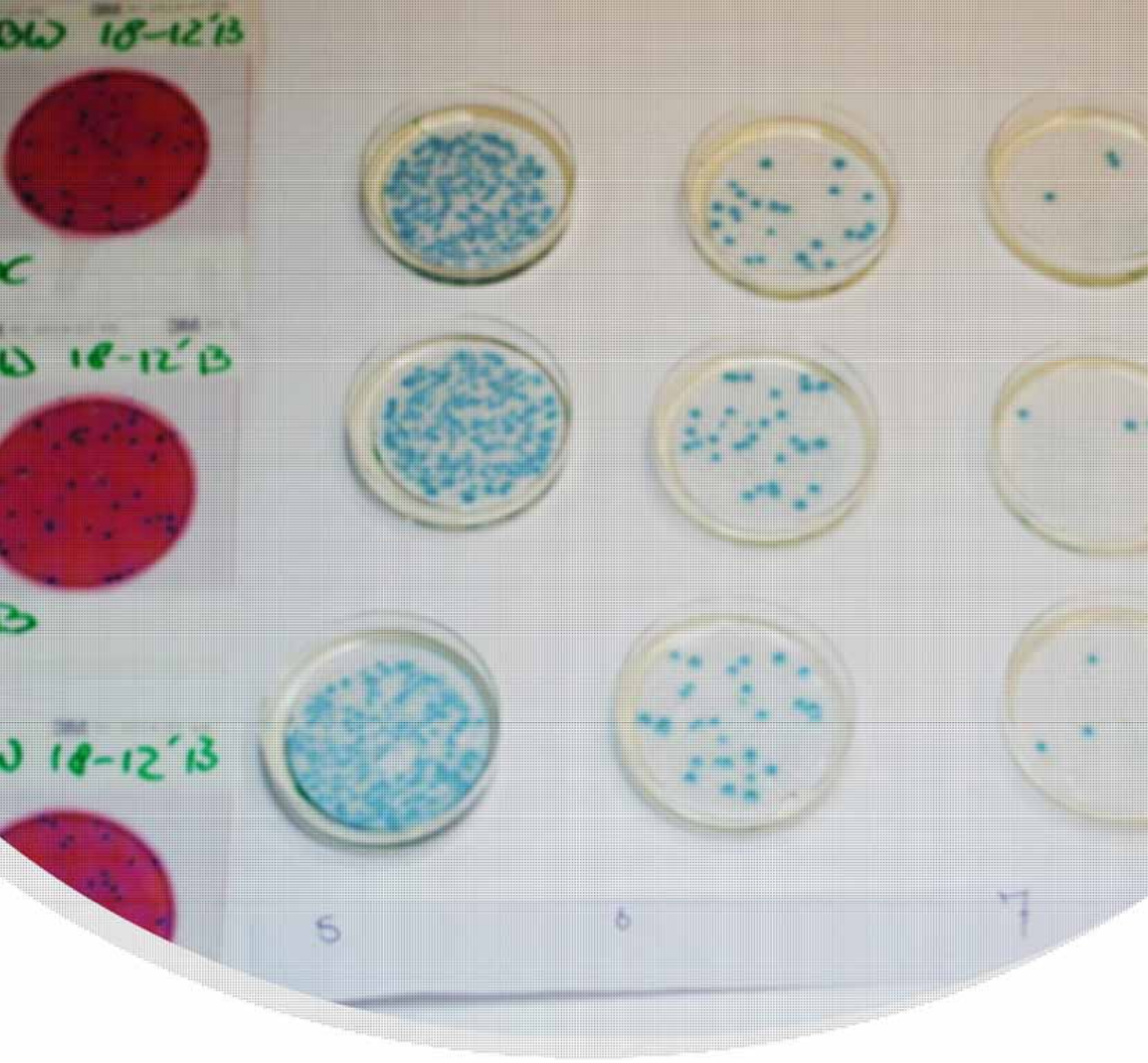

THE EFFICIENCY OF THE NIOO-KNAW PROPOSED DECENTRALIZED SANITATION SYSTEM IN REMOVAL OF COLIFORMS AND ESCHERICHIA COLI

How efficient is a 55 °C Thermophilic UASB followed by an algae based photo bioreactor in removal of Coliforms and Escherichia coli

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Summary

With increasing world population, more wastewater is produced which all need purification treatment. Removal of pathogenic organisms is important because they can cause problems to human health. This experiment will focus on the removal of human bacterial pathogens. The problem of human bacterial pathogens is that infections can emerge when there is contact between wastewater and humans. A bacterium is defined pathogenic when it causes disease to a human; therefore not all bacteria are pathogenic.

In the Netherlands the water boards start cooperating together in order to conduct energy from the wastewater treatments instead of consuming energy for the treatment of wastewater. The demand for new sanitation systems developed several new techniques including a decentralized sanitation system. The Black Water (BW) of the Netherlands Institute of Ecology (NIOO KNAW) is highly concentrated waste water. The BW is treated in a 55 ˚C thermophilic up flow Anaerobic Sludge Blanket reactor (UASB). The BW from The NIOO-KNAW only consist of urine, faeces and 1 litre of groundwater flush. The effluent of the UASB is referred to as Digested Black Water (DBW). The NIOO-KNAW wants to use a thermophilic UASB operating at 55 ˚C because it is supposed that the pathogen removal is higher compared to a mesophilic UASB 20-42 ˚C due to higher temperatures. In the UASB mostly biological activity takes place and the organic carbons are converted into biogases including methane (CH_4) which are collected and reused for heating up the UASB. The NIOO-KNAW does not has a UASB system operational and therefore 25 ˚C mesophilic effluent samples are derived from Sneek. Because the NIOO KNAW does not have a 55 ˚C thermophilic UASB in operation 55 ˚C thermophilic effluent is mimicked by incubating the 25 ˚C mesophilic UASB at 55 ˚C for 4 days.

The effluent of the UASB flows directly towards algea filled photo bioreactors (PBR). The algal filled PBR's are used because of the bactericidal properties of algae. Therefore the NIOO-KNAW desires to implement an algal based photo bioreactor. The algal specie inoculated in the PBR's of the NIOO-KNAW is *Chlorella sorokiniana,* this species is commonly used in the scientific world because of broad growth spectrum. Identification of all the human pathogen bacteria species is expensive, therefore indicator species are used. The Total Coliform (TC) and the *Escherichia coli (*E. coli) bacteria are most commonly used as indicator species. The E. coli bacteria is most preferred as indicator species because this species is exclusively found in faeces and E. coli can outlive high temperatures and circumstances compared to other bacterial species.

In the experiment there are three PBR's filled with *Chlorella sorokiniana*. Each PBR present a different Hydraulic Retention Time (HRT), respectively 12 hours, 21 hours and 30 hours. The HRT is the variable for the removal of coliforms and E. coli's. These HRT's are based on the growth rate of *Chlorella sorokiniana.* The HRT times are based on the growth of the algae because the algae wash out with the effluent of the PBR.

To detect TC and E. coli a culture plating technique is used. Three different mediums are selected: The m-Endo LES medium, the 1604 medium, and the 3M coliform/E-coli Petri films. The incubation time and temperatures are the same for the 3 mediums, 35 °C± 0, 5 ˚C and the time is set at 24± 2 hours, for the 3M Petri film the coli forming units other than E. coli are counted after 24 hours incubation time, and on the same Petri film the E. coli after 48 hours of incubation time.

According to the data the mimic 55 ˚C thermophilic UASB effluent still contain TC bacteria and E. coli bacteria. The results from the effluent of the PBR's filled with *Chlorella sorokiniana* do not contain any E. coli bacteria, However, they do contain TC other than E.coli. The PBR with a HRT of 12 hours has the lowest amount of TC other than E. coli compared to the two other HRT's. However, the effluent of the PBR with a HRT of 12 hours contains more TC than the effluent of the mimic thermophilic UASB. In the blank experiment only the 3M Petri films are used due to lack of time. The results was almost no growth at all. However it could be that the uv-light eliminated the bacteria because there were no algae to block the light. The overall conclusion is that the 55 ˚C thermophilic UASB could be efficient in removing TC and E. coli bacteria but more data is required. The PBR removes E. coli bacteria; however the algae enriches the effluent with TC growth. The amount of data is not sufficient enough to calculate approved statistical analyses. Although sufficient data is not available for approved statistical analyses, further data collection would make it possible to make a steady conclusion. With this data not a steady conclusion can be drawn, further investigation at this subject is necessary.

Definitions

Abbreviation	Full words	Definitions
BW	Black Water	Faeces+ urea+ 1 Litre of groundwater flush
COD	Chemical Oxygen Demand	The amount of oxygen which is acquired to perform chemical transformations
DBW	Digested Black Water	Black water which is digested, by the process where the water lost a lot of Carbon compounds
DESAR	Decentralized Sanitation and Reuse	In this thesis a wastewater treatment system which consists out of a UASB and a PBR followed by a helophyte filter.
DNA	Deoxyribonucleic acid	A nucleic acid that carries the genetic information in the cell and is capable of self-replication and synthesis of RNA
E-coli	Escherichia coli	A member of the coliform bacteria, distinguished from coliforms by fermenting lactose at 44 °C
Effluent		A water stream that flows out of another body of water
EPA	Environmental Protection Agency	An agency of the United States government that is created by an act of Congress and is independent of the executive departments
GW	Grey Water	Wastewater from kitchen, bathroom (not the toilet), and laundry cycles. This water can be reused or recycled, also called sanitary water
HRT	Hydraulic Retention Time	Is a measure of the average length of time that a soluble compound remains in a constructed bioreactor
Influent		Water flowing in or into
MDBW	Mesophilic Digested Black Water	In this thesis MDBW reveres specific to a 25 ˚C UASB positioned in Sneek
NIOO-KNAW	The Netherlands institute of Ecology	Carries out fundamental and strategic ecological research. The researchers studying organisms, populations, communities and ecosystems or Ecology

PAR	Photosynthetic Active Radiation	Watt/m^2
PBR	Photo bioreactor	A bioreactor is an installation for the production of microorganisms outside their natural habitat, however, inside an artificial environment
TDBW	Thermophilic Digested Black Water	In this thesis TDBW reveres to MDBW incubated MDBW for 4 days at 55˚C, which means the TDBW is a mimic
TNTC	Too Numerous to Count	CFU overgrowing each other, hence too numerous to count
TC	Total Coliform	Coliform bacteria are microbes found in the digestive systems of warm-blooded animals, in soil, on plants, and in surface water.
TCFU	Total Colony forming Units	coliform bacteria colony bigger than 1-3 mm.
UASB	Up flow Anaerobic Sludge Blanket reactor	Operates at different temperatures, produces biogas in conditions without oxygen and specific useful bacteria
UV-Light	Ultra violet light	UV-Light is electromagnetic radiation with a wavelength shorter than that of visible light. Ranging from 10 nm-380 nm

Content

1 Introduction

1.1 Problem description

1.1.1 Pathogens

With increasing world population, more wastewater is produced which all needs purification treatment. General pathogens are classified as protozoa's, parasites, fungi, helminths, viral pathogens and bacterial pathogens (Leclerc & Moreau, 2002). Removal of pathogenic organisms from waste water is important because they can become a potential hazard to human health. This experiment will focus on human bacterial pathogens. The common human bacterial pathogens found in wastewater are: Campylobacter *jejuni, Leptospira sp., Clostridium perfringens, Escherichia coli, Legionella pneumophila, Mycobacterium tuberculosist, Pseudomonas aeruginosa, Salmonella enterica, Shigella flexneri, Staphylococcus aureus, Vibrio cholerae,* and *Yersinia enterocolitica* (Awuah 2006; Lubberding, & Gijzen, 2001; Cai & Zhang, 2013). The potential hazard of human bacterial pathogens is that humans with a low resistance are vulnerable for infections when they come into contact with contaminated water.

A bacterium is defined pathogenic when it can cause diseases to a human; therefore not all bacteria are pathogenic, there are strains in DNA of bacteria which define if a bacterium is pathogenic. For example the E.coli strain O157:H7 causes severe diarrhoea, however, other strains of E.coli are essential in contribution of food digestion (Leclerc & Moreau, 2002). Most pathogenic bacteria are excreted by warm-blooded animals like humans, some bacteria could also have their origin in surface water or in sediments (Oshiro, 2002; W. Ahmed, 2006). Contaminated water can infect people with dramatically consequences. Therefore it is essential that all the pathogenic organisms are removed from the final effluent in a wastewater treatment system. For example, in some country's water is chlorinated or radiated by uv-light in order to remove pathogenic bacteria. The uv-light treatment is expensive and adding chlorine to water is not healthy for the environment. It is not precisely known how efficient water sanitation systems are in removing pathogenic bacteria, and the worldwide demand for less expensive and environmental friendlier wastewater treatment makes research for sustainable removal of pathogenic organisms necessary (Ansa, 2013;Awuah et al., 2001; Cai & Zhang, 2013).

1.1.2 Decentralized sanitation system

A typical conventional centralized sanitation systems consists of a preliminary treatment followed by a primary and a secondary treatment, and sometimes a tertiary treatment. Preliminary treatment incorporates the removal of large floating objects, followed by the primary removal which involves removal of sediments by settling or chemical coagulation. The purpose of the secondary treatment is to remove nutrients from the wastewater, this is necessary because in the surface water the amount of nutrients is less abundant. If many nutrients leave the sanitation system in the surface water the aquatic environment will be influenced by for example overgrowth of algae. In most cases, biological techniques are used to eliminate nitrogen and phosphate by using aerobic and anaerobic bacteria. The tertiary treatment is used to upscale the water quality from the effluent of the secondary treatment by using sand filtration or reverse osmoses. These conventional systems do not always retrieve all the nutrients, water or other important resources efficiently. The demand for more natural effluent water implemented a major difference in the way of wastewater treatment. Within the upper description of wastewater treatment water harmonicas are used in the tertiary treatment. Which means daphnia are commonly used for disinfection and helophyte filters for polishing the wastewater from nutrients. This way of treatment makes the effluent more similar to the surface water because the effluent contains oxygen and biological activity such as daphnia. This is a short explanation of wastewater treatment, there are many different treatment manners. In the Netherlands the water boards start cooperating together in order to conduct energy from the wastewater treatments instead of consuming energy for the treatment of wastewater (waterschappen, 2013).

The demand for new sanitation systems developed several new techniques including a decentralized sanitation system. Depending on the scale, decentralized sanitation systems are less expensive, use less water and extracts biogases like methane (CH_4.), compared to the conventional systems which just excrete the biogases into the atmosphere(Kujawa-Roeleveld, 2005). Therefore research at Decentralized Sanitation and Reuse (DESAR) can be fertile and useful for sustainability. An example of a DESAR is an Up flow Anaerobic Sludge Blanket Reactor (UASB). Containing a tank which includes a sludge bed in the bottom coated with anaerobic bacteria which convert organic compounds into methane (CH_4) and carbon dioxide (CO_2) (Verbyla, Oakley, 2013). These gases are collected as biogases and can be reused to heat up the UASB. The UASB can operate under three circumstances. The psychrophilic condition of 0-20 ˚C, mesophilic conditions of 20-42 ˚C and thermophilic conditions of 42-75 ˚C. A mesophilic condition is commonly implemented because it is effective in removing organic matter and the respectively low temperature enables a self-sustainable heating system.

