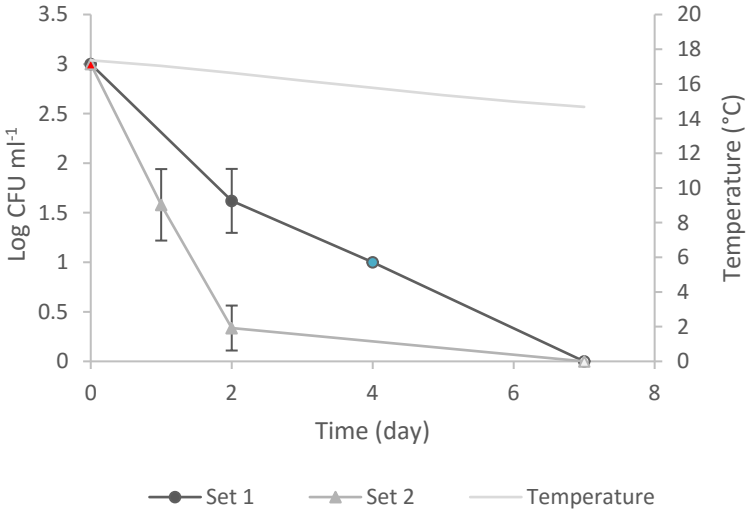

Figure 16. E.coli log CFU inactivation and temperature over time including error bars to show standard deviation

In order to compare the log reductions of the two sets the data was normalized (Figure 17). The fast inactivation of the *E.coli* is reflected in the D_r - value, which was calculated by fitting a linear regression. If the D_r – value were based on the initial concentration and the first sampling day that gave 0 CFU (day 7), the D_r - value for set 1 and 2 would be 2.3 days. When the calculations are based on the regression lines, the D_r – value for set 1 becomes 2.4 days and the D_r – value of set 2 changes to less than 1 day

(approximately 18 hours). This is because the linear regression line of set 2 has a steeper slope when day 7 is not included, which shows that CFU per ml would reach 0 between days 2 and 3, and not first on day 7 (Figure 17). This is different to set 1, which has a more accurate trendline (greater R^2 value) when day 7 is included.

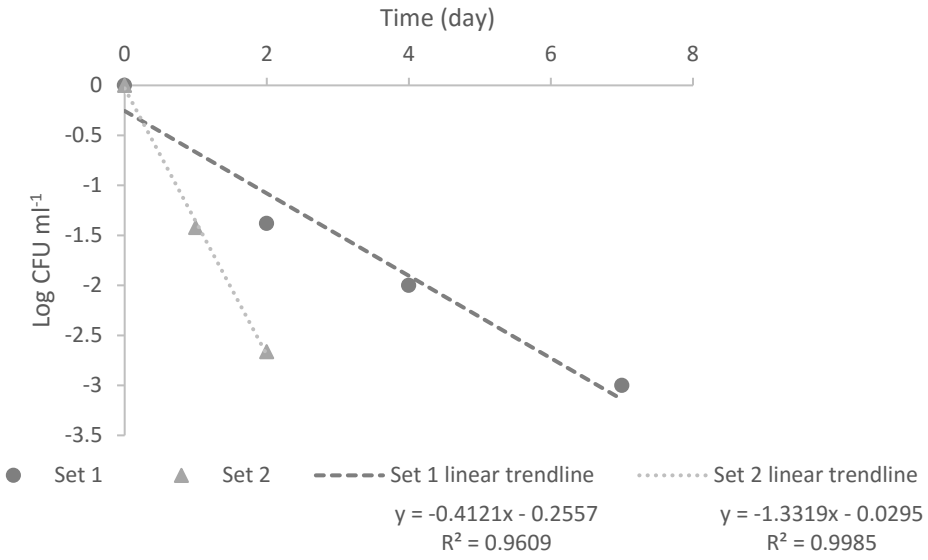

Figure 17. Normalized log reduction of E. coli over time

Enterococcus spp.

The single factor ANOVA test of the *Enterococcus* spp. concentrations between the three layers showed that there was no significant difference in both sets except for on day 21 from set 1. On this day there was no significant difference between the three layers, but there was a significant difference between the layers and the concentration of *Enterococcus* spp. after the blackwater had been mixed. So the difference is most likely due to microbes that had settled out being redistributed in the blackwater, resulting in higher concentrations throughout the mixture. So, since there was no difference between the layers the daily averages were used to show *Enterococcus* spp. inactivation over the 21 day time period (Figure 18). The outlier caused by the re-mixing in set 1 on day 21 was not used in the daily average, as shown by the red circle in Figure 18.

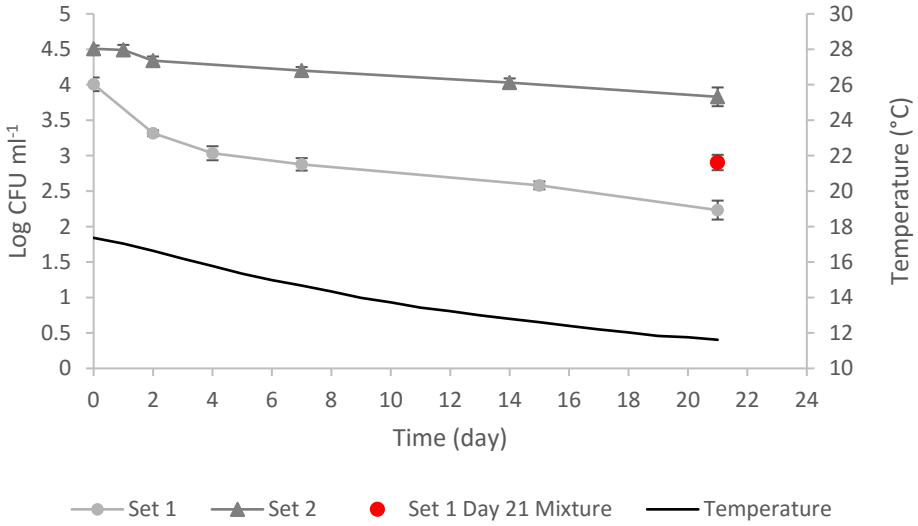

Figure 18. Enterococcus spp. inactivation and temperature over time including error bars to show standard deviation

A linear regression analysis was performed on the two sets to see if there was any significance in their reduction over the 21 days. In set 1 there was no significant reduction (p-value = 0.061), while in set 2 there was (p-value = 0.000). The insignificance in set 1 can be explained by the low log reduction by day 21 (approximately 0.6 log reduction), while over a 1 log reduction was reached at the same time in set 2 (Figure 19). In order to compare the log reductions of the two sets the data was normalized (Figure 19). The rather slow inactivation of *Enterococcus* spp. is reflected in the D_r - value, which is 14.6 days for set 1 and 31.9 days for set 2.

