Inactivation of pathogens in urine nitrification reactors

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Abstract

Through nutrient recovery by urine separation, the VUNA project aims to develop an effective sanitation system that simultaneously helps address important issues such as environmental pollution and water scarcity. Refinement of the nutrient recovery process presents several challenges: at a technical level, as nitrifying bacteria are sensitive to several parameters; and from a public health perspective, as nutrient recovery from urine implies exposure to pathogens.

As part of this problematic, the present study focused on four main objectives: operating nitrification reactors, characterising both virus and bacteria inactivation, and evaluating different inactivation mechanisms.

Indicator organisms (bacteriophages and bacteria) were used as surrogates for human pathogens. In a first set of experiments, the bacteriophage MS2 was spiked continuously over 60 days in a continuous flow nitrification reactor. Nitrification did not affect the bacteriophage concentration within the reactor. The second set of experiments consisted of the operation of small scale batch and semi-batch reactors to test in total three different bacteriophages (ΦX147, MS2, Qbeta) and two bacteria (Salmonella typhimurium, Enterococcus spp.) under varying parameters.

Pathogens can be inactivated or affected by different mechanisms. Four possible inactivation mechanisms were further evaluated: 1) effect of biological activity in a nitrification treatment system, 2) effect of the air-water interface in a buffer (PBS) and nitrified urine, 3) effect of the complexity of solution, 4) effect of ambient temperature.

Results indicated that nitrification caused bacterial inactivation but did not influence the bacteriophages concentration. Air-water interface did not affect bacteria and showed mixed results for bacteriophages. Ambient temperature was not an inactivating parameter for neither of the groups studied. Finally, the bacteriophages presented signs of resistance, possibly due to a protective effect of the complex solution in the nitrification reactor.
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1 Introduction

1.1 VUNA Project

The VUNA project, which stands for the Valorisation of Urine Nutrients in Africa, aims to promote sanitation and nutrient recovery through urine separation. The project was launched in 2010 by the Swiss Federal Institute of Aquatic Science and Technology (Eawag) with the University of KwaZulu-Natal (UKZN) and eThekwini Water and Sanitation (EWS) in South Africa, as well as the Swiss Federal Institute of Technology (ETHZ). Through the recovery of nutrient from urine in small decentralised reactors, the VUNA projects pursue several overarching objectives: improvement of sanitation, reduction of pollution of water resources, and promotion of entrepreneurship [1].

Urine contains the major fraction of nutrients found in human excreta: 80-90% of the nitrogen, 55-67% of the phosphorus and 50-80% of the potassium [4, 5, 6]. Because of its composition, the use of urine as fertilizer is an obvious application. Harvesting urine is not only interesting for developing the use of an alternative fertilizer. It would also reduce the effects of pollution from unsafe excreta disposal, and lower the ecological burden of the fertilizer production and surplus use of chemical fertilizers [4, 7]. Finally, it would promote wastewater management systems that reduce both water use and initial infrastructure investments [8].

Urine needs processing for several purposes: hygienisation, volume reduction, urine stabilisation, nutrient recovery (N, P), nutrient removal and handling of micropollutants [9]. Urine treatment and nutrient recovery pose many challenges in terms of technology but also in terms of health. Indeed, the handling and reuse of human waste always involves hygiene risks [6]. In order to have a successful alternative wastewater system and nutrient recovery process, the hygiene risk assessment is of utmost importance.

Research at Eawag has focused, amongst other technologies, on biological nitrification for urine stabilisation, followed by distillation for nutrient recovery. Nitrification reactor parameters have been optimised for nitrification performance and end product quality. The LCE at EPFL joined the VUNA project for research on pathogen inactivation during urine treatment. Pathogen inactivation during urine storage and struvite fertilizer production have already been studied [10, 11], and the mechanisms of inactivation by ammonia are under investigation. The steps where possible pathogen inactivation is to be assessed by VUNA researchers are: storage, struvite fertilizer production, electrolysis and nitrification.
1.2 Microbial health risks of source-separated urine

Urine of healthy people is usually sterile, but some pathogens (e.g. *Leptospira interrogans*, *Salmonella typhi*, *Salmonella paratyphi* and *Schistosoma haematobium*) can be excreted during infection [10]. On the other hand, human faeces contain high levels of disease-causing organisms, such as bacteria, viruses, parasitic protozoa and helminths, in concentrations depending on their prevalence within a given population [12]. These pathogens, principally affecting the gastrointestinal system, are of significant world-health concern. 1.7 million deaths per year world-wide, mainly caused by infectious diarrhoea, are due to poor water quality, sanitation and hygiene [13]. Overall, 99.8% of the sanitation-, water- and hygiene-related death occurs in developing countries and 90% are deaths of children under 5 years [14, 15].

Excreta-related pathogens are diverse and cause various infections. For example the gram-negative bacteria *Escherichia coli*, *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp. induce severe vomiting, diarrhea, typhoid fever and other infections [12]. Viruses, including hepatitis A and E, adenovirus, rotavirus, and norovirus are also found in excreta [12]. For example, hepatitis A causes severe liver damages, while rotavirus and adenovirus cause diarrheal diseases.

A main contamination pathway of enteric pathogens in urine is cross-contamination of source-separated urine with pathogens from faeces. Schönning et al. studied cross-contamination in Sweden and Australia and found up to 22-37% of contamination in their samples [6]. Recent sampling in urine storage tanks in Durban revealed the presence of ten pathogens: seven types of bacteria were detected in 11% to 94% of the samples (over 18 samples); adenovirus and rotavirus were detected in 31-34% of the samples (over 29 samples) and norovirus GI in 3% (over 29 samples) [16]. The need of protection against pathogens and further investigation is thus confirmed.

In order to develop a sustainable nutrient recovery process, the end-product should be safe to manipulate. A complete nutrient recovery system comprises several steps: household storage, transport, storage tank, nitrification reactor, distillation, fertilizer, and human exposure to pathogens is possible at each step. Applying unsafe fertilizer also presents risks for the environment [17]. To ensure complete safety, the risk of exposure to pathogens must be assessed at each step and appropriate protective measures must be taken.
1.3 Aim of the study

The overall aim of this study is to assess the inactivation of pathogens during biological nitrification, with the specific research objectives as follows:

1. Install, maintain and operate continuous flow nitrification reactors
2. Characterise virus inactivation
3. Characterise bacteria inactivation
4. Assess physical, biological and chemical inactivation mechanisms

To address these objectives, two continuous flow nitrification reactors and several batch and semi-batch reactors were set-up and monitored. Little is known regarding pathogen inactivation mechanisms during nitrification. Postulated inactivation methods are outlined in chapter 2.2.3. Indicator organisms (bacteriophages and bacteria) were used as surrogates for human pathogens to study the inactivation. Several types of bacteriophage and bacteria were tested, with varying experimental parameters, in order to evaluate causal connection between pathogen inactivation and reactor conditions.
2 Theory and literature review

2.1 Nitrification process

In fresh source-separated urine 75-90% of N is excreted as urea (CO(NH$_2$)$_2$) [5]. Fresh urine also contains salts, soluble organic matter and ammonia bound to urea [9]. Due to microbial activity, the urea in urine is hydrolysed during storage and transport. The overall reaction of urea hydrolysis, or ureolysis, is expressed as follows [18]:

$$\text{NH}_2\text{(CO)NH}_2 + 2\text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{NH}_4^+ + \text{HCO}_3^-$$

Due to its high urea concentration, urine is unstable [19]. Complete urea exhaustion is completed in a few days only [18] and total ammonia represents 90% of the nitrogen in stored urine [3]. The hydrolysis of urea causes a pH increase from pH 6 until around pH 9 [9]. This increase in pH has several consequences:

- The pKa of ammonia is 9.24, therefore at pH 9 the concentration of ammonia NH$_3$ is high (33-37%) [7, 20]. As ammonia NH$_3$ is volatile, losses can occur during transportation (agitation) or direct application as fertilizer [21]. NH$_3$ evaporation can also be detrimental to environment and human health [3, 5].