The main difference between a mesophilic and thermophilic condition is that with the thermophilic condition methanogenic bacteria utilize acetate (CH_3COO^-) more effectively and the Hydraulic Retention Time (HRT) is lower under thermophilic conditions. Because of the higher temperature it is assumed that the removal of pathogenic bacteria is more effectively (Cavinato, Bolzonella, 2013). The retrieval of resources is important because resources like phosphate are extracted from mines and they will be depleted within 50 years(Duley, 2010). The retrieval of resources is the drive force for research at decentralized systems.

1.1.3 The NIOO-KNAW decentralized sanitation system

The Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, the Netherlands, implemented a decentralized sanitation system for their new building. The toilets and sewer are adjoined through a vacuum system with the DESAR. Because the toilet water only consist out of faeces, urine, toilet paper and one litre of ground water per flushing, this water is referred to as Black Water (BW).Different BW can have different characteristics, depending on the source. BW is highly concentrated with organic compounds, nutrients, micro pollutants, and therefore human pathogens (Wilt, 2013). The BW is treated in a 55 ˚C thermophilic UASB, The effluent of the UASB is referred to as Digested Black Water (DBW). The advantage of separating wastewater into BW and Grey Water (GW) is that the amount of pathogens is mainly concentrated in the BW. Other wastewater different from BW contains shower, laundry, -kitchen wastewater and rain water is referred to as grey water (GW) (Lienert et al. 2007).

The NIOO-KNAW wants to use a thermophilic UASB operating at 55 ˚C because it is supposed that the pathogen removal is higher compared to a mesophilic UASB 20-42 ˚C (Cavinato et al., 2013;Wendland, Deegener, 2007;Skillman et al., 2009). The second reason is because in Sneek they are going to start a similar experiment with a UASB operating at 55 ˚C thermophilic conditions, the UASB of the NIOO-KNAW is inoculated with sludge from the UASB at Sneek. Therefore the NIOO-KNAW has decided to keep the same circumstances for their UASB. The third reason for the 55 ˚C choice is that other relative studies use a temperature close to 55 ˚C (Skillman et al., 2009). It is useful to keep obtained data comparable with other studies, together with the fact that 55 ˚C is still a thermophilic condition, yet not the highest temperature to be named thermophilic which reduces the amount of energy necessary for heating up the UASB. The (HRT) of the UASB at NIOO-KNAW is 4 days; this is based on a previous study. In this case study the optimal HRT and dimensions are calculated on a toilet survey (Guijt Anja, 2012). The effluent of the UASB continues through a photo bioreactor (PBR) based on the algae specie *Chlorella sorokiniana*. In the PBR the remaining nitrogen and phosphates are absorbed by the algae (Zimmer, 2003). The final sanitation process consists of a vertical constructed helophyte filter where the last remnant of nitrate and phosphate are removed. The GW of

the NIOO-KNAW is not treated in the UASB and goes directly towards the vertical helophyte filter. The problem of the effluent of the helophyte filter is that it still can contain human pathogens. At the moment the final effluent is not discharged on the local pond but in the sewer because the possibility of pathogenic contamination. The NIOO-KNAW desires their decentralized system to operate in conditions which allows them to discharge the effluent water on the local pond without any environmental or potential infection problems. If the final effluent is discharged on the surface water, the water can percolate in the ground water again and then the water cycle is closed. However, it is not known how a 55 ˚C thermophilic UASB and an algal based photo bioreactor combined system performs in removing pathogenic organisms.

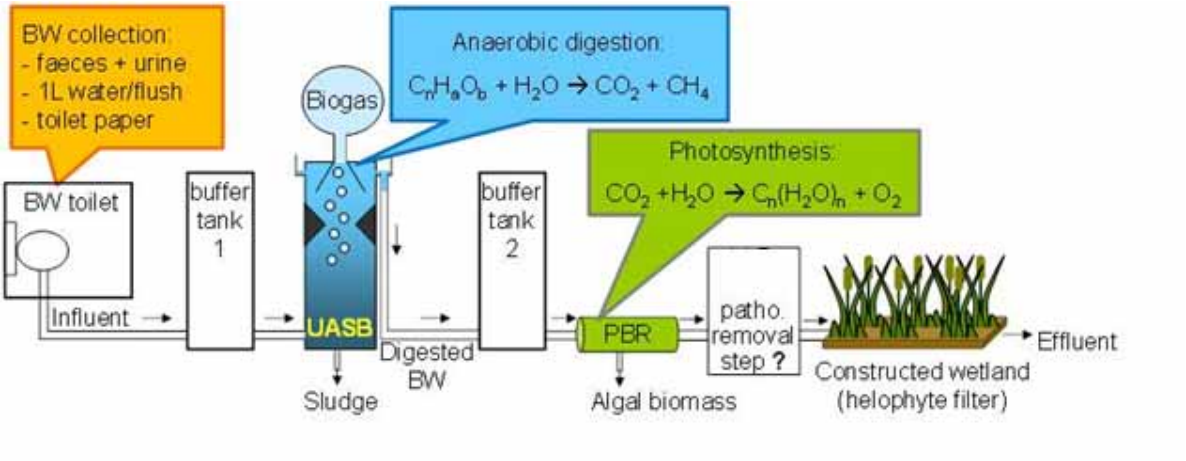


Figure: 1 proposed DESAR of NIOO-KNAW picture by Tania Fernandes, 2013

1.1.4 Photo bioreactor

An UASB conducts Chemical Oxygen Demand (COD) transformations through methanogenic bacteria, however the amount of phosphorus and nitrogen are still plentiful present in the effluent of the UASB, and therefore Photo bioreactors (PBR) are developed to extract these nutrients in form of algal growth. It is mentioned that a PBR filled with algae works bactericidal because the algae compete with bacteria for glucose(Awuah, 2006). If the thermophilic UASB is not efficient enough in removal of human bacterial pathogens, the performances of a PBR becomes more interesting for the removal of all human bacterial pathogens. According to Mara wastewater ponds and photo bioreactors are an inexpensive solution for the removal of bacterial pathogens and nutrients. Other scientist claim that algae ponds are better in removing bacterial pathogens compared to duckweed ponds, however García contradicts this statement(Mara, 2000; Ansa, 2013; Awuah et al., 2001; Zimmer, 2003; García et al., 2008). It is assumed that the pathogen removal is superior in algae based PBR due to the fact that duckweed ponds do not let the sunlight penetrate through the surface of the water, Ansa and Davies-Colley already explain that: pH, Dissolved oxygen (DO), temperature, starvation, predatory, and sunlight play a major role in the removal or deactivation of bacterial pathogens (Ansa, 2013; Dababneh & Shquirat, 2012; Davies-Colley,et al., 1999). The algae species inoculated in the PBR's of the

NIOO-KNAW is *Chlorella sorokiniana* because this species is commonly used in the scientific world, therefore much information is available for this species. Another advantage of this species is that *Chlorella sp*. grows rapidly and is viable in a wide spectrum of physical and chemical condition and therefore can reproduce under harsh conditions like in DBW. Because of the bactericidal effect of algae the NIOO-KNAW want to implement algae based photo bioreactor.

1.1.5 Detection of human pathogen bacteria

The utilization through the presence of bacteria as indicators for water quality has been used since the 1880. In that time it was theorised that only humans where responsible for the pollution of wastewater through bacteria (H. Heukelekian, 1964). Identification of all the human pathogen bacterial species is expensive, therefore indicator species are used. The *Escherichia coli (*E. coli) and Total Coliform (TC) bacteria are most commonly used. The E. coli bacteria is most preferred as indicator species because this species is exclusively found in faeces and E. coli can survive high temperatures compared to other bacterial species (Cai, 2013; Awuah, 2006). The allowed amount of E. coli and TC bacteria presence in surface water is qualified according Guidelines 2006/7/EG European Parliament and counsel (Unie, 2006; Skillman et al., 2009).

Several techniques are feasible for detecting indicator bacterial species in wastewater. For this project the most convenient type of experimenting is the culture plate based technique. The advantages are that the technique is widely spread in the scientific world. Culture plating is relatively inexpensive compared to other techniques; however the expenses are influenced by the medium composition (Noble, et al., 2010). With different compositions of mediums, different types of bacterial species are selected in growing, which makes them easy to count and exclude contamination of unwanted bacteria. Another advantage of the culturing technique is that only the healthy viable bacteria will grow, in comparison to the Polymer Chain Reaction (PCR) technique, which will just count DNA particles. This makes it uncertain if the DNA particle is from a viable bacterium or from a dead bacterium (Cai & Zhang, 2013). On the other hand, there is pendency of which strains are cultured. The aim in this thesis is to count the viable bacteria and not the dead bacteria. The purpose of the DESAR and the PBR is to eliminate the viable bacteria. Due to the unknown removal efficiency of TC and E-coli bacteria in the thermophilic UASB and in the photo bioreactor the main goal is established.

1.1.6 Coliform bacteria

Bacteria are defined as coliform bacteria when they are rod-shaped gram-negative and non-spore forming and can ferment lactose. Coliform bacteria inhabit the intestines of warm-blooded animals; however, they can also occur in the aquatic environment. Within the class of coliform bacteria there are also distinctions between faecal coliforms through the ability of reproducing by maximum temperature of 49. 5 ˚C within 24 hours. The distinguished faecal coliforms are Escherichia, Klebsiella and the Citrobacter and 90 % of the faecal bacteria consist out of Escherichia. (A.W.W.A, 2005)

1.1.7 Escherichia coli

The E. coli bacteria have been studied for more than 60 years. Hence, much information is available for this group of bacteria, E. coli occurs mainly in the intestines of warm blooded mammals, however, is capable for surviving outside the body for some time .The bacteria has a duplication time of 20 minutes and is considered a rapid grower.

Facultative means that the bacteria have the beneficial quality to be aerobic and anaerobic, within case of the E. coli the preference is to be anaerobic. The harmless strains of E. coli are important in the human intestines because they are responsible for production of vitamin K. because the harmless strains of E. coli inhibit the intestine the pathogenic E. coli has less chance to inhibit those places which are already occupied. Some strains of E. coli are pathogenic like the O157:H7 these can induce haemorrhagic diarrhoea through food which is contaminated with faeces and thereby possible pathogenic E.coli strains. E. coli bacteria outlive high temperatures and multiply successfully until a temperature of 49 ˚C (Fotadar, et al., 2005). Not many mesophilic bacteria can grow at these high temperatures. All these qualities makes E. coli the perfect indicator species for detecting possible contamination of faeces. If tested for E. coli bacteria and the test is negative, this determinates that other bacteria neither stand a chance of survival and there is no harmful faecal contamination.

1.2 Main goal

Implementing a 55 ˚C thermophilic UASB followed by an algae based photo bioreactor at the NIOO-KNAW

1.2.1 Main question

What is the TC and E.coli removal difference of a 25 ˚C mesophilic UASB compared to a 55 ˚C thermophilic UASB followed by a PBR filled with algae?

1.2.2 Sub questions

How efficient is a 55 ˚C UASB in TC and E.coli removal compared to a 25 ˚C UASB?

- How many TC and E.coli colonies does BW contain?
- How many TC and E.coli colonies does a 25 ˚C UASB effluent contain?
- How many TC and E.coli colonies does a 55 ˚C UASB effluent contain?
- What is the removal efficiency of TC and E.coli of a 25 ˚C?
- What is the removal efficiency of TC and E.coli of a 55 ˚C UASB compared to 25 ˚C UASB?

How efficient is an algae based PBR in TC and E. coli removal?

- What is the removal efficiency of TC and E.coli in a 12 hour HRT PBR filled with algae?
- What is the removal efficiency of TC and E.coli in a 21 hour HRT PBR filled with algae?
- What is the removal efficiency of TC and E.coli in a 30 hour HRT PBR filled with algae?
- Which HRT effluent contains the least amount of TC and E.coli?

How efficient is a non-algae based PBR in TC and E. coli removal?

- What is the removal efficiency of TC and E.coli in a 12 hour HRT PBR without algae?
- What is the removal efficiency of TC and E.coli in a 21 hour HRT PBR without algae?
- What is the removal efficiency of TC and E.coli in a 30 hour HRT PBR without algae?
- Which HRT effluent contains the least amount of TC and E.coli?

2 Material & Methods

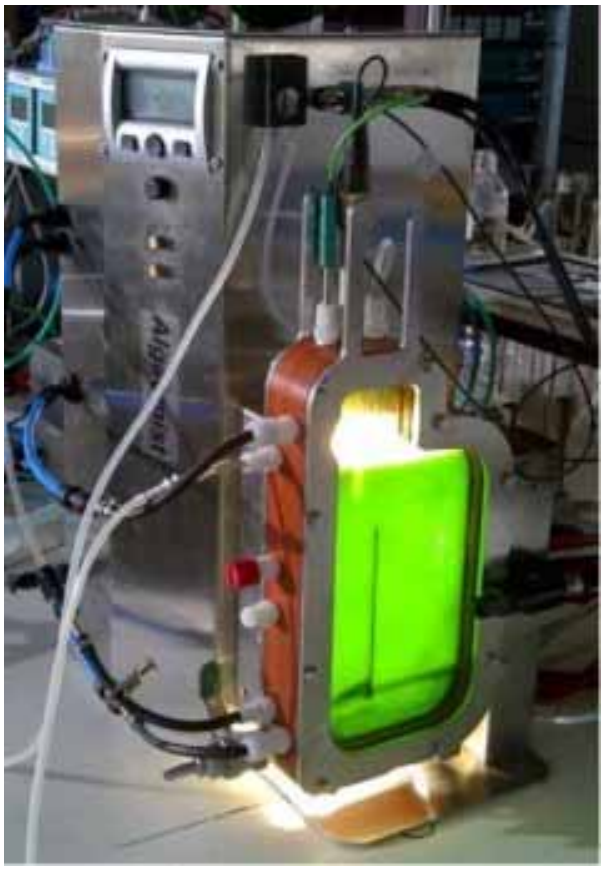
2.1 Sample locations

The BW and the Mesophilic Digested Black Water (MDBW) used in this experiment are derived from Sneek; the MDBW originates from a 25 ˚C mesophilic UASB demonstration site in Sneek (32 houses neighbourhood), Friesland the Netherlands. The UASB of NIOO-KNAW is out of function and therefore the MDBW samples from Sneek will be used in these experiments. The samples originated from Sneek are collected in 4 tanks of 5 litres each; the tanks are kept in a climate room of 4 ˚C.