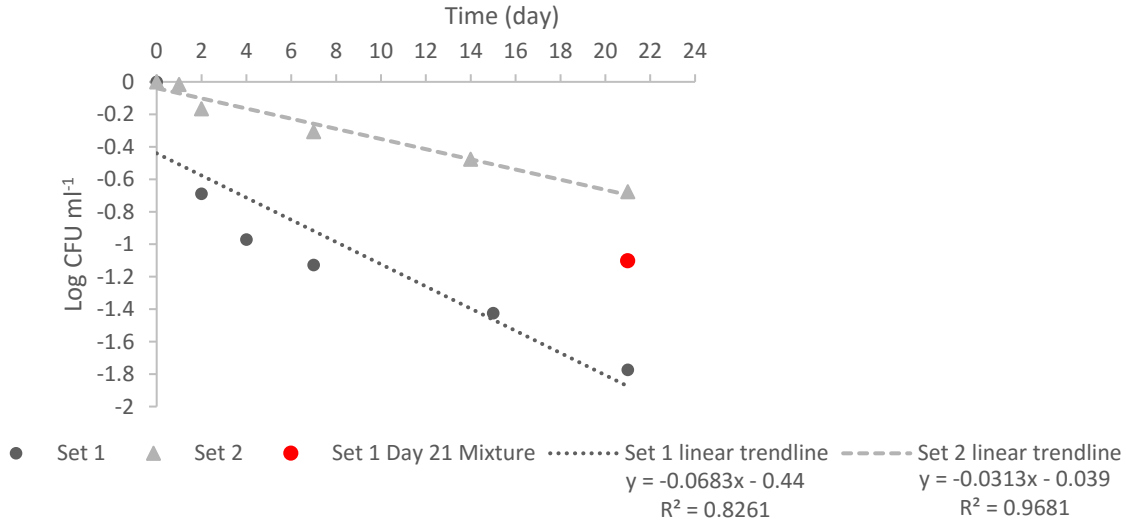

Figure 19. Normalized log reduction of Enterococcus spp.

F-RNA bacteriophages

Due to a lack of proper host bacteria, no f-RNA bacteriophages could be cultivated and counted from set 1, which is why there is only data for set 2. The single factor ANOVA test for the f-RNA bacteriophage concentrations between the three layers of set 2 showed that there was no significant difference on any of the sampling days. The concentration of the f-RNA bacteriophages reached below the detection limit by day 14 (0 PFU per ml). A linear regression analysis of the data did not find that the $2\log_{10}$ reduction within 14 days was significant (p-value = 0.087). The inactivation of f-RNA bacteriophages is reflected in the D_r - value, which was calculated as 6.8 days for set 2.

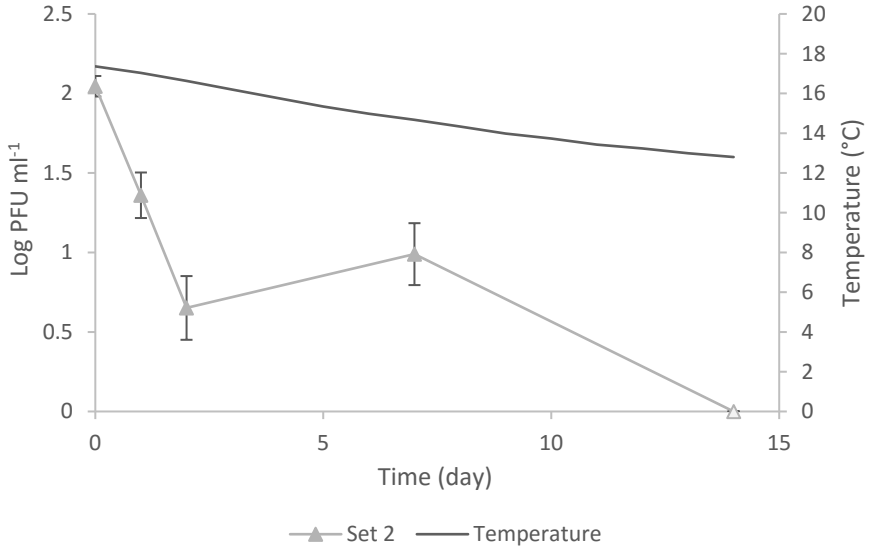

Figure 20. F-RNA inactivation and temperature over time

Somatic coliphages

The single factor ANOVA test of the somatic coliphage concentrations between the three layers showed that there was only a significant difference on day 14 from set 2. Here the somatic coliphage concentrations varied between the top (6.0 log PFU ml^{-1}), the middle (5.6 log PFU ml^{-1}), and the bottom (5.7 log PFU ml^{-1}) layers. ANOVA showed that there was no significant difference between the top and the bottom layer and the middle and the bottom layer, there was however a significant difference between the top and the middle layer. This significant difference is most likely due to the fact that there was strong microbial growth on the surface of the media which made it difficult to both identify and count the plaques. This occurred throughout set 2 and can be seen in Figure 21 by its fluctuating somatic coliphage concentrations. Since the discrepancy could be explained, the daily averages along with the standard deviations from the means, were used for all sampling days (Figure 21).

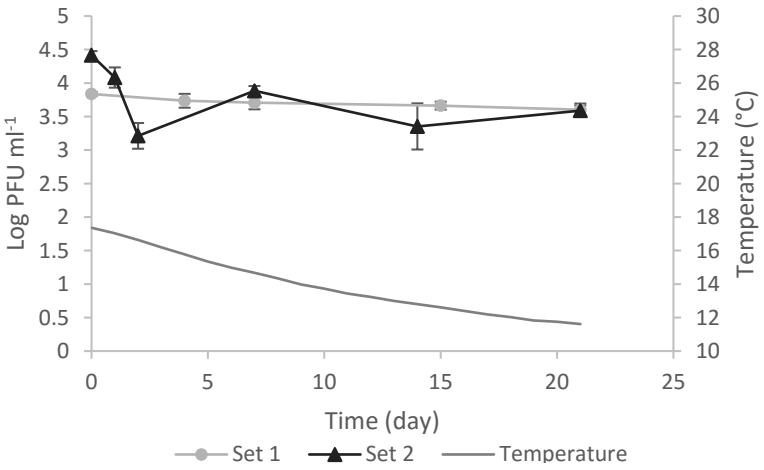

Figure 21. Somatic coliphage reduction and temperature over time

A linear regression analysis was performed on the two sets to see if there was any significant reduction over the 21 days. In set 1 the reduction was found to be significant (p-value = 0.012), while in set 2 it was not significant (p-value = 0.347). The insignificance in set 2 is possibly caused by the fluctuating concentration of the somatic coliphages due to the inability to properly count the plaques because of the strong microbial growths. In order to compare the log reductions of the two sets the data was normalized (Figure 22). The rather slow inactivation of the somatic coliphages is reflected in their D_r - value, which was 102 days for set 1 and 39.2 days for set 2.