- High pH values trigger precipitation of calcium phosphate, struvite and calcite [21]. The precipitation of calcium phosphate lowers the available P concentration in urine, which is undesirable if the urine is to be used as fertilizer.

The stabilisation of urine is therefore necessary in order to prevent ammonia volatilisation and convert ammonia into a less volatile form, to reach the goal of nutrient recovery. There are two options to stabilise urine: acidification or biological nitrification [7]. pH in the collection tank should be below 4 to prevent urea hydrolysis [9]. Acidification is an efficient method and such low pH value has an adverse effect on pathogens. However, it is a costly method due to the use of chemicals, and as ureolysis is a fast reaction, the technical realisation is complicated [8].

Biological nitrification is a suitable method for urine stabilisation. It prevents ammonia evaporation, but also the strong urine odour [9]. Nitrification involves two groups of nitrifying bacteria: ammonia oxidising bacteria (AOB) and nitrite
oxidising bacteria (NOB). AOB oxidise the ammonia to nitrite and NOB oxidise nitrite into nitrate. The nitrification reaction is divided in two steps:

1. Ammonia oxidation : \( \text{NH}_3 + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}^+ + \text{H}_2\text{O} \)

   The first step is actually divided into two steps where hydroxylamine (\( \text{NH}_2\text{OH} \)) is the intermediate product.

2. Nitrite oxidation : \( \text{NO}_2^- + 0.5 \text{O}_2 \rightarrow \text{NO}_3^- \)

Nitrification lowers the pH to around 6. As half of the \( \text{NH}_3 \) can be oxidised, the remaining \( \text{NH}_3 \) is converted to \( \text{NH}_4^+ \), which is non volatile. Udert et al. tested different types of reactor: a moving bed biofilm reactor (MBBR), a continuous flow stirred reactor (CSTR) and a sequencing batch reactor (SBR) [21]. In all cases the maximal conversion of ammonia into nitrate was of 50%. Apparently ammonia oxidation does not occur at pH values far below 6 [21]. Biological nitrification requires a good coordination between AOB and NOB, which is difficult to maintain especially because source-separated urine is a difficult environment for nitrifying bacteria. The parameters influencing nitrification are listed in the table below (Table 1):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AOB</th>
<th>NOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>Higher affinity to oxygen</td>
<td>Lower affinity to oxygen</td>
</tr>
<tr>
<td>Temperature</td>
<td>Faster increase with T°</td>
<td>Slower increase with T°</td>
</tr>
<tr>
<td>Substrate inhibition and limitation</td>
<td>Substrate for AOB = ammonia ( \text{NH}_3 )</td>
<td>Substrate for NOB = nitrous acid (( \text{HNO}_2 ))</td>
</tr>
<tr>
<td>Product inhibition</td>
<td>Strongly inhibited by ( \text{HNO}_2 )</td>
<td>( \text{NO}_3^- ) inhibition usually negligible</td>
</tr>
<tr>
<td>Inhibition by intermediates</td>
<td></td>
<td>Inhibition by ( \text{NH}_2\text{OH} ), the intermediate of ammonia oxidation</td>
</tr>
</tbody>
</table>

pH is a main parameter for AOB and NOB activity, as the concentration of their substrate depend on the pH: \((\text{NH}_3/\text{NH}_4^+)\) for AOB, \((\text{HNO}_2/\text{NO}_2^-)\) for NOB, with \(\text{pK}_a=9.25\) and 3.29 respectively [21]. High pH fluctuations can break down the process, and high pH values have more sever consequences. The pH must therefore be carefully regulated [21].
2.2 Pathogens

2.2.1 Viruses

Viruses are microorganisms that can infect all kinds of living organisms. They have their own genome, but are not dynamic systems and depend on the mechanical system of their host cell for replication. Replication can be damaging for the host cell, which is why viruses can be pathogenic [22].

There are various types of viruses: single- or double-stranded RNA, positive- or negative-sense RNA ((+/−)ss-RNA, (+/−)ds-RNA), and single- or double-stranded DNA (ss-DNA, ds-DNA), which confer different replication strategies. There are different types of virus structure (size, morphology, chemical composition), but in all cases the nucleic acid is protected by a protein coat called capsid. This structure permits the virus to survive in the environment for long periods. Some viruses have in addition a membrane structure, or envelope. Viruses are amongst the smallest organisms, tens to hundreds of nm in size (Fig.2) and are infective at low dose [23].

![Relative sizes of cells and their components](image)

Figure 2: Size of different organisms [2]

Heterogeneity and infectivity of viruses make them an important matter of concern in sanitation. Viruses are important enteric pathogens, therefore of major public health concern, especially in developing areas.

In order to study the behaviour of viruses in nitrification reactors, three different bacteriophages were used. Bacteriophages are often used in studies as surrogate for human viruses because of their similar structure. Additionally they are safe and easier to manipulate than human viruses [24].
**MS2**

MS2 is a (+)ss-RNA bacteriophage infecting *Escherichia Coli*. It has a genome of 3569 bases, an icosahedral symmetry [25], a diameter of 26nm and an isoelectric point of 3.9 [26]. MS2 has a similar structure to enteric viruses. It is known to be more hydrophobic than the bacteriophage ΦX147 [10].

**Qbeta**

Qbeta is a (+)ss-RNA bacteriophage infecting *Escherichia Coli*. It has a genome of 4217 bases [27], an icosahedral symmetry [28], a diameter of 24nm and an isoelectric point of 5.3 [26]. It has a similar structure and size to bacteriophage MS2, thus they are expected to have similar behaviours.

**ΦX147**

ΦX147 is a ss-DNA bacteriophage, infecting *Escherichia Coli*. It has a genome of 5386 bases [22], a icosahedral symmetry [29], a diameter of 27nm and an isoelectric point of 6.6 [26]. ΦX147 may be a relatively conservative model virus, because of its low hydrophobicity and high stability [30].

### 2.2.2 Bacteria

Bacteria are prokaryote microorganisms. Ranging in size from 0.2-2µm in width to greater than 50µm in diameter [22] (see Fig.2). In general, small cells grow faster than large cells. This is a reflection of their large surface to volume ratio that allows a rapid exchange with the external environment.

The diversity in the Bacteria domain is very large. However, they can be distinguished as gram-positive or -negative cells which indicates differences in the cell wall structure [22]. Bacteria have a rigid cell wall: gram-positive bacteria have a thick peptidoglycan layer, while gram-negative bacteria have a thin peptidoglycan layer and an outer membrane composed of lipopolysaccharide and protein [22]. The vulnerability of gram-positive and -negative bacteria differs according to this variation in membrane composition and structure.

Enteric bacteria (e.g. *Clostridium difficile*, *Salmonella enterica*), responsible for various diseases, are of concern in sanitation. They cause infections, gastroenteritis, and can have lifethreatening consequences.

In order to study the behaviour of bacteria in nitrification reactors, two different bacteria, one gram-negative and one gram-positive were used.
Enterococci
Enterococcus is a gram-positive bacteria. Enterococci are facultative anaerobic organisms that can survive and grow in many environments [22]. Enterococcus faecalis and Enterococcus faecium are two human pathogens frequently found. They are responsible for infections, endocarditis and septicemia and are also commonly used as indicators of faecal pollution in the environment.

Salmonella
Salmonella is a gram-negative bacteria. It is sensitive to acidic pH [22]. Salmonella are generally pathogenic for human beings, causing gastroenteritis and typhoid fever.