At the time of sampling, the NIOO-KNAW thermophilic UASB was out of order, which means no Thermophilic Digested Black Water (TDBW) samples could be obtained. Therefore the thermophilic conditions are simulated by modifying the MDBW. The MDBW from Sneek is held in an incubator set at 55 ˚C for 4 days in order to obtain simulated TDBW. The samples are held in an incubator because mainly the high temperature of a thermophilic UASB is held responsible for the bacterial die-off. These mimic samples cannot completely reassemble TDBW because the biological processes in the sludge of the UASB's are also different due to temperature differences. Also, the BW of Sneek has a different composition compared to the BW from NIOO-KNAW, this has to do due to the fact the NIOO-KNAW BW only consist of urine, faeces and 1 litre of groundwater flush, while the BW of Sneek is not only the toilet water but also the water from the sink and shower. Therefore the BW from Sneek has different characterizations for the pathogenic composition. As mentioned before TC and E. coli are mainly occurring in faeces, and therefore TC and E.coli are less abundant in the BW from Sneek because of dilution with GW. However, the addition of kitchen waste is desired because more biogas is produced in the Mesophilic UASB due to high nutrient concentrations (Kujawa-Roeleveld ;2005). See the Annex table 1 for the characterization of the MDBW from Sneek.

2.2 Design of the UASB at NIOO-KNAW

The BW of NIOO-KNAW will be treated in a thermophilic UASB which operates at 55 ˚C. HRT of the UASB is 4 days and the volume of the reactor is 893 Litres. The diameter of the UASB is 0.66 m and the height is 2.75 m, with 5 taps installed each 0.46 m apart from each other (Yixing, 2012). The sludge enters from the bottom of the UASB and on a sludge blanket bacteria will grow including methanogenic bacteria. Here the biological activity takes place and the organic carbons are converted into biogases like carbon dioxide (CO_2) and methane (CH_4). However hydrogen sulphide (H_2S) which is toxic for organisms is also produced, this gas is entrapped from the effluent through a GLS separator. The baffles keep the sludge in a downwards flow preventing inoculated bacteria washing out. Because the thermophilic UASB is not implemented, only the mesophilic UASB from Sneek is characterized. In the future the effluent of the thermophilic UASB continues into a yet to be designed PBR.



Picture 1: Algaemist PBR environment source: Algeamist manual, Tim de Nooij, 2011

2.3 Design PBR parameters

The mimic TDBW is the influent for the PBR experiments. The experiments are conducted in algaemists. This device is designed for continues experiments where the parameters are artificially set. There are three algaemists available at NIOO-KNAW for running experiments. The air inflow, CO_2 inflow, pH, temperature and light intensity are controlled by the algaemist. There are two experiments which are performed within the PBR's. The first experiment is conducted with the algaemists filled with the algal species *Chlorella sorokiniana*. The second experiment is performed with the same conditions, except that in this experiment the reactors are running without the algae, because all the characteristics are the same except for the presence of the algae this experiment is considered as the control group.

Table 1: The parameter setup for the algaemist, Tim de Nooij, 2011

Parameters in the algaemist	In continuous
Temperature	35 ˚C
Air/CO_2	360/40 ml/min

pH	7±0.02
Average light intensity	150 µmoll/m^2s
Medium	TDBW
Algae sp.	*Chlorella sorokiniana*

2.3.1 Temperature

The temperature for the algaemist is set at 35 ˚C; this is the optimum growth temperature for *Chlorella sorokiniana.* The influent within this experiment originates directly from the mimic TDBW stored in the 4 ˚C refrigerator. Through mixing the temperature of 35 ˚C remains homogeneous (Maria Cuaresma, 2009).

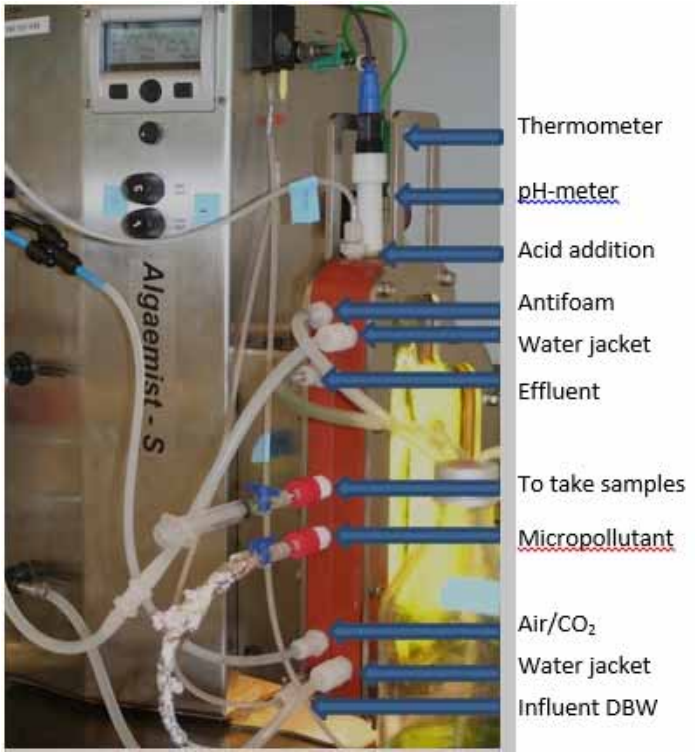
2.3.2 Air and CO_2 inflow and pH

A ratio of 20% air (which also contains 21 % oxygen) and 80 % CO_2 inflow is used. Extra adding of air and CO_2 is important because the algae use CO_2 for photosynthetic reactions and the aeration prevent conglomerating or clogging of the algae. If there is no CO_2 added Carbon limitation will occur and the algae will die-off because they cannot photosynthesize. Secondly, if the CO_2 values become too low, the pH will rise to a basic environment which also causes the algae to die-off, the algae can grow up to a pH 9. If the CO_2 inflow is too high the pH can drop. The algae can grow from a pH 4 value, below this value the algae die-off. The CO_2 inflow is regulated by measuring the pH parameter and adjusted to demand controlled by the algaemist. The algae can reproduce between a range of pH 4-9, however the pH is maintained at 7±0.02(Maria Cuaresma, 2009).

2.3.3 Light

The steady state of *Chlorella sorokiniana* cells is set at 3.8*10^8cell/ml for in the algaemist. For determining the biomass, algae cells are counted in a Multisizer 3 Coulter counter (Beckman Coulter). The coulter counter can only count sizes below 100 µm. Because the samples from the PBR's can conglomerate and clog, the coulter counter samples are filtered with a 70 µm filter. The difference in solids is computed in the calculations. While the algae are still growing to achieve 3.8*10^8cell/ml, the Photosynthetic Active Radiation (PAR) is set at 100 µmoll/m^2s. The density of the algae cells is at that moment underneath the threshold of 3.8*10^8cell/ml. When the cell density reaches 3.8*10^8 cell/ml the Photosynthetic Active Radiation (PAR) is set at 150 µmoll/m^2s because then the

light needs to reach every algae cell. (Maria Cuaresma, 2009).



Picture 2: Design of the Algaemist, source: Alba de Agustin Camacho, 2013

2.3.4 Hydraulic retention time

In the first experiment there are three reactors running filled with algae. Each reactor has a different HRT respectively 12 hours, 21 hours and 30 hours. These HRT are chosen for the algae in order to maintain the $3.8*10^8$cell/ml amount together with the different influent of mimic TDBW. The PBR's are also used for another parallel experiment running at the same time in the same PBR's, this experiment is based on the removal of pharmaceuticals by algae. The HRT of the mimic TDBW is the variable for the removal of TC and E. coli. These HRT's are based on the growth rate of *Chlorella sorokiniana (*Kliphuis et al., 2012). The following formula is used to calculate the inflow of TDBW in ml/h. The 0.8 is the fraction of TDBW, the remaining 0.2 volume fraction is addition of the pharmaceuticals.

$$Qdbw = \frac{Volume}{hours} \times 0{,}8$$

2.4 Experimental setup

The PBR experimental setup is displayed in figure 2 There are 5 different kind of samples measured.

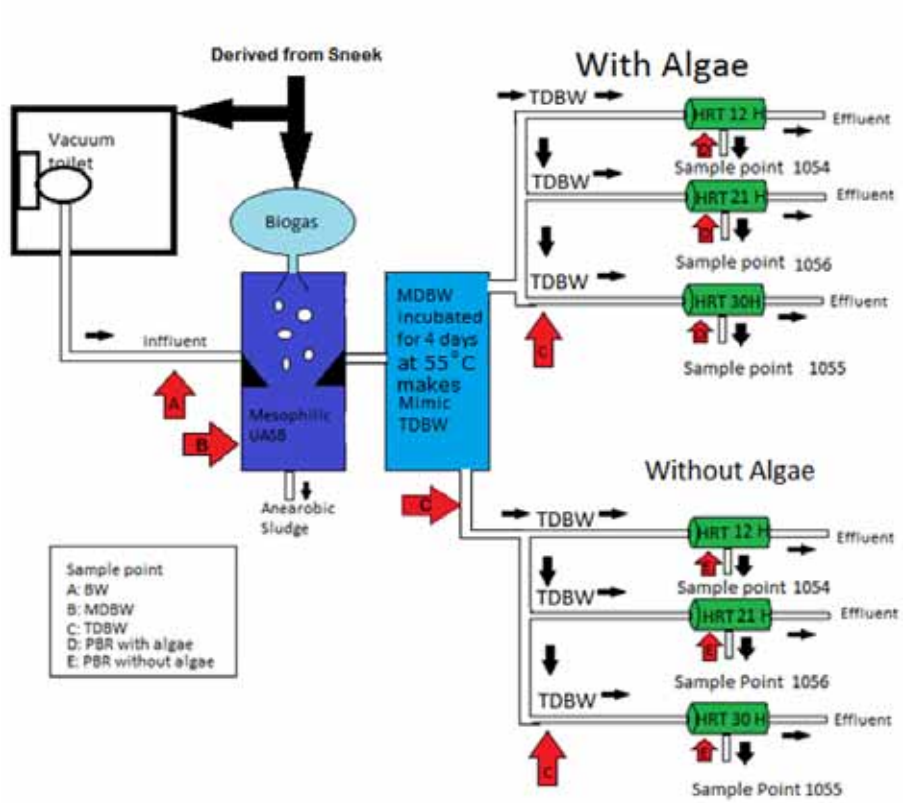


Figure 2: Setup design for the experiments source: Wendy van Kooten, 2013

2.4.1 Sample collecting

The MDBW samples are derived from the tanks stored in a climate room at 4 ˚C which is arrow B, this tank is homogenised before sampling by shaking the tank gently. The samples are 40 ml each and taken in duplicate. The mimic TDBW samples are taken from a 5 litre tank positioned in a refrigerator at 4 ˚C. From this tank a tube runs towards the 3 PBR's. The TDBW samples are taken directly by disconnecting the tube running towards the PBR's and 30 ml TDBW duplicate samples are collected from arrow C. For each PBR duplicate samples of 4 ml each are collected at the beginning of the experiment, and at end of the experiment and these are represented by the arrows D and E. The experiment begins when the PBR is in steady state. The PBR has achieved steady state when the amount of algal cells is around 3, $8*10^8$ cells/ml in a PBR. The start time samples for the pathogen bacteria testing are taken before the pharmaceuticals are inoculated. The samples are obtained directly from the reactor by use of a syringe and injected unfiltered into a sterile conical 15 ml tube and sealed off with the cap and marked. For end time samples the same procedure is used, which is the end of the HRT of a PBR. The running time for each experiment is 2 weeks, which is based on the reduction time of the pharmaceuticals. The samples are stored in a 4 ˚C refrigerator until necessary. Sample collecting procedure is performed according to the following method(A.W.W.A, 2005).

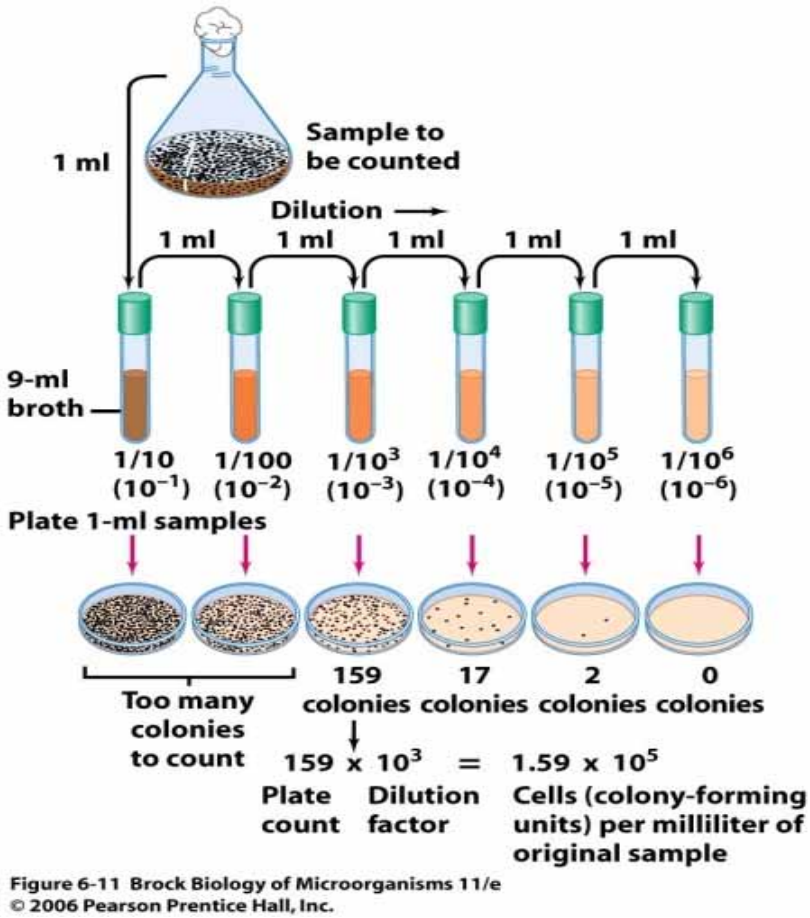
2.5 The vacuum system

This experiment is conducted in a Microbiologic Laboratory safety class 2 (ML-2) due to the biohazard potential of the pathogens. The air flow is a closed circuit with overpressure preventing outside air flowing inside the cabinet due to biohazard safety and keeping the cabinet sterile. Secondly, unlike the fume hood the Bio safety cabinet exhaust air is filtered through a HEPA filter and detains potential hazards like pathogens. In this case the vacuum tube is connected in another cabinet because the safety cabinet does not contain an entrance for a vacuum system. Before use of the complete vacuum system, be sure that the complete glass setup is sterilized by autoclaving all the exits covered with aluminium foil. Look for the complete description in the Annex, Method 1604 manual

Picture: 3 Setup of experiment in biosafety flow cabinet, source: Wendy van Kooten, 2013

2.5.1 Dilutions

The samples are diluted according to the decimal dilution technique. Dilutions are necessary for BW and MDBW, because a mesophilic UASB is not developed for pathogen removal(Wacka, 2012). The plate is declared uncountable if more than 250 Coli forming Units (CFU) are growing. The units over-grow each other and become Too Numerous to Count (TNTC). Some samples are diluted up to 10^{-7} times; each dilution is produced in triplicate for validity. The colonies are plated and counted according to the EPA Method 1604. For making the dilutions first fill the 9 conical tubes up to 9 ml with the working solution by use of the electrical pipette and close the caps. Then vortex the original sample until homogenized, Pipette 1 ml of the original sample into the first three conical tubes, for this step use the same 1 ml pipette tip, close the caps again. Vortex the first dilution until homogenized and pour 1 ml into the second dilution use a new 1 ml pipette tip for every dilution from now on. Continue this procedure for all the dilutions.