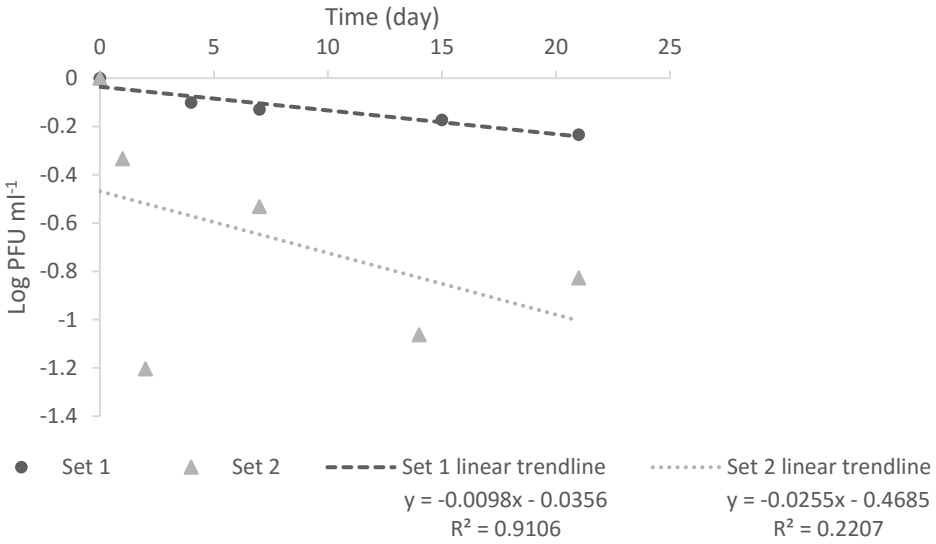

Figure 22. Normalized log PFU reduction of somatic coliphages

6 Discussion

The aim of this study was to test how effectively 1% urea (ww) would sanitize low temperature blackwater within a 21 day period. The inactivation of pathogenic and indicator microorganisms, along with physiochemical properties such as pH, temperature, TAN, total Nitrogen and TS were tested for in the blackwater samples at three different heights. This was done to observe if stratification took place. However, with the exception of a few outliers, there was no significant difference found between the three measured heights for all physiochemical properties and microorganisms. This contradicts findings by Höglund *et al.*, (2000) where the pH, TS, Nitrogen compounds, and microorganisms were found in higher concentrations at the bottom of the urine containing tank than at the top by the end of their 126 day study period. The major difference between the study by Höglund *et al.*, (2000) and the study performed in this paper is the length of the study period. The study period from Höglund *et al.*, (2000) is over 100 days longer with sampling occurring at much larger intervals. Due to the brevity of the study at Hölö presented in this paper (21 days), gravitational settlement of physiochemical properties and microorganisms did not occur in any significant manner. Extending the study period at Hölö might result in a similar stratification occurring as in the study by Höglund *et al.*, (2000) .

Physiochemical properties

Overall, the addition of urea increased the TAN concentration by approximately 5 times from its initial concentration; reaching full urea degradation around day 14. The pH was stable starting around day 7, indicating that enough TAN and carbonates have formed to buffer the pH. Finally, the total nitrogen concentration was stable, with the exception of some small insignificant variations, throughout the study. This indicates that any ammonia losses were minor despite the fact that the container was opened for sampling 6 times over 21 day study period.

The addition of urea to the blackwater is expected to cause an increase in the pH both through urea itself, which is alkaline, and through the degradation of the urea into ammonia and carbonate, both of which cause microbial inactivation (OECD, 2005; Diez-Gonzalez *et al.*, 2000). The degradation of urea is dependent on a variety of factors, including the temperature, the initial pH, the TS, and the amount of urea added to the treated material (Fidjeland *et al.*, 2013a; Vinnerås *et al.*, 2008; Williams *et al.*, 1984). However, an increased TAN concentration is not always needed to increase the un-ionized ammonia concentration (Vinnerås *et al.*, 2008). Since pH, temperature, and the measured TAN concentration are used to calculate the un-ionized ammonia concentration, a higher pH or temperature along with a lower TAN concentration can give the same un-ionized ammonia concentration as a higher TAN concentration at a lower pH or temperature (Vinnerås *et al.*, 2008).

In this study the overall temperature of set 1 (no temperature data was collected for set 2) decreased by almost 6°C over the 21 day study period, but this did not appear to have a great impact on the urea degradation (affecting un-ionized ammonia concentration) and thus the inactivation rates of the microorganisms. Especially since *Salmonella* spp., TtC/*E.coli*, and the f-RNA bacteriophages were inactivated to below their detection limit. However, the inactivation of these microorganisms happened within a shorter time period, which means there was a smaller temperature decline.

The pH increased quickly during the first week and then reached its steady state, stabilizing above 9.2 in both sets. The TAN and un-ionized ammonia concentrations increased the pH, but only appeared to reach a steady state in the last week. The exception to an increase in the TAN and un-ionized ammonia concentrations was in set 2 on day 21, where the daily average decreased slightly from the daily average of day 14. The decrease can be explained by the fact that on day 14 one of the layers was significantly different than the others, which has an impact on the daily mean. Since the standard deviation was used to show the differences of the measured values to their daily mean, this was also compared, and the confidence intervals were shown to overlap quite a bit for days 14 and 21. So, while there was a decrease it wasn't very large and most likely due to randomness in sampling. If the decrease had been larger it could have been explained either through volatile losses of ammonia, which means the storage container was not airtight, or by the adhesion of the urea to organic matter (Nordin *et al.*, 2009b).

The concentration of organic matter in a material can be estimated using the VS. So, the organic matter makes up a certain percent of the TS, which means the TS concentration can also impact the sanitization of the treated material. The higher the TS, the longer it will take to inactivate microorganisms and thus sanitize the material, especially when the TS is greater than 20%, because it becomes difficult to evenly mix the urea in the material (Nordin, 2010; Nordin *et al.*, 2009b; Williams *et al.*, 1984). Since the 1% urea (ww) was mixed into the blackwater for an hour to ensure an even distribution and the TS of the blackwater in this study was very low (<1%), both the organic matter content and TS should not cause any significant negative effects on the inactivation of the microorganisms.

The total Nitrogen concentration available to be degraded into TAN and un-ionized ammonia is dependent on the intrinsic Nitrogen concentration of the material and the amount of urea added. Once the 1% urea (ww) was mixed into the blackwater and increased the total Nitrogen concentration, it was expected that the total Nitrogen concentration would remain constant which was confirmed by a linear regression analysis. While the changes in the total Nitrogen concentration were not showing any significant trend, there were some fluctuations. These variations could have been caused either by the loss of Nitrogen as volatile ammonia during sampling, or by the small sample size which may make the amount of occurring Nitrogen containing particles random. This arbitrariness which can occur during sampling,

as well as the unevenness in the distribution of the intrinsic Nitrogen containing particles can also explain why the concentration of the measured total Nitrogen is often greater than the theoretical Nitrogen concentration.