2.2.3 Potential inactivation mechanisms
Pathogenic organisms such as bacteria and viruses are exposed to different inactivation mechanisms. Virus inactivation is particularly complicated to anticipate. Indeed, two highly related viruses treated with the same biocide can follow different inactivation kinetics [31]. Although little is known about inactivation of pathogens during nitrification, several parameters are known to influence pathogens inactivation in other conditions.

Air-water interface
Thompson et al. studied the inactivation of bacteriophages MS2 and ΦX147 by air-water interface (AWI) [29]. They proposed that viruses in solution reach the AWI, via convection and diffusion, where they adsorb. The adsorption is controlled by several factors (electrostatic, hydrophobic and hydration forces; solution ionic strength; pH; and other). At the AWI, hydrophobic regions of the virus capsid partition out of the solution into the gas phase via reconfiguration of the capsid proteins, resulting in the loss of infectivity [29]. The location of inactivation is more exactly at the triple-phase-boundary (TPB), i.e. the interface between air, liquid and solid, rather than at the AWI [29]. The inactivation is therefore influenced by the hydrophobicity of the solid phase [29, 32]. As the step of adsorption depends on the surface properties of the virus [33], different inactivation efficiencies are expected. Also, viruses containing hydrophobic parts are more sensitive to AWI inactivation, because these parts tend to be located in the air phase [32, 33]. In their study, bacteriophage MS2 revealed to be subject to inactivation by AWI, but ΦX147 was apparently not affected [29].
As the nitrification reactors are aerated, the AWI is continuously regenerated, renewing the location for virus inactivation. Also the composition of the reactor wall, whether it is made of hydrophobic or hydrophilic material, could influence the inactivation efficiency. The presence of biofilm carriers (which are the support for nitrifying bacteria growth) made of polyethylene (PE), a hydrophobic material, in the reactors is to be remembered. Their contribution to the inactivation would probably be inversely proportional to the amount of biofilm attached, so directly proportional to the surface of PE remaining.

**Ammonia concentration, pH and temperature**

Free ammonia (NH$_3$), pH and temperature are the three key parameters of urine storage. Inactivation of pathogens in stored urine has been the subject of various studies [6, 10, 14, 34, 35]. Stored urine presents hard conditions: high NH$_3$ concentrations and high pH. NH$_3$ is a known biocide for most organisms as are high pH values [36]. Several studies have demonstrated that the survival rate of pathogens is low at high temperatures [34, 35]. Gram-negative bacteria are more rapidly inactivated in stored urine than gram-positive bacteria and viruses were the most persistent group [6].

In this study, NH$_3$ in the reactors is oxidised and pH is lowered by the nitrification, and the temperature is not controlled. Therefore the conditions are less threatening to pathogens than in stored urine. However, localised inactivation might happen to the place where urine enters the reactor.

**Effect of biological treatment system**

Activated sludge in wastewater treatment systems is capable of removing viruses by physicochemical and biological processes [37]. The virus can be adsorbed on the sludge floc [37], or the sludge microbes can ingest it [38]. Kim et al. demonstrated that the inactivation of virus is efficient during the first hour of contact and that adsorption of virus onto bacteria is reversible. Floc-forming bacteria have a higher capability of decreasing virus infectivity than non-floc forming bacteria [37].

Other virucidal and bacteriocidal parameters are described in the literature, but did not seem relevant for this study.
Tailing phenomenon

In addition to the potential inactivation effects listed above, a nitrification system can provide protection to pathogens. Sigstam et al. studied the tailing phenomenon during virus disinfection [31], in which the inactivation rate decreases over time. The virus can be protected by deposition of an adduct on the capsid protein. This deposition can be removed by washing, therefore the protection is reversible [31].

Nitrified urine is a complex solution, it is thus possible to expect deposition of urine constituents on the virus in this study.
3 Set-up, materials and methods

Two types of nitrification reactors were operated in this study: two main continuous flow moving bed biofilm reactors (MBBR) and several batch and semi-batch reactors.

The two continuous flow moving bed biofilm reactors (MBBRs) were run in parallel. After ensuring the stability of the reactors, one of the reactors (Reactor no. 2) was spiked continuously with bacteriophage MS2. The other continuous flow reactor (Reactor no. 1) remained clean of tested bacteriophage and bacteria. A series of batch and semi-batch MBBRs, of smaller volume than the continuous flow reactors, were set-up to test several bacteriophages and bacteria under varying experimental conditions. These batch and semi-batch reactors were fed with the content of the continuous flow Reactor no. 1 (biofilm carriers, nitrified urine).

3.1 Continuous flow MBBRs

Free suspended biomass processes are known to be efficient with fast growing organisms that have a short residence time. In the case of slow growing organisms, such as nitrifiers, retention or recirculation of biomass is required. Biofilms effectively retain biomass in the reactor and improve the volumetric conversion capacity [39]. Moving bed biofilm reactors are reactors where the biofilm grows on small particles, the carriers, that are mobile in the reactor. The advantages of this process are an enlargement of the biofilm specific area, a higher volumetric substrate conversion and a higher oxygen transfer rate. The Kaldnes® carriers were used in the MBBRs and their characteristics are listed in the table below (Table 2):

<table>
<thead>
<tr>
<th>Technical specification</th>
<th>Kaldnes®</th>
</tr>
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<tbody>
<tr>
<td>Material</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>460 m²/m³</td>
</tr>
<tr>
<td>Maximum fill</td>
<td>30% to 65%</td>
</tr>
<tr>
<td>Weight per m³</td>
<td>152 kg/m³</td>
</tr>
<tr>
<td>Surface per unit</td>
<td>4.68 cm²</td>
</tr>
<tr>
<td>Percentage of hollow space</td>
<td>93%</td>
</tr>
</tbody>
</table>

The specific parameters for operating continuous flow MBBR were determined by research led at Eawag on nitrification reactors.
3.1.1 Continuous flow MBBR set-up

The main components of a continuous flow reactor are (see Fig.3):

1. Influent tank: filled with stored urine
2. Effluent tank: receives overflow from reactor
3. Reactor: filled with Kaldnes® carriers (50% of volume) and nitrified urine (to full volume). Each reactor has a capacity of 7 L.
4. pH transmitter
5. Peristaltic pump: controlled by the pH transmitter
6. Dissolved oxygen (DO) transmitter
7. pH and dissolved oxygen probes: fixed at the top of the reactor.
8. Data logger: records continuously pH and DO values
9. Aeration device

<table>
<thead>
<tr>
<th>Table 3: Reference for material used for continuous flow MBBRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH transmitter</td>
</tr>
<tr>
<td>DO transmitter</td>
</tr>
<tr>
<td>Data logger</td>
</tr>
<tr>
<td>pH probe</td>
</tr>
</tbody>
</table>

The reactor spiked with bacteriophage MS2 had supplementary components (see Fig.4):

10. Syringe pump and syringe: the syringe contains the solution of MS2. The syringe pump discharges the solution continuously in the reactor

Initial set-up

Urine for the nitrification experiments was collected from the men’s NoMix storage tank at Eawag’s main building. 100L were fetched on September 10, 2013, transported by car in 50L containers, and subsequently stored at 4°C. Nitrified urine (appr. 25L) and Kaldnes® carriers with biofilm already grown (appr. 10L) were collected from the operating nitrification reactor at Eawag. They were stored at 4°C until the reactors were launched (October 1-3, 2013).

pH regulation

The pH within the reactors is controlled by urine input. The pH transmitter is configured to regulate the pH within the reactor, with a PID regulation (proportional-integral-derivative). The pH transmitter records the instant pH value of the reactor and activates the perisaltic pump that injects stored urine in
**Figure 3:** Continuous flow MBBR set-up. 1: Influent tank, 2: Effluent tank, 3: Reactor, 4: pH transmitter, 5: Peristaltic pump, 6: DO transmitter, 7: pH and DO probes, 8: Datalogger (not present in the picture), 9: Aeration device

**Figure 4:** Reactor no. 2, with 10: syringe pump and syringe

the reactor when needed. Based on the work on nitrification reactors at Eawag, the target lower and upper pH limits in the reactor are pH 6 and 6.1. In this
study, a proportional regulation (P) was used to control the urine input, meaning that the action of the regulator is proportional to the difference between the measured value and the target value. The parameters ($K_p$, $t_{min}$) were empirically determined to have a rapid reaction, but without an overshoot of the set-point:

- pH set-point = 6.1
- $K_p = 3$: proportional gain. It determines the response time.
- $T_{min} = 0.1s$: minimal length of response. This length should allow at least one drop of urine in the reactor.