Picture 4: An example of decimal dilution factor, source: Brock Biology of Microorganisms

2.5.2 Interpretation of results

The results are implemented in tables which display the amount of Total Colony forming Units (TCFU) and E.coli bacteria of 1 millilitre sample. In the Annex on page XXIV the 1604 protocol explains that the samples are calculated for 100 ml sample. However because of the quantities of growth on the plates 1 ml calculation is used. According to the plating technique the best countable dilution rate is selected and compared with other results. The dilution rate itself does not have an influence on the amount of bacterial cells per millilitre and therefore can be compared with another dilution rate. For removal percentage the following formula is used.

$$Percentage\ removal/growth = \frac{influent - effluent}{influent} \times 100$$

2.6 Culturing coliforms and E-coli

The Method 1604 Total Coliforms and Escherichia coli in Water by Membrane filtration using a Simultaneous detection technique (MI Medium) from the Environmental Protection Agency (EPA) is used. However, two other mediums are used for detecting and comparison of the dilution rates. The other two mediums are the m-Endo LES medium and the 3M coliform/E-coli Petri films. The incubation temperatures and time are the same for the 3 mediums, 35 ˚C± 0. 5 ˚C, and 24± 2 hours. Only for the 3M Petri film the TC other than E. coli are counted after 24 hours incubation time and the E. coli after 48 hours incubation time.

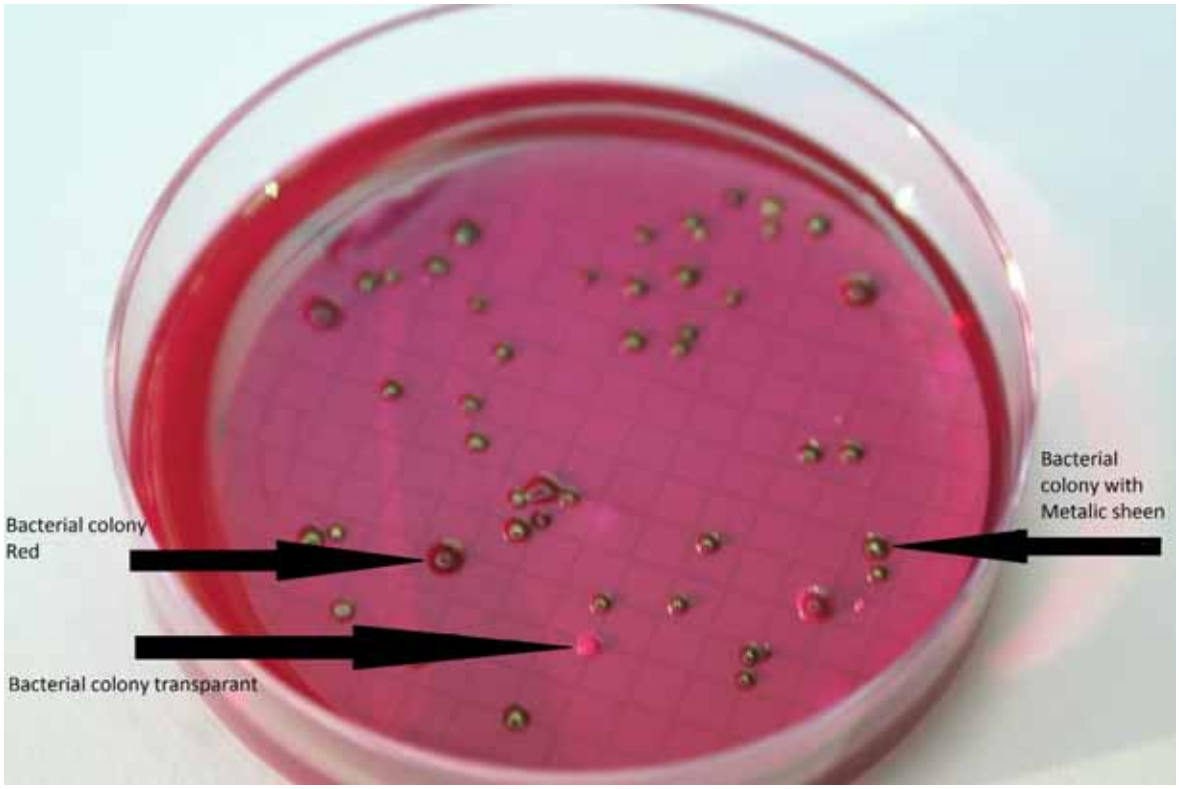
2.6.1 Enumeration and interpretation of petri dishes

For enumeration petri dishes countable up until 250 CFU are used. The ideal amount of CFU is between 20-80 units. Ideally this means the amount of CFU is divided by 200, 20 and 2 for the decimal dilutions. This means that only the dilution with 20 colonies is best to count. It is better to use only this dilution because the error of counting occurring is less big compared to a petri dish with 200 CFU or just 2. If a petri dish with just 2 colonies is used there could be an error because between 2 and 3 colonies is 50 % difference in amount of CFU, while the error difference between 20 and 21 is 5 %, and between 200 and 201 is 0, 5% however, the problem with a petri dish with 250 CFU is the counting error is bigger. Due to the fact that there are not many duplicates in this experiment and therefore not much data all 3 dilutions are taken in account.

Picture 5: M-Endo LESS medium with bacterial growth with decimal differences. Sample: BW 10^{-4} t/m 10^{-7} dilution in triplicate. Source: Wendy van Kooten, 2013

2.6.2 M-Endo LES medium and enumeration

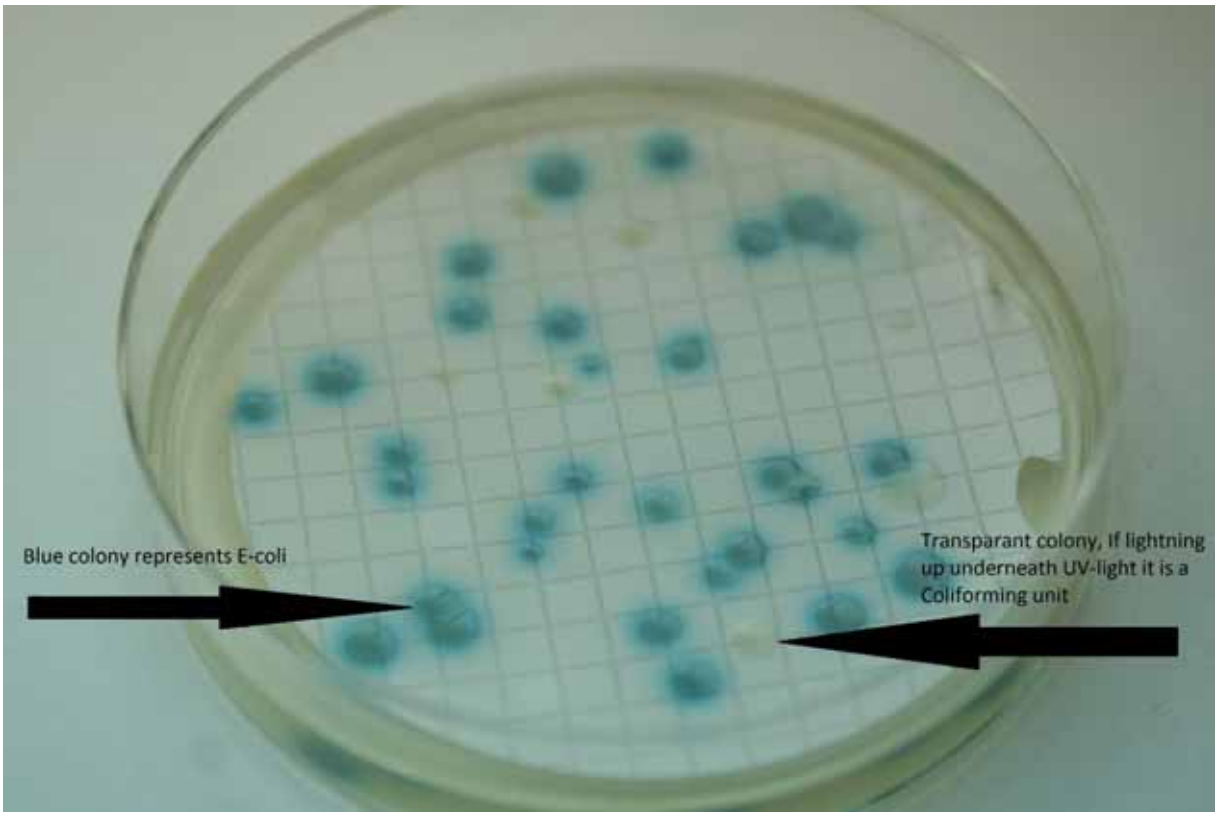
First the experiments are conducted with the m-Endo LES medium as this medium is less expensive compared to the medium from method 1604. The m-Endo LES medium reveals an evaluation for which dilutions are useful for plating on the 1604 medium. The main difference between the 1604 medium and the m-Endo LES medium is that the m-Endo LES medium does not distinguish E. coli colonies from TC colonies. With the m-Endo LES medium all the TC colonies produce a metallic sheen due to the fermentation of lactose and the basic fuchsine red dye. All other bacterial colonies that are not red and without sheen not counted as TC. For further information see the Annex.



Picture 6: m-Endo medium, metallic sheen and red colonies represent total coliform, pink colonies are not counted. MDBW 3th dilution, source: Wendy van Kooten, 2013

2.6.3 Medium 1604 and enumeration

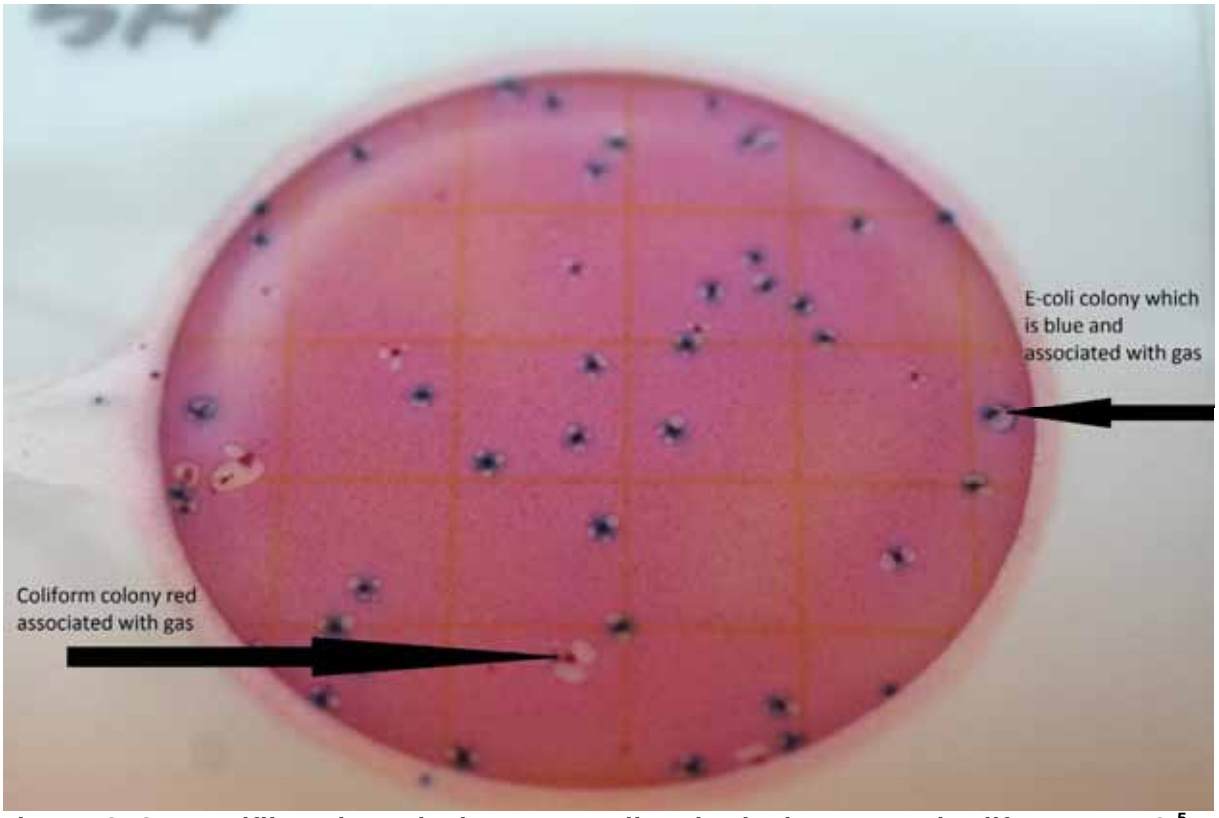
This medium is internationally used and known for water quality testing by plating the indicator species E. coli and TC. These are the typical faecal indicator species for detecting faecal contamination in water, and thereby a suitable method for this experiment(Oshiro, 2002). This is a sensitive medium for enumeration of TC and E. coli by use of a simultaneous detection medium. TC are visible with a UV-light (366 nm) the color of the TC colony illuminates bright white to a light blue. Count the blue colonies by ambient light for the total E. coli. Any other colonies which are not blue or not illuminated are not added to the count. For more information see interpretation guide for method 1604 in the Annex.



Picture 7: 1604 medium, blue colonies are E.coli, black colonies possible total coliform. MDBW 10^{-3} dilution source: Wendy van Kooten, 2013

2.6.4 3M Petri film and enumeration

This medium is used as a side line in the experiment. The medium is used in the food industry for testing TC and E. coli, however, the Petri films are also suitable on wastewater. The Petri films are directly ready for use and therefore there is no need to pour medium first. Secondly, there is just 1 ml sample necessary, and thirdly there is no need for the vacuum filter. Because the vacuum filter is not used this saves time and a contamination factor is eliminated. These tests are preformed according to the AOAC 991.14 test. For more information see the interpretation guide for 3M petrifilm total coliform/e-coli count plates in the Annex.