Microbiological inactivation

Overall, there was no stratification found between the three measured heights for all physiochemical properties. This contradicts findings by Höglund *et al.*, (2000)

The impact of the physiochemical properties, temperature, pH, TAN, total Nitrogen, and un-ionized ammonia, on the microbial inactivation could not be statistically evaluated because no control study was performed that could be used for comparison.

Ascaris suum eggs

According to the linear regression analysis of the *Ascaris suum* egg inactivation, with 1% urea (ww) and an average temperature of 14°C (the average temperature over the 21 days) it would take between 489 and 432 days to reach the ABP regulation and SEPA 2013 proposed 3log10 reduction (SwedishEPA, 2013; EU, 2011). This reduction time however does not take into account the initial lag phase that is known to be occur during *Ascaris suum* inactivation (Fidjeland *et al.*, 2013b; Nordin *et al.*, 2013; Nordin *et al.*, 2009a; Ghiglietti *et al.*, 1997). During the lag phase the inactivation is very low, however once it is over the *Ascaris* egg inactivation proceeds rapidly (Fidjeland *et al.*, 2013b). Since it is likely that the *Ascaris* eggs in this study are still in their lag phase, using the log reductions to calculate decimal reduction time would cause the value to be much greater than it actually is. Therefore, a model was used to calculate the time it would take to reduce *Ascaris* viability by 3log10 (Fidjeland *et al.*, 2015a). The model revealed that it would take 164 days for the necessary 3log10 reduction.

The lower the temperature (especially below 20°C) and ammonia concentration, the slower the *Ascaris* egg inactivation is (Fidjeland *et al.*, 2015b). When temperature and ammonia concentrations are increased the lag phase becomes shorter while the rapid inactivation phase after the lag phase does not appear to be influenced by the ammonia concentration (Fidjeland *et al.*, 2013b). The influence of the temperature on the *Ascaris* egg inactivation could be explained by the fact that the permeability of lipid layer in the egg shell increases with higher temperatures, allowing for certain substances, gases and lipid solvents (ammonia), to enter into the egg (Nordin *et al.*, 2009a; Wharton, 1980). So, in order to decrease the inactivation time of the *Ascaris* eggs in this study either a greater concentration of urea needs to be added and/or the temperature of the blackwater has to be increased. However, the SEPA 2010 plant regulations do not require an inactivation of *Ascaris suum* eggs since they are not prevalent in Sweden, as opposed to more tropical and subtropical countries in Africa and Asia (WHO, 2016).

Salmonella spp.

The current certification criteria at the plant require that *Salmonella* spp. is absent in 25 g of sample (SP, 2012). However, if the new proposal comes into force, the new regulations will require *Salmonella* spp. to be absent in 50 g of sample (SwedishEPA, 2013), which is why 50 g of sample were used in this study when testing for *Salmonella* spp. This criteria was reached by set 1 on day 4 and by set 2 on day 2, giving an average inactivation rate of 3 days.

The inactivation of *Salmonella* spp. is known to increase with a higher temperature, higher urea and un-ionized ammonia concentrations, and a lower TS (Fidjeland *et al.*, 2013a; Nordin *et al.*, 2009b; Ottoson *et al.*, 2008). A lower TS causes a decrease in the buffering capacity, which in turn allows for a higher pH and un-ionized ammonia concentration to be spread evenly throughout the mixture, using less urea than would be needed for a higher TS (Ottoson *et al.*, 2008). A study testing the inactivation of blackwater at ambient temperature (estimated to be between 10-20°C) with the addition of 0.1% urea (ww), detected no more *Salmonella* spp. after 28 days (Fidjeland *et al.*, 2015b). A study by Vinnerås *et al.* (2008) tested the inactivation of *Salmonella typhimurium* in undiluted urine, which had a similar ammonia concentration to the blackwater in this study; at 14°C they had a decimal reduction time of $<1.2 \pm 5$ days. This range is similar to the 2- 4 day reduction time needed in this study.

Thermotolerant coliform bacteria and *E.coli*

The current certification criteria plant where this study took place require an end product concentration of <1000 *E.coli* g^{-1} (TS) (SP, 2012). Both sets reached the detection limit (1 CFU per ml) within one week. Since the TS was not analyzed on either of these days, the lowest measured TS of each set was used to calculate and verify that 1 CFU per ml corresponded to <1000 CFU g^{-1} (TS).

It has been observed that TtC is less sensitive to inactivation than *E.coli* (Fidjeland *et al.*, 2015b). This was also observed in this study, because the number of TtC colonies was usually greater than *E.coli* colonies. Additionally, in set 1 on day 4 TtC was detected at the lowest dilution but no *E.coli* colonies were detected. However, both TtC and *E.coli* were inactivated in both sets by day 7. Studies have observed that *E.coli* is more sensitive to inactivation than *Salmonella* spp. (Vinnerås *et al.*, 2008; Mendez *et al.*, 2004). In this study however this was not possible to confirm since the starting and end concentration of *Salmonella* spp. was not known.

Enterococcus spp.

Enterococci strains are found naturally in the environment and can grow at a variety of temperatures (10 to 45°C) and at a high pH (9.6) (Byappanahalli *et al.*, 2012). Different strains could be observed while counting the colonies growing on the SlaBa agar since their appearance on the media varied. By the end

of the 21 day treatment, the number of *Enterococcus faecalis* colonies was usually much lower, if there were any growing at all, than the number of colonies of other *Enterococcus* strains, similar to what was observed in a study by Fidjeland *et al.* (2013a).

Studies have found using *Enterococcus* spp. as an indicator for microbial inactivation with ammonia often resulted in an overestimation of the risk since *Enterococcus* spp. are known to be more persistent than gram negative bacteria (e.g. *Salmonella* spp. and *E.coli*), especially at low temperatures (Fidjeland *et al.*, 2015b; Vinnerås *et al.*, 2008; Allievi *et al.*, 1994). However, current plant regulations require the concentration of *Enterococcus* spp. to be <1000 CFU g^{-1} (TS) (SP, 2012). ABP regulations require a 5 log10 reduction of *Enterococcus faecalis*, which is a strain that can be found in the human intestinal tract and can be a health risk (EU, 2011). According to the SEPA 2013 proposal, the concentration of *Enterococcus* spp. will not be regulated (SwedishEPA, 2013).

Over the 21 day period, the concentration of *Enterococcus* spp. never reached <1000 CFU g^{-1} (TS) in either of the sets. The inactivation of set 1 reached a reduction of 1.4 log10 by day 14, but increased again to 1 log10 reduction on day 21. This was likely due to active *Enterococcus* spp. bacteria settling at the bottom of the well, which were then stirred back into the blackwater when it was mixed. In set 2, this did not occur, but the inactivation also did not even reach a 1 log10 reduction by day 21.