With these values, the average pH in the reactors stayed stable at 6.05 (S.D. < 0.0001) throughout the entire sampling period, except for short periods of electronic malfunction. The P regulation allows a gentle approach of the target value, avoiding sudden pH changes.

Aeration
Compressed air was diffused from the bottom of the reactor to ensure the aeration of the reactor and the mixing of the carriers. An air diffuser (bought in Qualipet store) was fixed at the bottom of a plastic rod, which was secured against the wall of the reactor. The compressed air went first through an empty bottle to avoid backwash in the compressed air supply system, then through a bottle filled with water in order to moisturise the air before entering the reactor.

The open parts at the top of the reactors were clogged with paper towel to avoid too much evaporation and escape of foam from the reactor.

Maintenance
The pH and DO probes were calibrated weekly, according to the manufacturer instructions.

Monitoring
The weights of the influent and effluent tanks were recorded daily. In case of sampling, the amount, date and time were recorded. When nitrified urine and Kaldnes® were removed from the reactor for other experiments, the same volume of material was replaced, using the stock stored at 4°C.

The data logger recorded the values of pH and DO in each continuous flow reactor at a frequency of one data-point per minute. Temperature was recorded daily, based on the value displayed by the pH transmitter.
3.2 Batch and semi-batch MBBRs

In order to be able to test several types of pathogens as well as different parameters (such as aeration, composition of solution, etc.), small semi-batch and batch reactor tests were conducted. The volume of these reactors was more than 10 times smaller than the continuous flow MBBRs volume. Faster results could be obtained that way.

3.2.1 Batch and semi-batch set-up

Two sets of experiments with different types of reactor were conducted:
- semi-batch: input, no output, with aeration
- aerated batch: no input, no output, aeration
- non-aerated batch: no input, no output, no aeration

The main components of the set-up are (see Fig.5):
1. Batch or semi-batch reactor: 1L glass bottle, filled with carriers, previously sieved (250mL) and solution (500mL) as specified in Table 4.
2. Influent bottle: 1L glass bottle
3. Multichannel peristaltic pump
4. Aeration device

Figure 5: Batch and semi-batch MBBR set-up. 1 & 2: Reactors and influent bottles, 3: Multichannel peristaltic pump, 4: Aeration device
**Initial set-up**
Nitrified urine and biofilm carriers were collected from the operating continuous flow Reactor no. 1. Clean carriers (non-sterile) given by the Laboratory for Environmental Biotechnology (EPFL), were used in control batches (see exact composition in Table 4).

**pH regulation**
pH was not recorded constantly. The urine input flowrate was determined empirically by a previous test. Regular pH controls and nitrite strip tests allowed to check the batches stability.

**Aeration**
Compressed air was diffused from the bottom of the reactor to ensure the aeration of the reactor and the mixing of the carriers. An air diffuser was wedged against the wall of the reactor. The compressed air went first through an empty bottle to avoid backwash in the compressed air supply system, then through a bottle filled with water in order to moisturise the air before entering the reactor. The open parts at the top of the reactors were partially covered with parafilm to avoid contamination and too much evaporation.

**Filtration**
Filtered nitrified urine was used for some of the batch MBBRs (see Table 4). Nitrified urine was filtered first with a 0.7µm glass fiber filter (1825-047, Whatman) and subsequently with a 0.45µm cellulose nitrate filter (NC 045 50 BL, Albet LabScience), previously rinsed with 50mL of phosphate buffer saline (PBS).

As mentioned above, the batch and semi-batch reactors were used to assess the effect of different parameters on various pathogens in a short amount of time. To this extent, five types of reactors were prepared (Table 4).
Table 4: Composition of batch and semi-batch reactors

<table>
<thead>
<tr>
<th>Type</th>
<th>Name and purpose</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitrified urine 1 and 2</td>
<td>500mL nitrified urine (from Reactor no. 1) 250mL biofilm carriers (from Reactor no. 1) average pH measured = 6.2 aeration input = stored urine</td>
</tr>
<tr>
<td></td>
<td>Biologically active batch</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Filtered nitrified urine</td>
<td>500mL nitrified urine (from Reactor no.1) filtered at 0.45µm 250mL clean carriers (LBE, EPFL) average pH measured = 6.1 aeration input = nitrified urine filtered at 0.45µm</td>
</tr>
<tr>
<td></td>
<td>Sterile control</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PBS, aeration</td>
<td>500mL phosphate buffer saline (PBS) 250mL clean carriers (LBE, EPFL) pH controlled = 6.1 aeration no input</td>
</tr>
<tr>
<td></td>
<td>Simple matrix composition</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PBS, no aeration</td>
<td>500mL PBS pH controlled = 6.1 no aeration no input</td>
</tr>
<tr>
<td></td>
<td>Room temperature and AWI reference</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Nitrified urine, no aeration</td>
<td>500mL nitrified urine (from Reactor no. 1) pH controlled = 6.1 no aeration no input</td>
</tr>
<tr>
<td></td>
<td>Room temperature and AWI reference</td>
<td></td>
</tr>
</tbody>
</table>

Several inactivation mechanisms and inactivation influencing factors were estimated based on the following comparisons:

- **Reproducibility:** type 1 semi-batch is the closest replication of the continuous flow nitrification reactors. Two semi-batches of type 1 were run in parallel to assess the reproducibility of the measurements.

- **Effect of biological treatment system:** the comparison of type 1 and 2 semi-batches (Table 4) reveals the effect of the presence of nitrifying bacteria and nitrification reaction. Indeed, type 1 semi-batch composition is similar to the continuous flow MBBRs, while type 2 semi-batch undergo the same variation (solution input, temperature, ...) but contain no nitrifying bacteria (filtered solution and clean carriers).

- **Air-water interface:** the comparison of batches type 3 with 4, and type 1 with 5 should indicate whether the aeration, therefore a great air-water interface influences the die-off rate of bacteriophages and bacteria. Two
different solution compositions were assessed for this mechanism (nitrified urine, PBS).

- **Tailing phenomenon, solution composition:** the comparison of reactors type 1, 2 and 3 (two complex solutions and one simple solution) reveals whether the inactivation of pathogen indicators is affected by the composition of solution in aerated systems.

- **Room temperature:** if inactivation in batches types 1 to 3 is noticed, the effect of the room temperature on the survival of bacteria and bacteriophages needs to be known. Batches of type 4 and 5 were spiked with pathogen indicators and left in the same room as the other reactors, covered but not sealed. They can be used as reference. If the inactivation observed in the other batches is exactly the same as this reference, the exposure of the indicators to room temperature might be the cause of the die-off. Otherwise the presence of another inactivation mechanism is implied.

### 3.3 Analytical methods

#### 3.3.1 Chemical solutions

**PBS:** Virus and bacteria experiments were conducted using phosphate buffer saline (PBS: 5mM PO$_4^{2-}$, 10 mM NaCl, pH 7.5).

**Beef extract:** Beef extract (Merck) 6% containing glycine (pH 9.3) was used for a method development and for a desorption test.

#### 3.3.2 Sampling

**Monitoring of continuous flow reactors**

**Frequency:** Ammonium, nitrate, nitrite, total-nitrogen and COD were monitored periodically with cuvette tests. Over the period of this study (September 2013 to December 2013), 10 measurements were taken.