Picture 8: 3M petrifilm. Blue colonies are E-.coli, red colonies are total coliform. BW 10^{-5} dilution, source: Wendy van Kooten, 2013

3 Results

3.1 How efficient is a 55 ˚C UASB in TC and E.coli removal compared to a 25 ˚C UASB

3.1.1 TC and E.coli in BW

How many Total Colony forming Units (TCFU) and E.coli colonies does BW contain?

Table 1: The TCFU and E.coli distribution in BW samples calculated for 1 ml.

	Dilution rate		10^-5		10^-6		10^-7	
	Black Water		TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Medium 1604	Sample date: 17-12'13	1th experiment	TNTC	TNTC	3,8E+06	3,0E+06	4,0E+06	3,0E+06
	Plating date: 18-12-13	Duplicate	TNTC	TNTC	3,9E+06	3,4E+06	4,0E+06	3,0E+06
		Triplicate	TNTC	TNTC	4,3E+06	3,6E+06	4,0E+06	3,0E+06
Medium 1604	Sample date: 17-12-13	2th experiment	2,2E+06	2,0E+06	3,1E+06	2,8E+06	9,0E+06	7,0E+06
	Plating date: 9-1'14	Duplicate	2,5E+06	2,3E+06	4,2E+06	3,6E+06	1,0E+07	5,0E+06
		Triplicate	2,3E+06	2,0E+06	4,0E+06	2,8E+06	5,0E+06	4,0E+06
3M petrifilm	Sample date: 17-12-13	A	5,1E+06	3,8E+06				
	Plating date 18-12-13	B	5,3E+06	4,0E+06				
		C	5,6E+06	3,8E+06				
		Average 1th exp	-	-	4,0E+06	3,3E+06	4,0E+06	3,0E+06
		Average 2th exp	2,3E+06	2,1E+06	3,8E+06	3,0E+06	8,0E+06	5,3E+06
		Total average	-	-	3,9E+06	3,2E+06	6,0E+06	4,2E+06
		3M average	5,3E+06	3,9E+06				

Table 1 shows that the first experiment with the 5th dilution rate was TNTC. The most reliable dilution rate in this table is the 6th dilution because the TCFU on the grown on the plates is between 20 and 80 colonies which is considered the ideal counting circumstances. The 3M Petri film results are higher compared to the 1604 method. For the plate growth results look in the annex-BW Characterization.

3.1.2 TC and E.coli in 25 ˚C UASB

How many TCFU and E.coli colonies does a 25 ˚C UASB effluent contain?

Table 2: The TCFU and E.coli distribution in 25 ˚C UASB samples calculated for 1 ml.

	Dilution rate		10^-1		10^-2		10^-3	
	Mesophilic DBW		TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Medium 1604	Sample name: 18-2	1th experiment	2,3E+02	2,3E+02	2,1E+02	2,1E+02	0,0E+00	0,0E+00
	plating date : 26-2	Duplicate	2,4E+02	2,4E+02	1,6E+02	1,6E+02	1,1E+02	1,1E+02
		Triplicate	2,2E+02	2,2E+02	2,2E+02	2,2E+02	1,1E+02	1,1E+02
Medium 1604	Sample name: 18-2	2th experiment	1,4E+02	1,4E+02	6,7E+01	6,7E+01	2,0E+02	2,0E+02
	Plating date: 10-3	Duplicate	1,3E+02	1,3E+02	1,6E+02	1,6E+02	0,0E+00	0,0E+00
		Triplicate	1,4E+02	1,4E+02	6,7E+01	6,7E+01	1,0E+02	1,0E+02
3M Petrifilm	Sample name 18-2	A	2,5E+02	2,0E+01	1,0E+02	0,0E+00		
	Plating date: 7-4	B	4,4E+02	2,0E+01	1,0E+02	0,0E+00		
		C	3,8E+02	1,0E+01	2,0E+02	1,0E+02		
		Average 1th exp	2,3E+02	2,3E+02	2,0E+02	2,0E+02	7,4E+01	7,4E+01
		Average 2th exp	1,4E+02	1,4E+02	9,6E+01	9,6E+01	1,0E+02	1,0E+02
		Total average	1,8E+02	1,8E+02	1,5E+02	1,5E+02	8,7E+01	8,7E+01
		3M average	3,6E+02	1,7E+01	1,3E+02	3,3E+01		

Table 2 indicates that in the MDBW samples only E.coli were found. The 2th dilution is presumed the best for plating because the amount of colonies countable. For the 3M petrifilm the first dilution rate was best countable. Notice that the 3M petrifilm does differentiate TCFU and E.coli. For plate growth results look in the Annex-MDBW Characterization.

3.1.3 TC and E.coli in 55 ˚C UASB

How many TCFU and E.coli colonies does a 55 ˚C UASB effluent contain?

Table 3: The TCFU and E.coli distribution in 55 ˚C UASB samples calculated for 1 ml.

	Dilution rate		No Dilution		10^-1		10^-2	
	Thermophilic DBW		TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Method 1604	sample date: 12-3-14	1th experiment	1,0E+00	1,0E+00				
	date Plated: 13-3-14	Duplicate	1,1E+01	1,1E+01				
		Triplicate	8,9E+01	8,9E+01				
Method 1604	Sample 28-3-14	2th experiment	1,0E+00	0,0E+00	1,3E+00	0,0E+00	0,0E+00	0,0E+00
	Date plated 7-4-14	Duplicate	2,0E+00	0,0E+00	1,3E+00	0,0E+00	0,0E+00	0,0E+00
		Triplicate	1,0E+00	0,0E+00	0,0E+00	0,0E+00	0,0E+00	0,0E+00
3M Petrifilm	Sample 28-3-14	A	TNTC	TNTC	TNTC	TNTC	0,0E+00	0,0E+00
	Date plated 7-4-14	B	TNTC	TNTC	TNTC	TNTC	0,0E+00	0,0E+00
		C	TNTC	TNTC	TNTC	TNTC	0,0E+00	0,0E+00
3M Petrifilm	sample date: 12-3-14	A	TNTC	TNTC				
	date Plated: 13-3-14	B	TNTC	TNTC				
		C	TNTC	TNTC				
		Average 1th exp	3,4E+01	3,4E+01				
		Average 2th exp	1,3E+00	0,0E+00	8,3E-01	0,0E+00	0,0E+00	0,0E+00
		Total average	1,8E+01	1,7E+01	8,3E-01	0,0E+00	0,0E+00	0,0E+00
		3M average	-	-	-	-		

In table 3 it is noticeable that in the first experiment there is a major difference between the duplicate and triplicate, which can be explained due to the fact these samples were not properly homogenized. The 3M petrifilm however show a major growth of TCFU other than E.coli. The TCFU on the 3M petrifilm were smaller compared to the samples from BW for the 3M petrifilm growth. For the growth per plate data look in the Annex- TDBW characteristics.

3.1.4 TC and E.coli 25 ˚C UASB and BW comparison

What is the removal efficiency of TC and E.coli of a 25 ˚C UASB effluent compared to BW effluent?

Table 4: The TCFU and E.coli distribution in 25 ˚C UASB samples compared to BW samples for 1 milliliter.

Dilution rate	10^-5		10^-6		10^-7	
Black Water	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	TNTC	TNTC	4,0E+06	3,3E+06	4,0E+06	3,0E+06
Average 2th exp	2,3E+06	2,1E+06	3,8E+06	3,0E+06	8,0E+06	5,3E+06
Total average	-	-	3,9E+06	3,2E+06	6,0E+06	4,2E+06
3M average	5,3E+06	3,9E+06				
Dilution rate	10^-1		10^-2		10^-3	
MDBW	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	2,3E+02	2,3E+02	2,0E+02	2,0E+02	7,4E+01	7,4E+01
Average 2th exp	1,4E+02	1,4E+02	9,6E+01	9,6E+01	1,0E+02	1,0E+02
Total average	1,8E+02	1,8E+02	1,5E+02	1,5E+02	8,7E+01	8,7E+01
3M average	3,6E+02	1,7E+01	1,3E+02	3,3E+01		
Removal MDBW compared BW						
Average 1th exp	-	-	99,995%	99,994%	99,998%	99,998%
Average 2th exp	99,994%	99,993%	99,997%	99,997%	99,999%	99,998%
Total average	-	-	99,996%	99,995%	99,999%	99,998%
3M average	99,993%	100,000%	-	-	-	-

Table 4 displays the removal difference of the BW effluent and the MDBW effluent. The removal percentages is almost 100%. According to this data a 25 ˚C mesophilic UASB is sufficient in removal of TC and E.coli.

3.1.5 TC and E.coli 55 ˚C UASB and 25 ˚C UASB comparison

What is the removal efficiency of TC and E.coli of a 55 ˚C UASB effluent compared to 25 ˚C UASB effluent?

Table 5: The TCFU and E.coli distribution in 55 ˚C UASB samples compared to 25˚ C UASB samples for 1 milliliter.

Dilution rate	10^-1		10^-2		10^-3			
MDBW	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml		
Average 1th exp	229	229	196	196	74	74		
Average 2th exp	136	136	96	96	100	100		
Total average	182	182	146	146	87	87		
3M average	357	17	133	33				
Dilution rate			no dilution		10^-1		10^-2	
TDBW			TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp			34	34	0	0	0	0
Average 2th exp			1	0	1	0	0	0
Total average			18	17	1	0	0	0
3M average			4.967	0	37.333	0		
Removal TDBW compared MDBW								
Average 1th exp	-	-	82,849%	82,849%	100,000%	100,000%	-	-
Average 2th exp	-	-	98,615%	100,000%	99,167%	100,000%	-	-
Total average	-	-	88,038%	88,494%	99,043%	100,000%	-	-
3M average	-	-	-3625,000%	100,000%	-	-	-	-

The results in table 5 show that the 55 ˚C UASB should remove more TCFU and E.coli than the 25 ˚C UASB. However the 3M petrifilm displays completely different results. According to the 3M petrifilm there is a major growth of TCFU other than E.coli, however they are considered TNTC.

3.2. How efficient is an algae based PBR in TC and E. coli removal?

3.2.1 TC and E.coli removal in a 12 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 12 hour HRT PBR filled with algae?

Table 6: The amount of TCFU and E.coli colonies in the 12 hour HRT PBR.

Dilution rate	10^-1		10^-2		10^-3	
HRT 12 Begin	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	81	0	21	0	0	0
Average 2th exp	55	0	48	0	0	0
3M average	38	0	17	0	17	0
Total avarage	68	0	34	0	0	0
Dilution rate	10^-1		10^-2		10^-3	
HRT 12 End	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	5	1	13	0	0	0
Average 2th exp	4	0	4	0	0	0
3M average	7	0	0	0	0	0
Total average	4	0	8	0	0	0
% removal difference						
Average 1th exp	94%	-	40%	-	100%	-
Average 2th exp	93%	-	92%	-	-	-
3M average	83%	-	100%	-	100%	-
Tot average Removal	94%	-	77%	-	100%	-

Table 6 displays the removal efficiency of the start time samples and the end time samples. The amount of TCFU in the HRT 12 start time samples are considerably higher compared to the amount of TCFU in a 55 ˚C UASB (table 5). However the end time samples of HRT 12 demonstrates that the TCFU are effectively removed in the PBR, also the E.coli colonies are less dense in growth. For the HRT 12 start time samples the best dilution rate is the 1th dilution. For the end time samples no dilution at all should provide optimal counting results. For the plating numbers look in the Annex-PBR 12 hours HRT beginning and PBR 12 hours HRT end.

3.2.2 TC and E.coli removal in a 21 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 21 hour HRT PBR filled with algae?

Table 7: The amount of TCFU and E.coli colonies in a milliliter in the 21 hour HRT PBR.

Dilution rate	10^-1		10^-2		10^-3	
HRT 21 Begin	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	TNTC	0	121	0	111	0
Average 2th exp	TNTC	0	646	0	667	0
3M average	418	0	333	0	0	0
Total average	-	0	383	0	389	0
Dilution rate	10^-1		10^-2		10^-3	
HRT 21 End	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	TNTC	0	742	0	704	0
Average 2th exp	TNTC	0	422	0	500	0
3M average	515	0	417	0	500	0
Total average	-	0	582	0	602	0
%	Removal difference HRT 21 hours TCFU/E.coli/1ml					
Average 1th exp	-	-	-514%	-	-533%	-
Average 2th exp	-	-	35%	-	25%	-
3M average	-23%	-	-25%	-	-	-
Total average removal	-	-	-52%	-	-55%	-

Table 7 shows that the first dilution is declared TNTC. For both the start time samples and the end time samples. For the start time and end time the 2th dilution is presumed the optimum dilution rate. The experiments shows growth in the 21 hours HRT PBR end time samples. In the annex-PBR 21 hours HRT start time and end time the plate counts can be found.

3.2.3 TC and E.coli removal in a 30 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 30 hour HRT PBR filled with algae?

Table 8: The amount of TCFU and E.coli colonies in a milliliter in the 30 hour HRT PBR.

Dilution rate	10^-1		10^-2		10^-3			
HRT 30 Begin	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml		
Average 1th exp	TNTC	0	TNTC	0	5,5E+03	0		
Average 2th exp	TNTC	0	2,6E+03	0	2,4E+03	0		
3M average	1,4E+02	0	7,7E+02	0	5,7E+03	0		
Total avarage	-	0	-	0	4,0E+03	0		
Dilution rate			10^-1		10^-2		10^-3	
HRT 30 End			TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp			TNTC	0	9,6E+02	0	8,3E+02	0
Average 2th exp			TNTC	0	4,3E+02	0	5,0E+02	0
3M average			4,5E+02	0	1,1E+03	0	1,0E+03	0
Total average			-	0	6,9E+02	0	6,7E+02	0
%	Removal difference HRT 30 hours TCFU/E.coli/1ml							
Average 1th exp	-	-	-	-	82%	-		
Average 2th exp	-	-	-	-	82%	-		
3M average	-	-	41%	-	81%	-		
Total average removal	-	-	-	-	82%	-		

In table 8 the 3th dilution rate was the considered optimal for counting TCFU and E. coli units in the experiments. This dilution rate shows a clear removal between the start time samples and end time samples. The 30 hours HRT PBR start time samples contain more TCFU and E.coli compared to the 55 ˚C UASB. For the exact data view the Annex-PBR 30 hours HRT Beginning and-PBR 30 hours HRT end.

3.2.4 Optimum HRT for removal of TC and E.coli

Which HRT has the best removal efficiency of TC and E.coli?

Table 9: Removal performances of the PBR's compared.