The blackwater also did not meet the ABP regulation of a 5 log10 reduction of *Enterococcus faecalis* (EU, 2011). The decimal reduction time of the two sets ranged between 21 and 31 days, which means that according to ABP regulations the blackwater would need between 105 and 155 days to be sanitized. *Enterococcus* spp. have been known to have a lag phase, the time of which decreases when the temperature, pH, and amount of urea used are increased, causing an overall faster inactivation of Enterococci (Fidjeland *et al.*, 2013a; Ottoson *et al.*, 2008). So, the decimal reduction time may not be accurate since it does not take the lag phase into account, which means the required inactivation of *Enterococcus* spp. would be achieved more quickly than what was calculated. However, the reduction time may still be rather high, so it may be necessary to increase either the temperature, the dose of added urea, or both. A study by Fidjeland *et al.* (2015b) showed that at 14°C and doubling the 1% urea (ww) currently used in this study to 2%, would achieve the ABP required 5 log10 reduction in 75 days.

F-RNA bacteriophages and somatic coliphages

The current plant certification criteria (SPCR 178) do not require a log reduction of any viruses, however the 2013 SEPA proposal does (SwedishEPA, 2013; SP, 2012). It would require a 3 log 10 reduction of viruses. F-RNA bacteriophages, which are more sensitive to inactivation than somatic coliphages, were found to be a good indicator for estimating enterovirus inactivation (Vinnerås *et al.*, 2008; Cramer *et al.*, 1983). Additionally, the studies have shown that the inactivation of f-RNA bacteriophages and somatic coliphages are influenced by temperature; the lower the temperature the slower the inactivation,

likely due to a lower cell permeability (Vinnerås *et al.*, 2008; Cramer *et al.*, 1983). F-RNA bacteriophages inactivation is also influenced by the ammonia concentration; an increase in the ammonia concentration caused a linear increase in the inactivation (Cramer *et al.*, 1983).

There was only data for the reduction of f-RNA bacteriophages from set 2, where the concentration reached below the detection limit (1 PFU per ml) by day 14. The decimal reduction time was calculated to be 6.8 days, which means a 3 log10 reduction would be achieved in 20.4 days. This data differed to the more persistent somatic coliphages, where in both sets less than 1 log10 reduction was found by day 21.

Recommendations for further studies

A problem during this study was that after 21 days of treatment, some of the end product concentrations required by SPCR 178 had not been reached, i.e. *Enterococcus* spp. (SP, 2012). In fact, mixing the blackwater on day 21 of the treatment ended in an increase of the *Enterococcus* spp. level. Future studies might address this, if it still will be a requirement, by studying the effects of a higher usage of urea (1.5-2.0% based on wet weight) and testing if mixing on a regular basis can have an effect (daily or weekly). Additionally, it could be interesting to study if mixing different proportions of heated and unheated blackwater, in addition to different amounts of urea might influence inactivation rates. Lastly, while the urease activity was indirectly studied, in order to establish the rate of degradation the conversion rate between the total Nitrogen (urea) and TAN concentrations could have been measured more frequently at the beginning of the study.

7 Conclusions

In this thesis, the method testing an alternative combination of heating and ammonia treatment was only somewhat effective in the sanitization of the blackwater under SPCR 178 and the 2013 SEPA proposal. Over the 21 day treatment period the samples showed an even distribution of total-Nitrogen, TAN, pH, and temperature throughout the mixture, regardless of the depth. After an initial increase in the concentration due to the addition of urea, the total Nitrogen concentration remained constant, though usually higher than the calculated theoretical Nitrogen concentration. The pH increased at the beginning and then settled into a steady state. The measured TAN and un-ionized ammonia (NH_3), which makes up a portion of TAN, showed an increase in the concentration for the first 2 weeks and then appeared to become more steady from days 14 to 21. This partially confirms the first hypothesis, that the addition of 1% urea (ww) will increase the pH, total-Nitrogen, TAN, and un-ionized ammonia concentrations.

The combination of heated and ambient temperature blackwater resulted in a mixture whose temperature was approximately 17°C. Since the blackwater was in an insulated container, it was expected that there might be only a slight decrease in the temperature over the 21 days. Instead, the blackwater decreased by almost 6°C to approximately 11°C by day 21, which was however still higher than the temperature of the unheated blackwater. So, while the mixture of heated and ambient temperature blackwater did result in a higher temperature than that of the original blackwater, which was expected in the second hypothesis, there were no control to compare the inactivation of microorganisms in heated versus unheated blackwater.

The third hypothesis predicted that the reduction of the studied microorganisms within the 21 day study period would be in accordance with the legislative reductions required for these microorganisms of the production plant (SPCR 178). This means that in the final material on day 21, *Enterococcus* spp. would need to be <1000 CFU g^{-1} (TS), *Salmonella* spp. would need to be absent in 25 g (ww), and *E.coli* would need to be <1000 CFU g^{-1} (TS). While these concentrations for *Salmonella* spp. and *E.coli* were met, *Enterococcus* spp. was still far above the regulated concentration. Under the SEPA 2013 proposal, the requirements for validation of a method would also not be met, but in this case because of the *Ascaris suum* egg inactivation, which did not show the required 3 log10 reduction in the 21 day study period.

Based on these observations, further studies are suggested. These should focus on the influence of different physiochemical properties, such as different mixture ratios of heated and unheated blackwater in combination with different urea doses, on microbial inactivation in a full scale system. This way, the safest and most cost-effective treatment method for blackwater reuse can be established.

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Appendix I

1.14963.0001

Spectroquant®

Crack Set 20

1. Method

Organic and inorganic nitrogen compounds are transformed into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor.
The digestion is analogous to DIN EN ISO 11905-1.

2. Number of digestions

The reagents are sufficient to conduct 90 digestions.

3. Applications

Sample pretreatment for the determination of total nitrogen as nitrate

Sample material:
Groundwater, drinking water, and surface water
Spring water and well water
Mineral water
Industrial water, wastewater

The nitrogen content of the sample may not exceed 15 mg/l.

⚠ **The COD content of the sample may not exceed 350 mg/l.**
In the event of higher COD concentrations (up to 700 mg/l), the efficacy of the digestion can be maintained by doubling the quantity of reagent R-1. In the event of a COD content of 800 mg/l, the results obtained are false-low by 5 %.

4. Reagents and auxiliaries

Please note the warnings on the packaging materials!
The reagents are stable up to the date stated on the pack when stored closed at +15 to +25 °C.