**Methodology:** For each measurement the following quantities were sampled:

- **Influent:** 18mL removed (two times 9mL) from the influent tank with a sterile 10mL syringe.
- **Effluent:** 18mL pipetted (two times 9mL) directly from the reactor content with a sterile 10mL pipette.
**Hach and Dr.Lange cuvette tests:** Kit tests from the manufacturer Hach-Lange were used to measure the following parameters in the nitrification reactors:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dr. Lange kit reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>LCK 303</td>
</tr>
<tr>
<td>Nitrite</td>
<td>LCK 342</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>LCK 338</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD)</td>
<td>LCK 614</td>
</tr>
</tbody>
</table>

Samples analysed with the Dr.Lange kits were previously filtered using a 0.45\( \mu \text{m} \) cellulose nitrate filter (NC 045 50 BL, Albet LabScience). The first 9mL of the filtrate were discarded to avoid modification in nitrogen concentration due to the filter composition. Samples analysed were diluted with Milli-Q water.

Ion chromatography was used to analyse anions and cations in the nitrification reactors. However, due to technical issues, the data collected are not reliable enough, and will not be represented in the results.

**Monitoring specific to the spiked continuous flow Reactor no. 2**

MS2 spiking started on November 1st, 2013.

**Frequency:** Samples of the reactor content and syringe input reference were taken almost daily.

**Methodology:** A 1mL sample was pipetted directly from the reactor content, with a sterile 5mL pipette. All samples were taken in duplicates.

**Input syringe:** The syringe input solution had a concentration of 10^7 pfu/mL (±12%). To avoid possible decrease in bacteriophage concentration due to the exposure at room temperature, the input solution was renewed every 2 or 3 days. At each renewal, 0.5mL of the input solution was reserved in an eppendorf, and placed besides the Reactor no. 2, i.e. under the same temperature conditions. This solution was used as reference to assess the concentration in the syringe.
Monitoring of batch and semi-batch MBBRs

Frequency: Samples of were taken daily.
Methodology: A 1mL sample was pipetted directly from the reactor content, with a 5mL sterile pipette.

3.3.3 Bacteriophage enumeration

MS2
Bacteriophage MS2 (DSMZ 13767) and its Escherichia Coli host (DSMZ 5695) were used. The stock of MS2 was produced according to the method described in Pecson et al. [24]. The stock was conserved in PBS at 4°C. The E. Coli host was conserved in aliquots at -80°C, and was grown overnight at 37°C in streptomycin-containing LB broth immediately prior to use in batch experiments. The method of double-layer agar was used for enumeration [40].

ΦX147
Bacteriophage ΦX147 (DSMZ 4497) and its Escherichia Coli host (DSMZ 13127) were used. The stock of ΦX147 was produced according to the method described in Pecson et al. [24]. The stock was conserved in PBS at 4°C. The E. Coli host was conserved in aliquots at -80°C, and was grown overnight at 37°C in streptomycin-containing LB broth immediately prior to use in batch experiments. The method of double-layer agar was used for enumeration [40].

Qbeta
Bacteriophage Qbeta (DSMZ 13768) and its Escherichia Coli host (DSMZ 5695) were used. The stock of Qbeta was produced according to the method described in Pecson et al. [24]. The stock was conserved in PBS at 4°C. The E. Coli host was conserved in aliquots at -80°C, and was grown overnight at 37°C in streptomycin-containing LB broth immediately prior to use in batch experiments. The method of double-layer agar was used for enumeration [40].
3.3.4 Bacteria enumeration

Salmonella

_Salmonella typhimurium_ (M 1414) isolates grown on LB agar containing 100 $\mu g/ml$ ampicillin were provided by the Environmental microbiology group at Eawag, grown in LB broth with 100 $\mu g/ml$ ampicillin, and stored in aliquots with 15% glycerol at -80°C. Salmonella from stored aliquots were grown overnight in ampicillin-containing LB broth immediately prior to use in batch experiments. 100$\mu L$ of sample were plated using the spread-plate method on agar for enumeration.

Enterococci

_Enterococcus_ spp. (ENT) colonies were isolated from wastewater treatment plant influent on Bile Esulin Agar (Sigma Aldrich) and subsequently grown in Azide Glucose Broth (Sigma Aldrich). Aliquots of log-phase growth ENT were stored at -80°C with 15% glycerol. ENT from stored aliquots were grown overnight in azide glucose broth immediately prior to use in batch experiments. 100$\mu L$ of sample and 50mL PBS were filtered using the membrane filtration EPA method 1600 for enumeration.
4 Results and discussion

4.1 Continuous flow reactors

The two continuous flow MBBRs were launched on October 1st and 3rd, 2013. The material came from the operating reactor at Eawag, therefore the system was already at steady state. Several compounds and parameters in the reactors were regularly measured (see Chapter 3.3.2). The viability of a reactor can be estimated through the nitrification rate, which is calculated as follows:

\[
\text{Nitrification rate} \left[ \frac{g}{m^2 d} \right] = \frac{(NH_4, \text{in} \left[ \frac{gN}{L} \right] \cdot Q_{\text{in}} \left[ \frac{L}{d} \right]) - (NH_4, \text{out} \left[ \frac{gN}{L} \right] \cdot Q_{\text{out}} \left[ \frac{L}{d} \right])}{\text{Carriers total surface} \left[ m^2 \right]}
\]

Table 6: Average measured nitrification rate in continuous flow MBBRs

<table>
<thead>
<tr>
<th></th>
<th>Average nitrification rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor no. 1 ([gN/m^2d])</td>
<td>0.56 ± 0.14</td>
</tr>
<tr>
<td>Reactor no. 2 ([gN/m^2d])</td>
<td>0.49 ± 0.10</td>
</tr>
</tbody>
</table>

On the graphs below (Fig.6), the pH, DO and temperature data points are represented along with the nitrification rate. The disturbances represented by the red bars correspond to high perturbation events in the system. For Reactor no. 1, each event is a removal of the reactor content (and replacement from the stock material) for the batches experiments (see Chapter 3.2.1). The long disturbance in Reactor no. 2 is a period with no urine input, as a parallel experiment required the pH transmitter, and also because of technical issues.

The nitrification rate decreased over the period of the study in both reactors. pH and DO were relatively stable. The measurements of temperature were taken once a day, and at different times of the day. The temperature was in average lower than the temperature recorded during experiments led at Eawag, which may have affected the efficiency of the nitrification. The strongest variations appeared in Reactor no .1, where an important part of the reactor material (2L of nitrified urine, 1L of carriers) was removed and replaced by stored material. The important sampling might have had an effect on the stability of the system.

Along with the decrease in nitrification rate, the input and output flowrate decreased in both reactors. Again, the phenomenon is more pronounced in Reactor no. 1 (see Fig.7).
Figure 6: Continuous flow MBBRs recorded parameters (pH, dissolved oxygen) compared to nitrification performances. Reactor no. 1 at the top, Reactor no. 2 at the bottom.
The evolution of the MBBRs was followed by chemical analysis of the system. The concentrations measured in stored urine (Table 7) are consistent with other analysis of urine from the NoMix men’s storage tank at Eawag [8, 10].