Dilution rate	10^-1		10^-2		10^-3	
% removal HRT 12	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	94%	-	40%	-	100%	-
Average 2th exp	93%	-	92%	-	-	-
3M average	83%	-	100%	-	100%	-
Total average remov	94%	-	77%	-	100%	-
Dilution rate	10^-1		10^-2		10^-3	
% removal HRT 21	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	-	-	-514%	-	-533%	-
Average 2th exp	-	-	35%	-	25%	-
3M average	-23%	-	-25%	-	-	-
Total average remov	-	-	-52%	-	-55%	-
Dilution rate	10^-1		10^-2 compared to 10^-1		10^-3 compared to 10^-2	
% removal HRT 30	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml	TCFU/1 ml	E-coli/1ml
Average 1th exp	-	-	-	-	82%	-
Average 1th exp	-	-	-	-	82%	-
3M average	-	-	41%	-	81%	-
Total average remov	-	-	-	-	82%	-

The PBR with a HRT of 12 hours has the best TCFU removal efficiency. The PBR with a HRT of 30 hours also removes TCFU considerably. Take in account that the removal efficiency is not the same as the real actual amount of TCFU present in the PBR.

Table 10: actual growth of TCFU and E.coli on the plates.

Dilution rate		10^-1		10^-2		10^-3	
		TCFU/Plate	E-coli/Plate	TCFU/Plate	E-coli/Plate	TCFU/Plate	E-coli/Plat
HRT 12 End	Average 1th e	4	0	1	0	0	0
Method 1604	Average 2th e	4	0	0	0	0	0
3M petrifilm	3M average	1	0	0	0		
	Total average	4	0	1	0	0	0
HRT 21 End	Average 1th e:	TNTC	0	59	0	6	0
Method 1604	Average 2th e:	TNTC	0	38	0	5	0
3M petrifilm	3M average	52	0	4	0	1	0
	Total average	-	0	49	0	6	0
HRT 30 End	Average 1th e:	TNTC	0	77	0	7	0
Method 1604	Average 2th e:	TNTC	0	28	0	4	0
3M petrifilm	3M average	45	0	5	0	1	0
	Total average	-	0	58	0	5	0

Table 10 shows that the PBR with a HRT of 12 hours contains the least amount of TCFU and E.coli in the effluent. Table 9 displays that the PBR with a 30 hours HRT has a removal efficiency of 80 %, however table 10 displays that the amount of TCFU is denser compared to the PBR with a HRT of 12 hours.

3.3 How efficient is a non-algae based PBR in TC and E. coli removal?

3.3.1 TC and E.coli removal in a 12 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 12 hour HRT PBR without algae?

For the experiment with PBR's without algae only the 3M petrifilm is used because of lack of time.

Table 11: PBR with 12 hours HRT without algae.

Dilution rate		10^-1	
Bioreactors without algae		TCFU/plate	E. Coli/plate
HRT 12 begin	A	0	0
3M Petrifilm	B	0	0
Sample date: 28-3-14	C	0	0
plated: 10-4-14	Total average	0	0
HRT 12 end	A	0	0
3M Petrifilm	B	0	0
Sample date : 6-4-14	C	0	0
Plated 10-4-14	Total average	0	0

No TCFU or E. coli were observed on the 3M petrifilm.

3.3.2 TC and E.coli removal in a 21 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 21 hour HRT PBR without algae?

Table 12: The PBR with 21 hours HRT without algae.

Dilution rate		10^-1	
Bioreactor without algae		TCFU/plate	E. Coli/plate
HRT 21 Begin	A	0	0
3M Petrifilm	B	0	0
Sample date: 28-3-14	C	0	0
Plated: 10-4-14	Total average	0	0
HRT 21 End	A	0	0
3M Petrifilm	B	1	0
Sample date: 6-4-14	C	0	0
Plated: 10-4-14	Total average	0	0

Table 12 shows growth of 1 TCFU on 1 3M petrifilm.

3.3.3 TC and E.coli removal in a 30 hour HRT PBR

What is the removal efficiency of TC and E.coli in a 30 hour HRT PBR without algae?

Table 13: In the PBR with 30 hours HRT without algae.

Dilution rate		10^-1	
Bioreactor without algae		TCFU/plate	E. Coli/plate
HRT 30 Begin	A	0	0
3M Petrifilm	B	0	0
Sample date: 28-3-14	C	0	0
Plated: 10-4-14	Total average	0	0
HRT 30 End	A	0	0
3M Petrifilm	B	0	0
Sample date: 28-3-14	C	0	0
Plated: 10-4-14	Total average	0	0

Table 13 shows that no TCFU or E.coli have grown. However another observation was made. Tiny red dots were barely visible, according to the 3M interpretation guide these bacteria should not counted as TCFU. In the Annex the 3M petrifilm interpretation guide is available.

3.3.4 Optimum HRT for removal of TC and E.coli?

Which PBR without algae has the best removal efficiency?

According to the data the HRT of 12 hours and 30 hours have the optimum removal efficiency because the PBR with a HRT 21 hours contain 1 TCFU.

4 Conclusion

4.1 How efficient is a 55 ˚C UASB in TC and E.coli removal?

How efficient is a 55 ˚C Thermophilic UASB followed by an algae based photo bioreactor in removal of Coliforms and Escherichia coli

What is the removal efficiency of TC and E.coli of a 25 ˚C UASB effluent compared to BW effluent?

Table 4 in chapter 3.1.4 presents the amount of TCFU in 1 milliliter. The results show a removal efficiency of 99% plus. The overall conclusion is that a 25 ˚C UASB removes TCFU and E.coli efficiently from BW.

What is the removal efficiency of TC and E.coli of a 55 ˚C UASB effluent compared to 25 ˚C UASB effluent?

Table 5 in chapter 3.1.5 presents the amount of TCFU in 1 milliliter. These results display that the 55 ˚C UASB removes TCFU and E.coli more efficiently compared to the 25 ˚C UASB. However the 3M petrifilm gives completely different results. According to the 3M petrifilm used for the experiments with TDBW showed TNTC for TCFU other than E.coli which suggests that the E.coli is not present but the TCFU other than E.coli is. This information is contradicting with the results from the method 1604 plating technique. In the table the total average TCFU from the MDBW is less dense with each higher dilution rate. This could be explained if the mixing of the previous samples is not properly done and the samples are not completely homogenized. Because the TDBW is mimicked no steady conclusion can be made, however the information provides promising data for further experimenting with a 55 ˚C UASB. Additional information can be found in the annex, MDBW Characterizations.

4.2 How efficient is an algae based PBR in TC and E. coli removal?

What is the removal efficiency of TC and E.coli in a 12 hour HRT PBR filled with algae?

According to table 6 in chapter 3.2.1 the amount of TCFU in the start time samples are higher in density compared to the TDBW effluent results displayed in table 3. However in the TDBW the TCFU mainly exist out of the E.coli bacteria, which are not present anymore in the PBR with a 12 hour HRT. However, the 3M petrifilm used for the experiments with TDBW showed TNTC for TCFU other than E.coli which suggests that the E.coli is not present but the TCFU other than E.coli

is. This information is contradicting with the TDBW results obtained through the 1604 method. The overall conclusion is that the E.coli is not present any more in a 12 HRT PBR. But the amount of experiments made with TDBW is not enough to make a steady conclusion. However the results suggest that the 12 HRT PBR is capable in removal of E.coli bacteria.

What is the removal efficiency of TC and E.coli in a 21 hour HRT PBR filled with algae?

Table 7 displays the removal efficiency of TCFU and E.coli of a 21 HRT PBR. However the data acquired reveals that there is almost no TCFU or E.coli removal at all. Instead there is TCFU growth. The 1th dilution of the 21 hours HRT PBR start time samples and end time samples are TNTC. The 2th dilution is considered most reliable. The collected data for the start time samples 21 hours HRT PBR is not consistent, meaning between the first second and third dilution there are huge differences in the amount of TCFU growth, and this could be because the samples are not homogeneous enough. It can be suggested that the 21 hours HRT PBR is not efficiently enough in removal of TCFU. However, according to the data, in the 21 hours HRT PBR the E.coli bacteria appear not viable anymore. This data can be found in the annex in PBR 21 hours HRT.

What is the removal efficiency of TC and E.coli in a 30 hour HRT PBR filled with algae?

According to table 8 the 1th dilution and the 2th dilution of HRT 30 start time samples are both TNTC. In the 3M petrifilm the amount of TCFU stays between the correct counting boundaries for the 1th dilution for the start time samples and end time samples except for the start time samples of the 1th dilution second experiment. In 30 hours HRT the end time samples of the first dilution are also TNTC. The 3th dilution rate for the start time samples was the first correct countable for all the experiments and therefore the most reliable. The 2th dilution rate is most presentable for the end time samples. Compared to each other a removal of 80% is achieved. However, the amount of TCFU in the effluent of the 30 hours HRT PBR is considerably more compared to the effluent of the mimic TDBW. No viable E.coli bacteria were detected in the start time samples or end time samples. For the exact data view the Annex-PBR 30 hours HRT Beginning and-PBR 30 hours HRT end.

Which HRT of an algae based PBR has the best results in removing TC and E.coli?

In Table 9 chapter 3.1.9 the PBR with a HRT of 12 hours presents the best results in removing TCFU and E.coli. The PBR with a HRT of 21 hours presents moreover growth instead of removal. The 30 hours HRT PBR does remove TCFU except that the start and end time samples still contain more TCFU compared to

the PBR with a HRT of 12 hours. In this experiment the data represents that the E.coli bacteria are successfully removed. The data represents however that the amount of TCFU could be enhanced by the PBR, but there is no sufficient amount of data available to statistically prove this statement. Further investigation at this subject is necessary.

4.3 How efficient is a non-algae based PBR in TC and E. coli removal?

In the tables 11 to 13 only the 3M Petri film is used because there was no time to conduct the time consuming 1604 method. Which makes the amount of data not sufficient for statistical analyses. However, no growth was detected at all except for one TCFU in the 21 hours HRT PBR end time sample. Further investigation is necessary if the 55 ˚C UASB is sufficient enough for removal of CFU and E.coli.

4.4 How efficient is the NIOO-KNAW proposed decentralized sanitation system in removal of Coliforms and Escherichia coli?

The main goal of the NIOO-KNAW is to implement a DESAR capable of producing an effluent which can flow in the local pond. The data of the 25 ˚C mesophilic UASB and the 55 ˚C thermophilic UASB show that TCFU and E.coli could effectively be removed by use of a 55 ˚C thermophilic UASB compared to a 25 ˚C mesophilic UASB. However, because the TDBW is a mimic more data is required to make statistical analyses. The E. coli bacteria is not present any more after the PRB with algae treatment, however, the experiments of the PBR's without algae display complete removal of all the TCFU. But the data of the PBR's without algae is only obtained through the 3M Petri film and therefore needs more investigation. The overall conclusion is that the 55 ˚C thermophilic UASB could be very promising in the removal of TC and E.coli. The 12 hours HRT PBR is according to data in this report the most sufficient PBR filled with algae because no E.coli bacteria were detected. Further investigation is necessary because of lack of samples and time. And most importantly the mimic TDBW effluent cannot represent actual TDBW effluent.

5 Discussion & Recommendations

5.1 Samples

Because the 55 ˚C thermophilic UASB of the NIOO-KNAW is out of order therefore MDBW effluent was derived from Sneek. In order to mimic the TDBW the MDBW was put in an incubator for 4 days at 55 ˚C. The main difference between a mesophilic UASB and a thermophilic UASB is the temperature. According to the literature the temperature plays the key role in removal of TCFU and E. coli bacteria(Cavinato et al., 2013) (Wendland, Deegener, Behrendt, Toshev, & Otterpohl, 2007) (Skillman et al., 2009). A mesophilic UASB has a HRT of 4 days, if you incubate these samples 4 days for 55 ˚C in order to mimic the thermophilic property's this means there is an extra HRT of 4 days. Not only the temperature is different between the mesophilic and thermophilic UASB the sludge composition also is slightly different which can give different results, which are not implemented in this project with the thermophilic UASB. The HRT of a thermophilic UASB and a mesophilic UASB is different as well, the thermophilic UASB needs a shorter HRT. The HRT difference can influence the chemical composition of the DBW, which can also influence the growth environment of the TC and E. coli.

Another major difference which is not taken in account is the difference between the influent BW into the UASB. As mentioned in the introduction the BW of Sneek also consist out of grey water while the NIOO-KNAW only wants their BW to consist out of toilet water with 1 litre of groundwater flush. The BW of the NIOO-KNAW will therefore be more concentrated in TC and E.coli. The recommendation is that the TDBW samples are taken directly from the effluent from a 55 ˚C thermophilic UASB instead of mimicking the MDBW from Sneek.

5.2 Photo bioreactors

The steady state of a PBR depended on the amount of algae cells which was set at 3, $8*10^8$ cells/ml. A steady state was achieved if the PBR contained this amount of algal cells for a couple of days including outwash. This means the algal cells regenerated at a constant rate. After inoculation of the pharmaceuticals there was no actual steady state any more (Alba De Agustin Camacho). The amount of algae cells dropped, or the amount of algae cells was higher. The start time samples for the TC and E.coli testing were taken before inoculation of the pharmaceuticals. However the end time samples were after inoculation. If there is no steady state this could influence the supposed bactericidal capacity of the algae, and thereby influence the growth of pathogen bacteria. A solution to this problem can be solved by experimenting only on the pathogen bacteria and not adding pharmaceuticals which causes decrease of algae cells. Thereby take more samples between start time samples and end time samples in order to compare with each other. If the amount of algae cells drop and there can be a difference detected in the presence of TC or E. coli then there could be a connection between the amount of cells and the removal capacity.

5.3 Acquiring samples

The mesophilic DBW samples were maintained in 5 litre tanks in a 4 ˚C refrigerator. For acquiring 40 ml samples a syringe is used to collect the samples, however, the tank is homogenized only by shaking. Just shaking is not enough to homogenize the tank. If tank stood there for a month that means all the suspended solids settle on the bottom of the tank including the bacteria. Because the syringe takes the sample from the top it could be that most of the bacteria still reside on the bottom of the tank which influences the amount of bacteria in the samples. In a future experiment it would be recommendable to homogenize the tank better, or take samples directly from the effluent of the MDBW and of proportioned volume. The thermophilic samples were obtained directly from a tube which runs from a 5 litre tanks stored in a 4 ˚C refrigerator. Here is the same discussion point as for taking the MDBW samples. The tank is not homogenized and therefore it can be assumed there is a big difference between the bacterial condition at the bottom and the top of the tank. Both the MDBW and the mimic TDBW are stored in a 4 ˚C refrigerator, however the temperature shock can already kill most of the TC and E.coli bacteria instantly which can influence the obtained information from the results.