Package contents:
1 bottle of reagent R-1
1 bottle of reagent R-2

Other reagents and accessories:
MQuant™ Peroxide Test, Cat. No. 110011,
measuring range 0.5 - 25 mg/l H_2O_2
Sodium sulfite anhydrous for analysis EMSURE®, Cat. No. 106657

Empty cells 16 mm with screw caps (25 pcs), Cat. No. 114724
Pipette for a pipetting volume of 10 ml
Thermoreactor

5. Procedure

Pretreated sample	10 ml	Pipette into an empty cell.
Reagent R-1	1 level blue microspoon (in the cap of the R-1-bottle)	Add and mix.
Reagent R-2	6 drops[1]	Add, **close cell tightly**, and mix.
Heat the cell at 120 °C[2] in the preheated thermoreactor for 1 hour. Allow the closed cell to cool to room temperature in a test-tube rack. **Do not cool with cold water!**		
Shake the cell briefly after 10 min. (Turbidity or precipitation frequently occurs in the digestion solution.)		
Check the digested sample for freedom from peroxide with the MQuant™ Peroxide Test. If necessary, eliminate any peroxide by adding a spatula-tip of sodium sulfite.		

[1] **Hold the bottle vertically while adding the reagent!**
[2] A digestion temperature of 100 °C may result in false-low readings.

The digestion solution can be analyzed using the following test kits directly after being allowed to cool to room temperature:

Spectroquant® Nitrate Tests:
Cat. Nos. 109713, 114773

Spectroquant® Nitrate Cell Tests:
Cat. Nos. 114542, 114563, 114764

6. Note

Reclose the reagent bottles immediately after use.

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Tel. +49(0)6151 72-2440
www.analytical-test-kits.com
EMD Millipore Corporation, 290 Concord Road,
Billerica, MA 01821, USA, Tel. +1-978-715-4321

September 2013

1.14963.0001

Spectroquant®

Crack Set 20

1. Methode

Organische und anorganische Stickstoff-Verbindungen werden nach der Koroleff-Methode durch Behandeln mit einem Oxidationsmittel im Thermoreaktor in Nitrat überführt.
Der Aufschluss ist analog DIN EN ISO 11905-1.

2. Anzahl der Aufschlüsse

Die Reagenzien sind für 90 Aufschlüsse ausreichend.

3. Anwendungsbereich

Probenvorbereitung zur Bestimmung von Gesamt-Stickstoff als Nitrat

Probenmaterial:
Grund-, Trink- und Oberflächenwasser
Quell- und Brunnenwasser
Mineralwasser
Brauchwasser, Abwasser

Der Stickstoff-Gehalt der Probe darf 15 mg/l nicht überschreiten.

⚠ **Der CSB-Gehalt der Probe darf 350 mg/l nicht überschreiten.**
Bei höheren CSB-Gehalten (bis 700 mg/l) kann durch Verdoppelung der Menge an Reagenz R-1 die Wirksamkeit des Aufschlusses erhalten bleiben. Bei einem Gehalt von 800 mg/l CSB kommt es zu Minderbefunden von 5 %.

4. Reagenzien und Hilfsmittel

Gefahrenkennzeichnung auf den einzelnen Bestandteilen der Packung beachten!
Die Reagenzien sind - bei +15 bis +25 °C verschlossen aufbewahrt - bis zu dem auf der Packung angegebenen Datum verwendbar.

Packungsinhalt:
1 Flasche Reagenz R-1
1 Flasche Reagenz R-2

Weitere Reagenzien und Zubehör:
MQuant™ Peroxid-Test, Art. 110011,
Messbereich 0,5 - 25 mg/l H_2O_2
Natriumsulfit wasserfrei zur Analyse EMSURE®, Art. 106657

Leerküvetten 16 mm mit Schraubkappe (25 Stück), Art. 114724
Pipette für Pipettiervolumen 10 ml
Thermoreaktor

5. Durchführung

Vorbereitete Probe	10 ml	In eine Leerküvette pipettieren.
Reagenz R-1	1 gestrichener blauer Mikrolöffel (im Deckel der R-1-Flasche)	Zugeben und mischen.
Reagenz R-2	6 Tropfen[1]	Zugeben, **Küvette fest verschließen** und mischen.
Küvette 1 Stunde bei 120 °C[2] im vorgeheizten Thermoreaktor erwärmen. Verschlossene Küvette in einem Reagenzglasgestell auf Raumtemperatur abkühlen lassen. **Nicht mit kaltem Wasser kühlen!**		
Nach 10 min Küvette kurz umschütteln. (In der Aufschlusslösung treten häufig Trübungen oder Niederschläge auf.)		
Aufgeschlossene Probe auf Peroxid-Freiheit prüfen mit MQuant™ Peroxid-Test. Falls erforderlich, Peroxid durch Zugabe einer Spatelspitze Natriumsulfit zerstören.		

[1] **Flasche während der Zugabe des Reagenzes senkrecht halten!**
[2] Eine Aufschlusstemperatur von 100 °C kann u.U. zu Minderbefunden führen.

Die Aufschlusslösung kann direkt nach dem Abkühlen auf Raumtemperatur mit den folgenden Testsätzen analysiert werden:

Spectroquant® Nitrat-Tests:
Art. 109713, 114773

Spectroquant® Nitrat-Küvettentests:
Art. 114542, 114563, 114764

6. Hinweis

Flaschen nach Reagenzentnahme umgehend wieder verschließen.

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Appendix II

7.75778.0003-601543412 msp.

August 2013

1.09713.0001
1.09713.0002

Spectroquant®
Nitrate Test

NO_3^-

1. Method

In sulfuric and phosphoric solution nitrate ions react with 2,6-dimethylphenol (DMP) to form 4-nitro-2,6-dimethylphenol that is determined photometrically.
The method is analogous to DIN 38405-9.

2. Measuring range and number of determinations

Cell	Measuring range		Number of determinations
mm	mg/l NO_3-N	mg/l NO_3^-	
50	**0.10** - 5.00	**0.4** - 22.1	100 (Cat. No. 1.09713.0001) or 250 (Cat. No. 1.09713.0002)
20	0.5 - 12.5	2.2 - 55.3	
10	1.0 - **25.0**	4.4 - **110.7**	

For programming data for selected photometers / spectrophotometers see **www.service-test-kits.com**.

3. Applications

This test is not suited for the determination in waters with chloride contents exceeding 1000 mg/l and COD values exceeding 500 mg/l.

Sample material:
Groundwater, drinking water, and surface water
Spring water and well water
Mineral water
Wastewater and industrial water
Soils and fertilizers after appropriate sample pretreatment
This test is **not suited** for seawater.

4. Influence of foreign substances

This was checked in solutions containing 10 and 0 mg/l NO_3-N. The determination is not yet interfered with up to the concentrations of foreign substances given in the table.