Table 7: Average stored urine measured parameters in continuous flow MBBRs (n=8)

<table>
<thead>
<tr>
<th>Stored urine, Reactor no. 1</th>
<th>Average ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄ tot [mg/L]</td>
<td>3764 ± 182</td>
</tr>
<tr>
<td>N tot [mg/L]</td>
<td>3973 ± 436</td>
</tr>
<tr>
<td>COD [mg/L]</td>
<td>3870 ± 61</td>
</tr>
<tr>
<td>COD/N [mg O₂/mg N]</td>
<td>1.04 ± 0.12</td>
</tr>
<tr>
<td>pH [-]</td>
<td>9.04 ± 0.09</td>
</tr>
<tr>
<td>Stored urine, Reactor no. 2</td>
<td>Average ± S.D.</td>
</tr>
<tr>
<td>NH₄ tot [mg/L]</td>
<td>3573 ± 300</td>
</tr>
<tr>
<td>N tot [mg/L]</td>
<td>3780 ± 168</td>
</tr>
<tr>
<td>COD [mg/L]</td>
<td>3893 ± 264</td>
</tr>
<tr>
<td>COD/N [mg O₂/mg N]</td>
<td>1.06 ± 0.08</td>
</tr>
<tr>
<td>pH [-]</td>
<td>9.04 ± 0.1</td>
</tr>
</tbody>
</table>
The values measured within the reactors are shown in Table 8. From comparison with Table 7, we see that half of the total ammonia was oxidised during nitrification, which is consistent with the literature (see Chapter 2.1). NO$_3$ was measured by IC, but due to technical issues, the data are not reliable enough to be represented here.

<table>
<thead>
<tr>
<th>Effluent values, Reactor no. 1</th>
<th>Effluent values, Reactor no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2$ [mg/L]</td>
<td>Average ± S.D.</td>
</tr>
<tr>
<td>1.37 ± 0.43</td>
<td>NO$_2$ [mg/L]</td>
</tr>
<tr>
<td>NH$_4$ tot [mg/L]</td>
<td>1980 ± 129</td>
</tr>
<tr>
<td>4273 ± 330</td>
<td>NH$_4$ tot [mg/L]</td>
</tr>
<tr>
<td>N tot [mg/L]</td>
<td>419 ± 72</td>
</tr>
<tr>
<td>COD [mg/L]</td>
<td>COD [mg/L]</td>
</tr>
<tr>
<td>0.09 ± 0.01</td>
<td>COD/N [mg O$_2$/mg N]</td>
</tr>
<tr>
<td>pH [-]</td>
<td>pH [-]</td>
</tr>
</tbody>
</table>

In general, the two continuous flow MBBRs were operating properly. But after a certain time the nitrification rate decreased. The exact causes of this decrease are not fully understood. The removal of material is not the main cause, because the nitrification rate decreased in both reactors, and material was mostly removed from Reactor no.1. It may still have a impact on the stability of the reactor, as nitrification and flowrates in Reactor no. 1 were slower. The dimensions of the reactors (height, width) and force of aeration could contribute to the difference with the rates measured in other experiments led at Eawag.

In the table below (Table 9), we can note that the losses by evaporation were limited. The hydraulic retention time (HRT= V/Q) increased as the flowrate decreased. The average temperature in the reactors in approximately 5°C lower than the reported operating reactors temperature in the literature [8].

<table>
<thead>
<tr>
<th>Parameters, Reactor no. 1</th>
<th>Parameters, Reactor no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT [d]</td>
<td>Average ± S.D.</td>
</tr>
<tr>
<td>20.7 ± 8.2</td>
<td>HRT [d]</td>
</tr>
<tr>
<td>17 ± 3.7</td>
<td>Losses [%Vol]</td>
</tr>
<tr>
<td>1.1% ± 0.9</td>
<td>1.6% ± 0.9</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>Temperature [°C]</td>
</tr>
<tr>
<td>19.1 ± 1.8</td>
<td>20.2 ± 1.3</td>
</tr>
</tbody>
</table>
4.2 Continuous flow reactor spiked with MS2

The continuous flow Reactor no. 2 was spiked continuously with the bacterio-
phage MS2 for 50 days. The concentrations are expressed in plaques forming 
unit per mL (pfu/mL).

In a continuous flow reactor, the concentration of a tracer is calculated as follows:

\[
C_{\text{tracer}}[\text{pfu/mL}] = \frac{\left( (C_{R2,t-1}[\text{pfu/mL}] * V_{R2}[mL]) \right)}{V_{R2}[mL]} + \frac{\left( C_{\text{syringe}}[\text{pfu/mL}] * Q_{\text{syringe}}[mL/min] * \Delta t[\text{min}] \right)}{V_{R2}[mL]} - \frac{\left( C_{R2}[\text{pfu/mL}] * Q_{R2,\text{out}}[mL/min] * \Delta t[\text{min}] \right)}{V_{R2}[mL]}
\]

Where:
- \( C_{R2,t-1} \) = Concentration in the reactor at \( t = t-1 \)
- \( V_{R2} \) = Volume of reactor
- \( C_{\text{syringe}} \) = Concentration of MS2 in syringe
- \( Q_{\text{syringe}} \) = Flowrate of discharge of MS2 solution in the reactor
- \( Q_{R2,\text{out}} \) = Discharge out of the reactor

In a continuous flow reactor, the volume is constant. The general mass balance 
is: IN - OUT + CONVERSION = 0, as there is no accumulation.

In the equation to calculate the concentration of a tracer, \( C_{\text{tracer}} \), the first term 
of the numerator corresponds to the amount already present in the reactor, 
divided by the volume of the reactor. The second term is the amount of MS2 
discharged in the reactor during a period of time \( \Delta t \), divided by the volume of 
the reactor. The third term of the equation is the amount of MS2 leaving the 
reactor, divided by the volume of the reactor.

Based on this equation, a model based on the \( C_{\text{tracer}} \) equation in Excel using 
1-minute time steps was used. The steady-state in a CSTR (continuously stirred 
tank reactor) is reached after three HRT [41]. The Excel model confirmed that 
the period for the tracer to reach a steady state concentration within the reactor 
is very long. Injecting at first a high concentration of a tracer in a continuous 
flow reactor allows the concentration in the reactor to rise very fast. A lower 
concentration can later be spiked. The period of time required to reach the 
tracer steady state can be skipped this way.
This assumption was tested on the Reactor no. 2 by injecting:

- $10^{10}$ pfu/mL of MS2 at 0.001 mL/min for 1 hour; followed by
- approximately $10^7$ pfu/mL of MS2 at 0.001 mL/min continuously after this time.

These concentrations were determined with the Excel model. The values were manipulated by trial-and-error to establish the target concentration at steady-state in the reactor within a reasonable timeframe.

In Fig. 8, the evolution of MS2 concentration over time is displayed. Based on the measurements of the MS2 spiking solution (concentration, injection flowrate) and the reactor monitoring, the concentration of a tracer is calculated (blue line). The actual sampling results are represented by the red line. To date, the difference between these concentrations is not significant and MS2 reacts for the moment as a tracer within the nitrification reactor, suggesting that over the period of this experiment, the bacterial community in the reactor did not adapt to inactivate MS2 bacteriophage.

**Figure 8:** Evolution of bacteriophage MS2 vs. the concentration of a calculated tracer
4.3 Batch experiments

Smaller batch reactors avoid the constraint of the time to reach steady-state mentioned before. Inactivation of bacteriophages MS2, ΦX147 and Qbeta, as well as the inactivation of bacteria Enterococcus spp. and Salmonella was tested over 7 to 10 days.

The spiked concentrations were:
- *S. typhimurium* = $10^8$ cfu/ml
- ENT = $10^5$ cfu/ml
- MS2 = $10^6$ pfu/ml
- Qbeta = $10^6$ pfu/ml
- ΦX147 = $10^6$ pfu/ml

Concentrations are expressed in plaques forming unit per mL (pfu/mL) for bacteriophages and in colony forming unit per mL (cfu/mL) for bacteria.

The evaporation in the batches proved more significant with this set-up than in the continuous flow reactors. The aeration was difficult to adjust to the size of the batch reactors. Another difference with the continuous flow MBBRs is that the semi-batch MBBRs do not have an outflow, so the volume rises. The calculated concentrations were adapted to the evaporation and the input. In the batches containing nitrifying bacteria, pH and nitrite concentrations were measured daily to verify the batch reactors stability. pH values were stable around 6.2 and there was no nitrite accumulation.