The start time samples for the PBR's with a HRT of 12 and 30 hours are taken from the effluent bottle instead of directly from the PBR. This can majorly influence the growth of bacteria in the samples acquired. Because once in the effluent bottle it has already completed the HRT. And in the bottle the HRT continues because of the room temperature which could enhance duplication for the bacteria. The bottles were emptied on a regular basis and therefore no major

growth has occurred. However, because of the amount of bacteria this could be a significant problem. The acquired samples are preserved in a 4 ˚C refrigerator before experimenting. Some samples are kept in the refrigerator up to 4 months before experimenting. The time in the refrigerator could affect the viability of the bacteria because of the temperature shock. For the PBR with a HRT 12 and HRT 30 all the samples are saved for 4 months. However there is a major difference between the TC and E.coli growth between the 12 hours HRT and 30 hours HRT. On the other hand, for the TDBW a sample is tested just 1 day after sampling, and another non-duplicate sample was tested 8 days later. There was no significant difference in viability of the bacteria. For further investigation, take samples directly from the source and plate them directly for the best results, or keep refrigerator time as short as possible.

5.4 Dilutions and abundance of data

According to all the used interpretation guides the best abundance of bacteria on a petri dish/film is between 20-80 colonies. In this experiment a decimal dilution is used. Due to lack of time there are not enough duplicates and repeated experiments to perform significant statistical analyses on the data. For the next experiment the dilution rate could be altered in order to obtain better results. Make two times a dilution with 5 ml from the previous dilution, instead of 1 ml and in triplicate. The use of this technique produces more representative and useful data. The first and second experiment difference in TCFU amount an improvement would be to make triplicates instead of duplicates.

The 3M Petri film method is difficult to read. The bacteria colonies are sometimes small but abundant; following the interpretation guide it was not evident if the growth was considered to be TC, E. coli or another species of bacteria. Therefore the 3M Petri film is not taken in account. There is too much doubt about which kind of bacteria are cultured. Especially with the samples for MDBW and the TDBW effluent. Enumeration for the BW was not a problem the difference between TC and E. coli was obvious. According to the interpretation guide from 3M Petri film, the pathogenic E. coli strain O157:H7 does not grow beneath a temperature of 44. 5 ˚C and it does not produce glucuronidase. This temperature is not reached in the incubator and because no Beta-Glucuronidase enzyme is produced the E. coli will not turn blue but red, which means it will be counted as a TC bacteria colony instead of E. coli. This is the case in the 1604 medium and the 3M petrifilm, the temperature is not reached, and because the O157: H7 strain does not use the enzyme Beta-glucuronidase this E.coli will not turn blue in ambient light. For the next plating experiment it is advisable use the PCR technique to detect if the O157: H7 pathogen E. coli strain is present and influences the recovery on the plates.

5.5 Interpretation of the results

Due to lack of time no blank experiment has been fully executed. There are 3M petrifilm used on the blank experiments and they present promising data based on lack of growth of TC and E.coli bacteria. Because 3M petrifilm is not fully accounted in the experiment and the 1604 medium has not been utilized there is only speculation about the results. Experiment on more samples in the future which gives more data to compare. The blank experiment had resulted in almost no growth at all on the 3M petrifilm, an important cause for this result could be because the algae do not block the uv-radiation from the algaemist anymore. Therefore the uv-radiation could be the reason why the bacteria are not viable anymore.

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Zimmer, O. (2003). Nitrogen transformations and removal mechanisms in algal and duckweed waste stabilisation ponds.

II Annex

II.I M-Endo less medium manual

M Endo Agar LES

Intended Use

M Endo Agar LES is used for enumerating coliforms in water by membrane filtration.

Summary and Explanation

McCarthy, Delaney and Grasso[1] formulated Endo Agar LES (Lawrence Experimental Station) for testing water for coliform bacteria by a two-step membrane filter procedure using Lauryl Tryptose Broth as a preliminary enrichment. They recovered higher numbers of coliforms by this method compared with the one step technique using m Endo Broth.

The American Public Health Association specifies using m Endo Agar LES in the standard total coliform membrane filtration procedure for testing drinking water[2] and bottled water.[3] It is also specified for use in the completed phase of the standard total coliform fermentation technique.[2] The coliform bacteria are bacteria that produce a red colony with a metallic (golden) sheen within 24 hours incubation at 35°C on an Endo-type medium.

Principles of the Procedure

m Endo Agar LES contains peptones as sources of carbon, nitrogen, vitamins and minerals. Yeast extract supplies B-complex vitamins, which stimulate bacterial growth. Lactose is the carbohydrate. Phosphates are buffering agents. Sodium chloride maintains the osmotic balance of the medium. Sodium desoxycholate and sodium lauryl sulfate are added as inhibi- tors. Basic fuchsin is a pH indicator. Sodium sulfite is added to decolorize the basic fuchsin solution. Agar is the solidifying agent.

Lactose-fermenting bacteria produce acetaldehyde that reacts with the sodium sulfite and fuchsin to form red colonies. The development of a metallic sheen occurs when the organism produces aldehydes with the rapid fermentation of lactose. If the inoculum is too heavy, the sheen will be suppressed. Lactose-nonfermenting bacteria form clear, colorless colonies.

Formula

Difco™ m Endo Agar LES

Approximate Formula* Per Liter	
Yeast Extract:	1.2g
Casitone	3.7g
Thiopeptone ..	3.7g
Tryptose	7.5g
Lactose	9.4g
Dipotassium Phosphate ...	3.3g
Monopotassium Phosphate	1.0g
Sodium Chloride .	3.7g
Sodium Desoxycholate	0.1g
Sodium Lauryl Sulfate.	0.05g
Sodium Sulfite	1.6g
Basic Fuchsin.	0.8g
Agar .	15.0g

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 51 g of the powder in 1 L of purified water containing 20 mL of 95% ethanol. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. DO NOT AUTOCLAVE.
3. Test samples of the finished product for performance using stable, typical control cultures.

Procedure

1. Place a membrane filter absorbent pad inside the cover of a Petri dish.
2. Add 1.8-2.0 mL Lauryl Tryptose Broth or Lauryl Sulfate Broth to each pad.
3. Run the water sample through a membrane filter.
4. Place the filter, top side up, onto the pad containing Lauryl Tryptose Broth or Lauryl Sulfate Broth. Use a rolling motion to avoid entrapping air bubbles.
5. Incubate at 35 ± 0.5°C for 1.5-2.5 hours. Transfer the membrane from the pad to the surface of the m Endo Agar LES medium in the Petri dish bottom, keeping the side on which the bacteria have been collected facing upward.
6. Leave the filter pad in the lid and incubate the plates in the inverted position at 35 ± 0.5°C for 22 ± 2 hours.
7. Observe and count all colonies that are red and have a metallic sheen.

Expected Results

All colonies that are red and have the characteristic metal- lic sheen are considered coliforms. The sheen may cover the entire colony, may only be in the center or may appear only around the edges.

Limitations of the Procedure

Occasionally, noncoliform organisms may produce typical sheen colonies. Coliform organisms may also occasionally produce atypical colonies (dark red or nucleated colonies without sheen). It is advisable to verify both colony types.[2]

User Quality Control

Identity Specifications

Difco™ m Endo Agar LES

Dehydrated Appearance:	Purple, free-flowing, homogeneous.
Solution:	5.1% solution, soluble in purified water containing 2% ethanol upon boiling. Solution is pinkish-red, slightly opalescent to opalescent with precipitate.
Prepared Appearance:	Rose colored, slightly opalescent, with precipitate.
Reaction of 5.1% Solution at 25°C:	pH 7.2 ± 0.2

Cultural Response

Difco™ m Endo Agar LES

Prepare the medium per label directions. Use the membrane filter technique to inoculate filters and preincubate on pads saturated with Lauryl Tryptose Broth or Lauryl Sulfate Broth at 35 ± 0.5°C for 1.5-2 hours. Transfer filters to plates of m Endo Agar LES and incubate at 35 ± 0.5°C for 22 ± 2 hours.

References

1. McCarthy, Delaney and Grasso. 1961. Water Sewage Works 108:238.
2. Eaton, Rice and Baird (ed.). 2005. Standard methods for the examination of water and wastewater, 21st ed., online. American Public Health Association, Washington, D.C.
3. Kim and Feng. 2001. In Downes and Ito (ed.), Compendium of methods for the microbiological examina- tion of foods, 4th ed. American Public Health Association, Washington, D.C.

Availability
Difco™ m Endo Agar LES
COMPF SMD SMWW
Cat. No. 273610 Dehydrated – 100 g
273620 Dehydrated – 500 g

II.II 3M petrifilm manual

3M

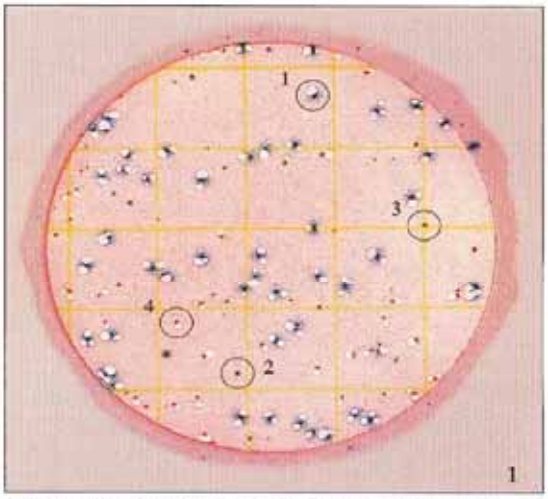
Interpretation Guide

Petrifilm™

E. coli and Coliform Count Plates

This guide should familiarize you with results on 3M™ Petrifilm™ E. coli and Coliform Count plates (EC). For more information contact the official 3M Microbiology Products representative in your area.

Petrifilm EC plates contain VRB nutrients, a cold-water-soluble gelling agent, an indicator of glucuronidase activity BCIG, and a tetrazolium indicator that facilitates colony enumeration. The top film traps gas produced by the lactose fermenting Coliforms and *E. coli*.



- ***Time and Temperature of incubation as well as interpretation of the Petrifilm EC plates vary by method. Therefore, this may give slightly different results. Temperatures of incubation used in the most common methods are mentioned in the package insert, and examples are shown in the technical section (pages 2 and 3) of this Guide.***
- ***Do not count colonies on the foam dam since they are removed from the selective influence of the medium.***
- Follow the time and temperature usually used into the laboratory. These are the most common used temperatures (*E. coli* and coliform) : 35, 37, 42, 44 or 44.5°C during 24 to 48h.

E. coli :

E. coli are able to grow on media containing Violet Red Bile (VRB) nutrients. Most *E. coli* (about 97%) produce beta-glucuronidase which reacts with a BCIG indicator dye in the Petrifilm EC plate that makes the colony turn blue to red-blue. About 95% of *E. coli* produce gas from lactose, this is indicated by colonies associated (within approximately one colony diameter) with entrapped gas. See Circle 1. *E. coli* colonies appear blue to red-blue and produce gas, confirm blue to red-blue colonies without gas. See Circle 2.

In some validation processes, this interpretation has been modified : the following organizations AOAC, AFNOR, NMKL, EMMAS have validated or assessed the use of the Petrifilm EC plates under specific conditions. See pages 2 and 3 of this Interpretation Guide.

Remark :

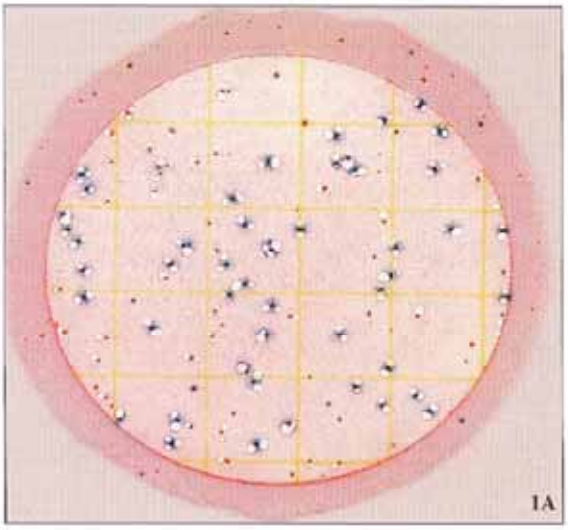
Because most *E. coli* O157:H7 strains are atypical : they do not grow at temperatures ≥ 44.5°C, are glucuronidase negative, and therefore will not produce a blue precipitate. They will appear as non-*E. coli* Coliforms (red with gas).

Coliforms :

The Petrifilm EC plates can also be used to search for Coliforms.

- **ISO** defines Coliforms by their ability to grow in method-specific, selective media. **ISO method 4832**, enumerating Coliforms by the colony count technique, defines Coliforms by colony size and acid production on VRB with lactose (VRBL) agar. On Petrifilm EC plates, these acid-producing Coliforms are indicated by red colonies with or without gas (within approximately one colony diameter). See Circle 3. **ISO method 4831**, enumerating Coliforms by the Most Probable Number (MPN) method, defines Coliforms by their ability to grow and produce gas from lactose in a selective broth. On Petrifilm EC plates these Coliforms are indicated by red colonies associated (within approximately one colony diameter) with gas. See Circle 4.
- **AOAC INTERNATIONAL** and U.S. FDA Bacteriological Analytical Manual (BAM) define Coliforms as Gram-negative rods which produce acid and gas from lactose during metabolic fermentation. Coliform colonies growing on the Petrifilm EC plate produce acid which deepen the gel color. Gas trapped around Coliform colonies (within approximately one colony diameter) indicates confirmed Coliforms. See Circle 4.

Technical section presenting interpretations of 3M Petrifilm EC Plates according to protocols described by the following bodies : AOAC, AFNOR, NMKL, EMMAS

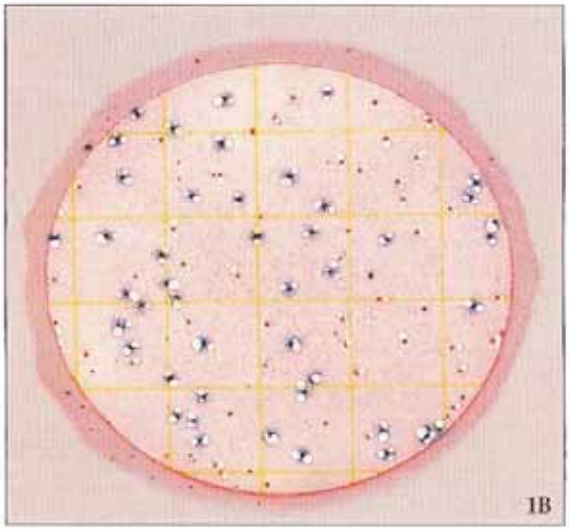


53 *E. coli*, **AFNOR** approved method

Reading following AFNOR approval (certificate 3M 1/4-09/92) :

Incubation :
24h +/- 2h at 42°C +/- 1°C

Interpretation :
• *E. coli* : Count all blue colonies with and without gas.