Concentrations of foreign substances in mg/l or %					
Al^{3+}	1000	Hg^{2+}	100	Surfactants [2]	1000
Ca^{2+}	500	Mg^{2+}	1000	**COD (K-hydrogen phthalate)**	**500**
Cd^{2+}	250	Mn^{2+}	1000		
Cl^-	**1000**	NH_4^+	1000	**Organic substances (glucose)**	**500**
CN^-	100	Ni^{2+}	500	Na-acetate	25 %
Cr^{3+}	500	**NO_2^-**	**5** [1]	NaCl	0.2 %
$Cr_2O_7^{2-}$	**50**	Pb^{2+}	100	Na_2SO_4	25 %
Cu^{2+}	500	PO_4^{3-}	1000		
F^-	1000	SiO_3^{2-}	500		
Fe^{3+}	100	Zn^{2+}	1000		

[1] In cases of higher concentrations, eliminate nitrite ions acc. to section 6.
[2] tested with nonionic, cationic, and anionic surfactants

5. Reagents and auxiliaries

Please note the warnings on the packaging materials!
The test reagents are stable up to the date stated on the pack when stored closed at +15 to +25 °C.

Package contents:
1 bottle of reagent NO_3-1
1 bottle of reagent NO_3-2
1 AutoSelector

Other reagents and accessories:
MQuant™ Chloride Test, Cat. No. 110079,
measuring range 500 - ≥3000 mg/l Cl^-
MQuant™ Nitrite Test, Cat. No. 110007,
measuring range 2 - 80 mg/l NO_2^- (0.6 - 24 mg/l NO_2-N)
Amidosulfuric acid for analysis EMSURE®, Cat. No. 100103
MColorpHast™ pH-indicator strips pH 0 - 6.0, Cat. No. 109531
Sulfuric acid 25 % for analysis EMSURE®, Cat. No. 100716
MQuant™ Nitrate Test, Cat. No. 110020,
measuring range 10 - 500 mg/l NO_3^- (2.3 - 113 mg/l NO_3-N)
Spectroquant® CombiCheck 20, Cat. No. 114675
Nitrate standard solution CRM, 0.500 mg/l NO_3-N, Cat. No. 125036
Nitrate standard solution CRM, 2.50 mg/l NO_3-N, Cat. No. 125037
Nitrate standard solution CRM, 15.0 mg/l NO_3-N, Cat. No. 125038

Empty cells 16 mm with screw caps (25 pcs), Cat. No. 114724
Pipettes for pipetting volumes of 0.50 and 4.0 ml
Rectangular cells 10, 20, and 50 mm (2 of each), Cat. Nos. 114946, 114947, and 114944
Semi-microcells 50 mm (2 pcs), Cat. No. 173502

6. Preparation

- Analyze immediately after sampling.
- Check the chloride content with the MQuant™ Chloride Test.
  Samples containing more than 1000 mg/l Cl^- must be diluted with distilled water.
- Check the nitrite content with the MQuant™ Nitrite Test.
  If necessary, eliminate interfering nitrite ions (stated amounts apply for nitrite contents of up to 50 mg/l):
  To 10 ml of sample add approx. 50 mg of amidosulfuric acid and dissolve.
  The pH of this solution must be within the range 1 - 3. Adjust, if necessary, with sulfuric acid.
- Check the nitrate content with the MQuant™ Nitrate Test.
  Samples containing more than 25.0 mg/l NO_3-N (110.7 mg/l NO_3^-) must be diluted with distilled water.
- Filter turbid samples.

7. Procedure

Reagent NO_3-1	4.0 ml	Pipette into a dry test tube [1].
Pretreated sample **(5 - 25 °C)**	0.50 ml	Add with pipette, **do not mix!**
Reagent NO_3-2	0.50 ml	Add with pipette (**Wear eye protection! The mixture becomes hot!**) and mix, holding **only the upper part** of the tube!
Leave the hot reaction solution to stand for 10 min (reaction time). **Do not cool with cold water!** Fill the sample into the rectangular cell and measure in the photometer.		

[1] Empty cells Cat. No. 114724 are recommended that can be sealed with screw caps, thus enabling the sample to be mixed safely.

For measurement in the **50-mm cell** both the sample volume as well as the quantities of reagents NO_3^-1 and NO_3^-2 must be doubled. Alternatively, the semi-microcell Cat. No. 173502 can be used.

Notes on the measurement:

- **Certain photometers may require a blank** (preparation as per measurement sample, but with distilled water instead of sample).
- For photometric measurement the cells must be clean.
  Wipe, if necessary, with a clean dry cloth.
- Measurement of turbid solutions yields false-high readings.
- The color of the measurement solution remains stable for 30 min after the end of the reaction time stated above. (After 60 min the measurement value would have increased by 5 %.)

8. Analytical quality assurance

recommended before each measurement series
To check the photometric measurement system (test reagent, measurement device, handling) and the mode of working, the nitrate standard solutions CRM, 0.500 mg/l NO_3-N (Cat. No. 125036), 2.50 mg/l NO_3-N (Cat. No. 125037), and 15.0 mg/l NO_3-N (Cat. No. 125038) or Spectroquant® CombiCheck 20 can be used. Besides a **standard solution** with 9.0 mg/l NO_3-N, CombiCheck 20 also contains an **addition solution** for determining sample-dependent interferences (matrix effects).
Additional notes see under **www.qa-test-kits.com**.

Characteristic quality data:
In the production control, the following data were determined in accordance with ISO 8466-1 and DIN 38402 A51 (10-mm cell):

Standard deviation of the method (mg/l NO_3-N)	± 0.10
Coefficient of variation of the method (%)	± 0.82
Confidence interval (mg/l NO_3-N)	± 0.3
Number of lots	25

Characteristic data of the procedure:

	Measuring range mg/l NO_3-N	
	0.10 - 5.00	1.0 - 25.0
Sensitivity: Absorbance 0.010 A corresponds to (mg/l NO_3-N)	0.04	0.2
Accuracy of a measurement value (mg/l NO_3-N)	max. ± 0.12	max. ± 0.6

For quality and batch certificates for Spectroquant® test kits see the website.

9. Notes

- Reclose the reagent bottles immediately after use.
- **Information on disposal can be obtained at www.disposal-test-kits.com.**

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Appendix III

7.75731.0004-xxxxxxxxx msp.

December 2013

1.00683.0001

Spectroquant®

Ammonium Test

NH_4^+

1. Method

Ammonium nitrogen (NH_4-**N**) occurs partly in the form of ammonium ions and partly as ammonia. A pH-dependent equilibrium exists between the two forms. In strongly alkaline solution ammonium nitrogen is present almost entirely as ammonia, which reacts with hypochlorite ions to form monochloramine. This in turn reacts with a substituted phenol to form a blue indophenol derivative that is determined photometrically. Due to the intrinsic yellow coloration of the reagent blank, the measurement solution is yellow-green to green in color.
The method is analogous to EPA 350.1, APHA 4500-NH_3 F, ISO 7150-1, and DIN 38406-5.

2. Measuring range and number of determinations

Cell	Measuring range		Number of determinations
mm	mg/l NH_4-N	mg/l NH_4^+	
10	**2.0** - 75.0 5 - **150**	**2.6** - 96.6 6 - **193**	100

For programming data for selected photometers / spectrophotometers see www.service-test-kits.com.