The following graphs display the fraction of surviving organisms ($C/C_0$) over time, $C_0$ being the initial spiked concentration.
4.3.1 Inactivation of bacteriophages

Reproducibility (Fig.9) The reproducibility was assessed by comparing the results of the two semi-batches of "type 1" that are the closest representation of the continuous flow nitrification reactors (see Table 4). Fig.9 shows that the reproducibility between two batches was strong for each of the three bacteriophages. Two-tailed t-test assuming equal variance was calculated for the three bacteriophages experiments by comparing the full data set of batch 1 to that of batch 2 for each organism: ΦX147 (p=0.84), MS2 (p=0.77), Qbeta (p=0.71).

![Figure 9: Reproducibility of inactivation of bacteriophage (ΦX147, MS2, Qbeta) in semi-batch nitrification MBBR](image-url)
**Effect of biological treatment system (Fig.10)**  
Semi-batches containing an operating nitrification system (type 1 in Table 4) were compared to sterile controls, semi-batches containing filtered nitrified urine, i.e., no bacteria larger than 0.45\(\mu\)m (type 2 in Table 4). The composition of the solution is complex in both cases and the same conditions were applied (input of solution, either urine or filtered nitrified urine).

![Graphs showing inactivation of bacteriophage](image)

**Figure 10:** Effect of nitrification in semi-batch MBBR on the inactivation of bacteriophage (\(\Phi X147\), MS2, Qbeta)

For each phage, the concentration of bacteriophage followed the same evolution, regardless of the batch type (active biological system or sterile control). This result indicates that the biological activity in the nitrification reactors does not cause bacteriophage inactivation.
Air-water interface (Fig. 11 and 12)  The air-water interface influence was evaluated by comparing inactivation in aerated and non-aerated systems, in both PBS (Fig.11) and nitrified urine (Fig.12), corresponding to a comparison between batches of type 3 and 4 and of type 1 and 5 from Table 4, respectively.

In the buffer, a strong inactivation of ΦX147 was observed in the batch in the presence of aeration. A second test in buffer with aeration was conducted to confirm this decrease, but in this second test, the concentration remained stable (Fig.11, ΦX147: PBS, aeration 2). The result of either tests in PBS aerated could be an isolated event. It would be interesting to repeat this test to confirm the result.

The aeration seemed to have an inactivation effect on Qbeta in PBS. MS2 was not disturbed at all. The interpretation for ΦX147 is difficult due to the results mentioned above.

**Figure 11:** Effect of air-water interface on inactivation of bacteriophage (ΦX147, MS2, Qbeta) in PBS

Based on the results of the repeated test of ΦX147 in buffer (aerated batch) duplicates of the experiments with all three bacteriophages are recommended.
In nitrified urine, neither ΦX147 nor MS2 were affected by the aeration. The concentration of Qbeta in nitrified urine without aeration decreased importantly and went even lower than that concentration in aerated nitrified urine. Therefore, the inactivation of Qbeta could not be explained by aeration alone. Duplicates of the experiments with Qbeta in nitrified urine without aeration are recommended.

The comparison of the batches with nitrified urine with and without aeration was meant to highlight the effect of the aeration in a complex media. Nevertheless, the interpretation is difficult due to the presence of nitrifying bacteria in both types of batch, but under different conditions: in the first type of batch (aerated), nitrifying bacteria are grown in biofilm on the carriers and the system receives urine by input, therefore there is an active nitrification; whereas in the second batch (non aerated) nitrifying bacteria are in suspension and there is no urine input.
**Matrix effect (Fig. 13)** Batches containing PBS (type 3, Table 4) were compared to nitrified urine (type 1, Table 4) and filtered nitrified urine (type 2, Table 4) which are two complex solutions. All batches compared were aerated.

Based on the results displayed in the graphs below, one can conclude that in the complex solutions (nitrified urine and filtered nitrified urine) the inactivation of bacteriophage is either the same or even less pronounced than in the buffer. This phenomenon might be due the protective effect of the solution and the tailing phenomenon described above (Chapter 2.2.3), that would result in the protection of the bacteriophage.

![Graphs showing inactivation of bacteriophage in different solutions](image)

**Figure 13:** Effect of composition of solution on inactivation of bacteriophage (ΦX147, MS2, Qbeta) in aerated systems

As a plateau was observed in the Qbeta batches, a test was conducted to see if despositions on the capsid were the cause of the decrease in inactivation. According to the method described by Sigstam et al. [31], the bacteriophage
was washed, spiked in a new solution. The concentration spiked should follow a new inactivation curve. Unfortunately, because the concentration of Qbeta remaining in solution was low, the volume of washed solution was not large enough to properly conduct the experiment, and no result could be obtained.

The inactivation curve observed for Qbeta in nitrified urine can be divided into the exponential decrease and the plateau. The rate constants \( k \) were calculated for the exponential decrease:

**Table 10:** Inactivation rate constants \( k \) for first step of inactivation of Qbeta in nitrification semi-batch MBBR, \( n=4 \)

<table>
<thead>
<tr>
<th>Nitrified urine semi-batch 1</th>
<th>Nitrified urine semi-batch 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k = 1.344 \text{ [d}^{-1}\text{]} ) (( R^2 = 0.76 ))</td>
<td>( k = 1.290 \text{ [d}^{-1}\text{]} ) (( R^2 = 0.71 ))</td>
</tr>
</tbody>
</table>

The monitoring was done daily, so by taking only the first part if the inactivation the number of samples is limited.
**Room temperature (Fig.14)** Viruses are affected by temperature and the exposure to room temperature of the bacteriophages over the period of time of the tests might have had an influence on their inactivation. In order to estimate the proportion of inactivation due to the temperature, batches with PBS and nitrified urine without aeration (type 4 and 5, Table 4) were installed in the same room as the other batches. The batches were not completely closed, to be in the same condition as the other batches. The temperature in the LCE laboratory at EPFL is 20°C.

**Figure 14:** Effect of room temperature on inactivation of bacteriophage (ΦX147, MS2, Qbeta) in non-aerated systems

Bacteriophages ΦX147 and MS2 were not affected by the ambient temperature over 8 days. The concentration of Qbeta decreased of five log in nitrified urine over 8 days, but remained relatively stable in PBS. The presence of nitrifying bacteria in nitrified urine might contribute to this decrease. This measurement should be repeated to confirm the result.
Adsorption on the biofilm carriers  An assessment of the adsorption on the carriers permitted to estimate whether the observed inactivation of the bacteriophages could be partially due their adsorption the plastic carriers. A two-step desorption test was conducted. One carrier was placed in 15mL PBS and shaken vigorously for 2 minutes. The carrier was then placed in 15mL of beef extract and mixed for 20 minutes. The PBS and beef extract were plated to calculate the desorption. In parallel, the amount of liquid retained in the void of a carrier unit was estimated by drying 10 carriers separately. Half of the carriers tested contained a large amount of biofilm grown on them, and the other half was almost free of biofilm.

The recovered concentration from the desorption method corresponded to the concentration due to liquid retained by the carrier. This parameter can therefore be ruled out for bacteriophages. The test was not conducted for bacteria.
4.3.2 Inactivation of bacteria

Reproducibility (Fig.15) The curves of inactivation of Enterococcus spp. (ENT) and S.typhimurium were similar between two identical batches (type 1, Table 4). Two-tailed test (t-test) assuming equation variance was calculated for the two bacteria: S.typhimurium (p=0.996) and ENT (p=0.943).

Figure 15: Reproducibility of inactivation of bacteria (ENT, S.typhimurium) in semi-batch nitrification MBBR
Effect of biological treatment system (Fig. 16)  The concentrations of ENT and 
*S.typhimurium* were stable in the absence of bacteria, i.e. in filtered nitrified 
urine (batch type 2, Table 4). On the contrary, in nitrification batches (batch 
type 1, Table 4) the inactivation was clear and reached a 3-log inactivation for 
ENT and a 5-log inactivation for *S.typhimurium*.