3. Applications

This test measures both ammonium ions and dissolved ammonia.

Sample material:
Groundwater and surface water, seawater
Drinking water
Wastewater
Nutrient solutions for fertilization
Soils and food after appropriate sample pretreatment

4. Influence of foreign substances

This was checked in solutions containing 40 and 0 mg/l NH_4-N. The determination is not yet interfered with up to the concentrations of foreign substances given in the table.

Concentrations of foreign substances in mg/l or %					
Al^{3+}	1000	Mn^{2+}	100	EDTA	1000
Ca^{2+}	1000	Ni^{2+}	250	**Primary amines**[1]	**0**
Cd^{2+}	1000	NO_2^-	1000	Secondary amines[2]	250
CN^-	100	Pb^{2+}	1000	**Aminophenols**	**10**
Cr^{3+}	100	PO_4^{3-}	1000	Aniline	50
$Cr_2O_7^{2-}$	1000	S^{2-}	50	Triethanolamine	1000
Cu^{2+}	1000	SiO_3^{2-}	1000	Surfactants[3]	1000
F^-	1000	Zn^{2+}	500	Na-acetate	10 %
Fe^{3+}	25			NaCl	20 %
Hg^{2+}	500			$NaNO_3$	20 %
Mg^{2+}	500			Na_2SO_4	20 %

Reducing agents interfere with the determination.

[1] tested with methylamine
[2] tested with dimethylamine
[3] tested with nonionic, cationic, and anionic surfactants

5. Reagents and auxiliaries

Please note the warnings on the packaging materials!

The test reagents stable up to the date stated on the pack when stored closed at +15 to +25 °C.

Package contents:
1 bottle of reagent NH_4-1
1 bottle of reagent NH_4-2 (contains granulate + desiccant capsule)
2 AutoSelectors

Other reagents and accessories:
MQuant™ Ammonium Test, Cat. No. 110024,
measuring range 10 - 400 mg/l NH_4^+ (8 - 310 mg/l NH_4-N)
MColorpHast™ Universal indicator strips pH 0 - 14, Cat. No. 109535
Sodium hydroxide solution 1 mol/l TitriPUR®, Cat. No. 109137
Sulfuric acid 0.5 mol/l TitriPUR®, Cat. No. 109072
Spectroquant® CombiCheck 70, Cat. No. 114689
Ammonium standard solution CRM, 6.00 mg/l NH_4-N, Cat. No. 125025
Ammonium standard solution CRM, 12.0 mg/l NH_4-N, Cat. No. 125026
Ammonium standard solution CRM, 50.0 mg/l NH_4-N, Cat. No. 125027

Pipettes for pipetting volumes of 0.10, 0.20, and 5.0 ml
Rectangular cells 10 mm (2 pcs), Cat. No. 114946

6. Preparation

- Rinse glassware ammonium-free with distilled water. **Do not use detergent!**
- Analyze immediately after sampling.
- Check the ammonium content with the MQuant™ Ammonium Test. Samples containing more than 150 mg/l NH_4-N must be diluted with distilled water.
- **The pH must be within the range 4 - 13.** Adjust, if necessary, with sodium hydroxide solution or sulfuric acid.
- Filter turbid samples.

7. Procedure

Measuring range 2.0 - 75.0 mg/l NH_4-N (2.6 - 96.6 mg/l NH_4^+):

Reagent NH_4-1 (20 - 30 °C)	5.0 ml	Pipette into a test tube.
Pretreated sample (20 - 30 °C)	**0.20 ml**	Add with pipette and mix.
Reagent NH_4-2	1 level blue microspoon (in the cap of the NH_4-2 bottle)	Add and shake **vigorously until the reagent is completely dissolved**.
Leave to stand for 15 min (reaction time), then fill the sample into a 10-mm cell, and measure in the photometer.		

Measuring range 5 - 150 mg/l NH_4-N (6 - 193 mg/l NH_4^+):

Reagent NH_4-1 (20 - 30 °C)	5.0 ml	Pipette into a test tube.
Pretreated sample (20 - 30 °C)	**0.10 ml**	Add with pipette and mix.
Reagent NH_4-2	1 level blue microspoon (in the cap of the NH_4-2 bottle)	Add and shake **vigorously until the reagent is completely dissolved**.
Leave to stand for 15 min (reaction time), then fill the sample into a 10-mm cell, and measure in the photometer.		

Notes on the measurement:

- Due to the strong temperature dependence of the color reaction, the temperature of the reagents should be between 20 and 30 °C.
- **Certain photometers may require a blank** (preparation as per measurement sample, but with distilled water instead of sample).
- For photometric measurement the cells must be clean. Wipe, if necessary, with a clean dry cloth.
- Measurement of turbid solutions yields false-high readings.
- Ammonium-free samples turn yellow on addition of reagent NH_4-2.
- The pH of the measurement solution must be within the range 11.5 - 11.8.
- The color of the measurement solution remains stable for at least 60 min after the end of the reaction time stated above.
- In the event of ammonium concentrations exceeding 2500 mg/l, other reaction products are formed and false-low readings are yielded. In such cases it is advisable to conduct a plausibility check of the measurement results by diluting the sample (1:10, 1:100).

8. Analytical quality assurance

recommended before each measurement series
To check the photometric measurement system (test reagent, measurement device, handling) and the mode of working, the ammonium standard solutions CRM, 6.00 mg/l NH_4-N (Cat. No. 125025), 12.0 mg/l NH_4-N (Cat. No. 125026), and 50.0 mg/l NH_4-N (Cat. No. 125027) or Spectroquant® CombiCheck 70 can be used. Besides a **standard solution** with 50.0 mg/l NH_4-N, CombiCheck 70 also contains an **addition solution** for determining sample-dependent interferences (matrix effects).
Additional notes see under **www.qa-test-kits.com**.

Characteristic quality data:

In the production control, the following data were determined in accordance with ISO 8466-1 and DIN 38402 A51:

	Measuring range mg/l NH_4-N	
	2.0 - 75.0	5 - 150
Standard deviation of the method (mg/l NH_4-N)	± 0.48	± 1.0
Coefficient of variation of the method (%)	± 1.2	± 1.2
Confidence interval (mg/l NH_4-N)	± 1.2	± 2
Number of lots	23	23

Characteristic data of the procedure:

	Measuring range mg/l NH_4-N	
	2.0 - 75.0	5 - 150
Sensitivity: Absorbance 0.010 A corresponds to (mg/l NH_4-N)	0.3	1
Accuracy of a measurement value (mg/l NH_4-N)	max. ± 1.7	max. ± 3

For quality and batch certificates for Spectroquant® test kits see the website.

9. Notes

- Reclose the reagent bottles immediately after use.
- **Information on disposal can be obtained at www.disposal-test-kits.com.**

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