![Graph showing inactivation of bacteria](image)

**Figure 16:** Effect of nitrification in semi-batch MBBR on the inactivation of bacteria (ENT, 
*S.typhimurium* )

Biological activity appeared to have an effect on the survival of bacteria. The 
difference between the two types of batches was clear.

The inactivation rate constants $k$ in both type 1 semi-batch reactors were calcu-
lated:

**Table 11:** Inactivation rate constants $k$ for inactivation of *S.typhimurium* and ENT in bi-
ologically active reactors

<table>
<thead>
<tr>
<th></th>
<th>Nitrified urine semi-batch 1</th>
<th>Nitrified urine semi-batch 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S.typhimurium</em></td>
<td>$k = 0.570$ [d$^{-1}$] ($R^2 = 0.85$)</td>
<td>$k = 0.665$ [d$^{-1}$] ($R^2 = 0.82$)</td>
</tr>
<tr>
<td>ENT</td>
<td>$k = 0.400$ [d$^{-1}$] ($R^2 = 0.91$)</td>
<td>$k = 0.400$ [d$^{-1}$] ($R^2 = 0.83$)</td>
</tr>
</tbody>
</table>

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**Air-water interface (Fig.17)** Batches containing nitrified urine and PBS were compared with and without aeration.

It is important to note that the batch containing nitrified urine without aeration did not contain carrier and was not fed by urine. Nevertheless, nitrifying bacteria were present in suspension. On the other hand, the batch aerated with nitrified urine was set-up as a nitrification reactor, with urine input and biofilm grown on the carriers. The comparison is still interesting in order to assess the effect of bubbling.

![Graphs showing the effect of air-water interface on inactivation of bacteria (ENT, S.typhimurium) in PBS and nitrified urine.](image)

**Figure 17:** Effect of air-water interface on inactivation of bacteria (ENT, S.typhimurium) in PBS and nitrified urine

Based on the graphs in Fig.17, the aeration did not affect the inactivation of bacteria neither in PBS nor in nitrified urine, as all the slopes followed the same path.
**Matrix effect (Fig.18)** Fig.18 shows the comparison of inactivation in an aerated system in a simple solution (PBS) and in complex solutions (nitrified urine and filtered nitrified urine). The concentration of ENT stayed unchanged in filtered nitrified urine and PBS, but decreased in nitrified urine (nitrification batch). *S.typhimurium* reacted in a similar way.

![Graph showing inactivation of Enterococcus and Salmonella](image)

**Figure 18:** Effect of composition of solution on inactivation of bacteria (ENT, *S.typhimurium*) in aerated systems

The complexity of the solution did not protect bacteria as it could do for bacteriophage. The difference in inactivation in Fig.18 seemed to be due to the biological activity in semi-batch type 1 (Table 4). Fig.18 shows that the complexity of solution does not provide a protective or inactivating effect, as the same concentrations were measured in PBS and filtered nitrified urine. The composition of solution as factor of inactivation could be ruled out and the result displayed in Fig.16 (effect of active nitrification) confirmed.
Room temperature (Fig.19) In order to estimate the proportion of inactivation due to the temperature, batches with PBS and nitrified urine without aeration (type 4 and 5, Table 4) were installed in the same room as the other batches.

There was no inactivation of the tested bacteria in PBS at room temperature. In nitrified urine, however, the concentration of ENT lowered of 2-log and 4-log for *S.typhimurium*, this inactivation is likely to be due to the presence of nitrifying bacteria in suspension in nitrified urine.

![Graph showing inactivation of ENT and S.typhimurium in PBS and nitrified urine](image)

**Figure 19**: Effect of room temperature on inactivation of (ENT, *S.typhimurium*) in non-aerated systems
4.3.3 Summary of inactivation mechanisms

Tests performed on bacteriophages and bacteria are summarised in the table below (Table 12). As the effect of air-water interface and room temperature in nitrified urine is unclear due to the presence of the nitrifying bacteria in solution, the results are not part of the recapitulative table (Table 12).

<table>
<thead>
<tr>
<th></th>
<th>ΦX147</th>
<th>MS2</th>
<th>Qbeta</th>
<th>S.typhimurium</th>
<th>ENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active biological treatment system</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Air-water interface in PBS</td>
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<td>X/?</td>
<td>√/?</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Protective matrix effect</td>
<td>possible</td>
<td>possible</td>
<td>possible</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Room temperature in PBS</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Remarks can be drawn based on these comparisons:
- The presence of active nitrification affects bacteria relative to controls but does not affect bacteriophages.
- Bacteriophages might be protected by the nitrified urine (filtered or not) complex solution. Further tests according to the method described by Sigstam et al. [31] can test this supposition. This protective effect was not observed on the tested bacteria.
- Bacteriophage Qbeta appeared to be sensitive to the effect of air-water interface in PBS. MS2 was apparently not affected by the AWI. But based on contradictory results with ΦX147, duplicates of the effect of AWI in PBS with all three bacteriophages should be done.
- Neither bacteriophages nor bacteria were sensitive to exposure at room temperature in PBS.

In general, bacteriophages ΦX147 and MS2 appeared to be the most resistant. It is interesting to point out that despite the fact that MS2 and Qbeta have a similar structure, their reactions were not always similar. Bacteria were less resistant and were apparently affected mainly by biological activity.

The results of the tests run in batch and semi-batch MBBRs cannot be extrapolated directly to the continuous flow MBBRs. Several parameters were not the same (reactor dimensions, aeration rate, etc.). Nevertheless, this kind of test permits to evaluate the inactivation mechanisms of the bacteria and bacteriophage and to inform further experiments on continuous flow MBBRs.
5 Conclusion

The present study focused on four main points: operating nitrification reactors, characterising virus and bacteria inactivation with the use of surrogate virus and estimate different types of inactivation mechanisms.

Two continuous flow nitrification reactors were successfully set-up. Yet, the nitrification efficiency decreased over time. Given the relatively short period of this study, it is difficult to determine if the nitrification will stabilise at an operating rate or if the decrease will continue. Also the frequent and important sampling might have influenced the reactors stability. With a longer study, a more stable system can provide information about the effect of the dimension of the reactor in order to optimise large scale applications.

Inactivation of bacteriophage MS2 in a continuous flow MBBR was monitored over 60 days. To date, MS2 underwent no inactivation. Based on semi-batch experiments, MS2 revealed resistant to nitrifying bacteria and to most of the inactivation mechanisms tested. Therefore it would be interesting to continue the study over a longer period and to run tests with additional indicator organisms.

Mechanisms of inactivation of bacteria and bacteriophage were estimated through several tests. Small batch and semi-batch tests were set-up to provide information faster than with a larger reactor. The results of these experiments can be used as a guideline to run tests in larger continuous flow MBBRs. In these tests only bacteria were inactivated during active biological nitrification. The other mechanisms tested were not efficient at inactivating bacteria. It appeared that bacteriophages were in general more resistant than bacteria, as expected. In addition, a protecting effect of the solution composition is suspected.
A step of the nutrient recovery process is the storage of urine. Several studies showed that storage is an efficient way to inactivate many pathogens. However, pathogens can be detected in stored urine used for nitrification, either due to the resistance of some pathogens to inactivation during storage or because of insufficient storage time. During storage, bacteria are known to inactivate more rapidly than viruses. Thus viruses are more likely to be found in nitrification reactors. Based on the results of this study, they are likely to be also resistant to the conditions in nitrification reactors. In addition, viruses may receive protection from components of the nitrified urine.

Therefore, as the VUNA project moves forward, urine treatment for the inactivation of viruses remains an important challenge to address.
References