47 *E. coli*, **AOAC** official method
87 confirmed Coliforms, **AOAC** official method

Reading following AOAC INTERNATIONAL validated methods

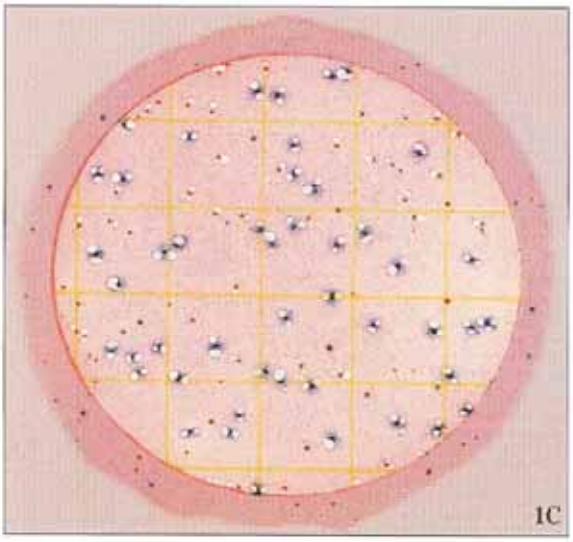
All foods (Method 991.14)

Incubation :
• Coliforms in all foods : incubate 24h +/– 2h at 35°C +/– 1°C.
• Enumeration of *E. coli* in all foods, except those here under : incubate 48h +/– 2h at 35°C +/– 1°C.

Meat, poultry and seafood (Method 998.08)

Incubation :
• Enumeration of *E. coli* in Meat, Poultry and Seafood, and Coliforms in all foods : incubate 24h +/– 2h at 35°C +/– 1°C.

Interpretation (both methods) :
• *E. Coli* : blue colonies with gas.
• Confirmed Coliforms : all colonies with gas (blue and red).



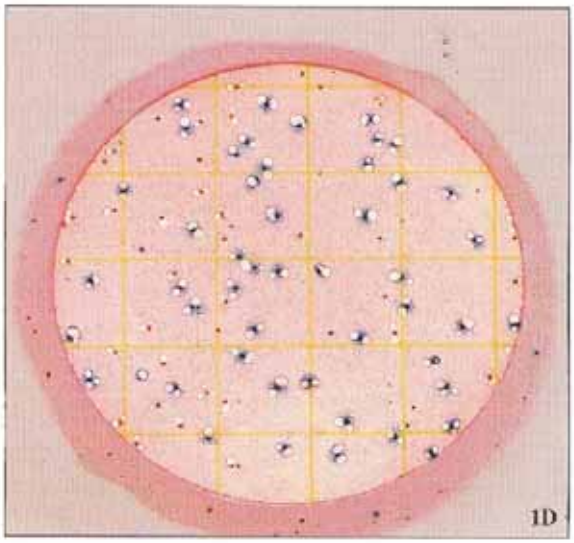
53 *E. coli*, NMKL method
95 Total Coliforms, NMKL method.

Reading following NMKL validated method (Method 147.1993) :

Incubation :
37°C +/- 1°C

Interpretation :

- *E. coli* : Count all blue colonies, with and without gas after 48h +/- 2h of incubation.
- Coliforms : Count red colonies with gas and all blue colonies with or without gas after 24h +/- 2h of incubation.



53 E. coli, EMMAS assessed method.

Reading following EMMAS assessed method :

Incubation :
48h +/- 2h at 37°C +/- 1°C

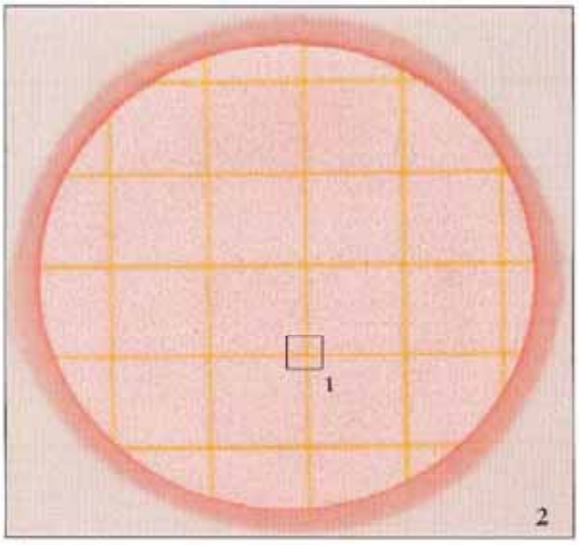
Interpretation :

- *E. coli* : Count all blue colonies with and without gas.

It is advisable to confirm blue colonies without gas, particularly when they are present in high proportion.

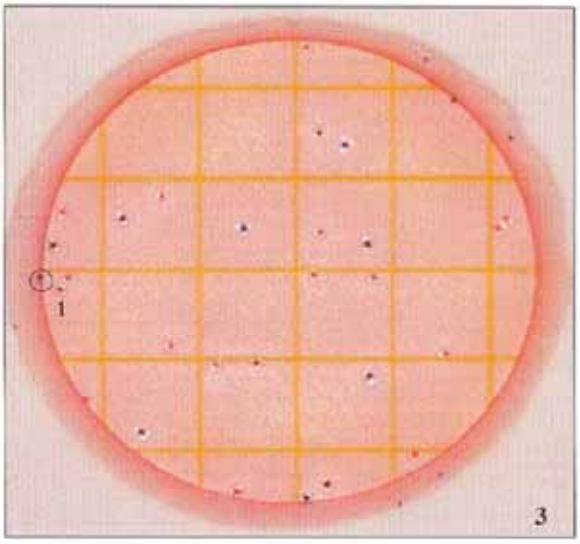
3M Petrifilm E. coli and Coliform Count Plates

Notice the change in gel color in figures 2 through 8. As the E. coli or Coliform count increases, the color of the gel turns to dark red or purple-blue.



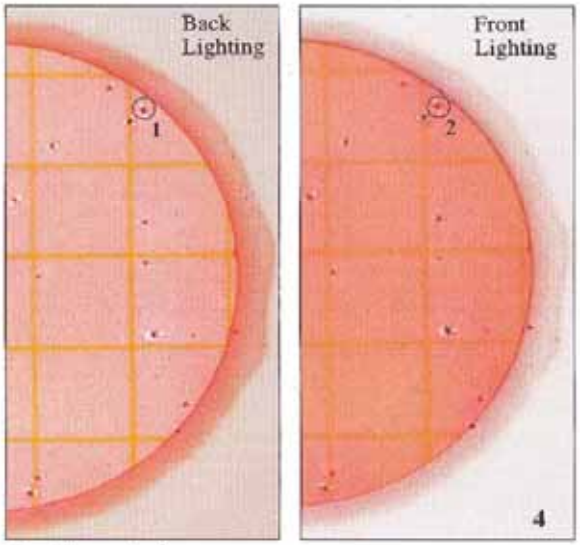
No growth
***E. coli* count = 0**

Background bubbles are a characteristic of the gel and are not a result of *E. coli* or Coliform growth. Background gas bubbles are small to pin-point in size, regular in shape and do not have a colony associated with them. See Square 1.



***E. coli* count = 13**
Gas producing Coliforms count = 28

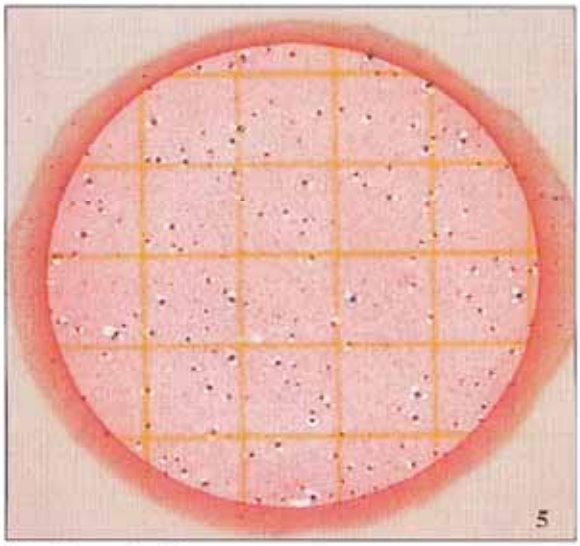
As with VRB agar plates, the preferable counting range (total colony population) on Petrifilm EC plates is 15 - 150. Do not count colonies that appear on the foam dam since they are removed from the selective influence of the medium. See Circle 1.



***E. coli* count = 3**

Any blue in a colony (blue to red-blue) indicates the presence of *E. coli*. Front lighting may enhance the detection of blue precipitate formed by a colony.

- Circle 1 shows a red-blue colony using back lighting.
- Circle 2 shows the same colony with front lighting. The blue precipitate is more evident in this case.



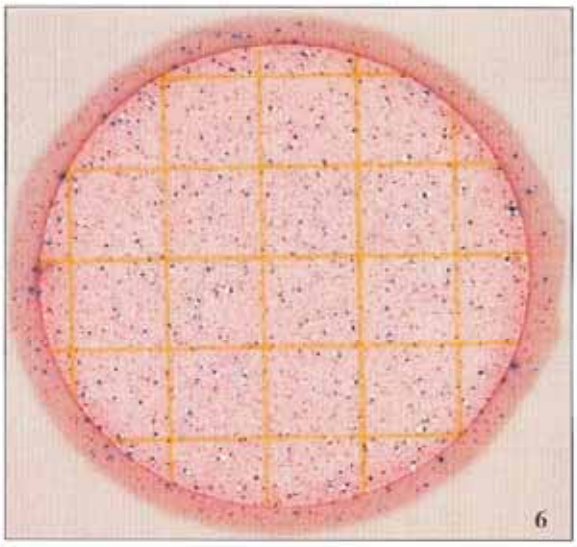
E. coli count = 20
Estimated total count = 150

The Petrifilm EC plate circular growth area is approximately 20 cm^2. Estimates can be made on plates containing greater than 150 colonies by counting the number of colonies in one or more representative squares and determining the average number per square. Multiply the average number by 20 to determine the estimated count per Petrifilm EC plate.

To obtain an accurate count, dilute the sample further.

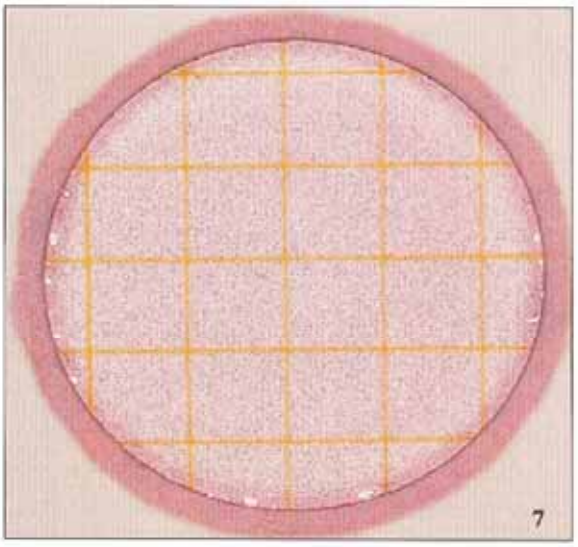
TNTC (Too Numerous To Count) plates

To obtain an accurate count, dilute the sample further.



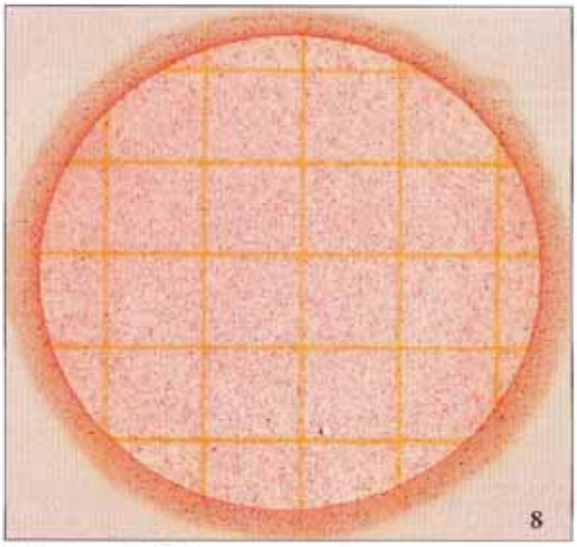
Actual count ~ 10^6

Petrifilm EC plates with colonies that are TNTC have one or more of the following characteristics: many small colonies, many gas bubbles, and a deepening of the gel color from red to purple-blue.



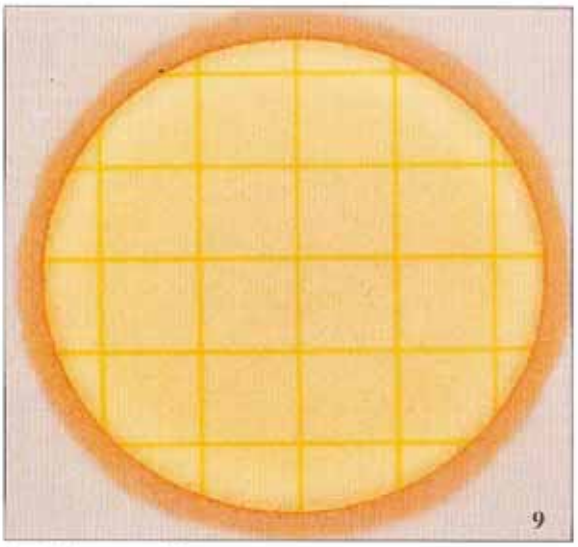
Actual count ~ 10^6

High concentrations of *E. coli* will cause the growth area to turn purple-blue.



Actual count ~ 10^5

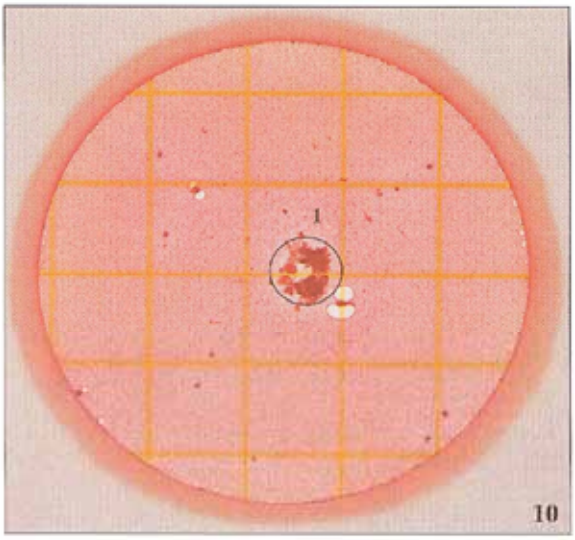
High concentrations of Coliforms (non *E. coli*) will cause the growth area to turn dark red. Additional dilutions are required to determine if *E. coli* are present.



Actual count ~ 10^6

When high numbers of non-Coliforms organisms such as *Pseudomonas* are present on Petrifilm EC plates, the gel may turn yellow.

Bubbles



Food particles are irregularly shaped and are not associated with gas bubbles. See Circle 1.

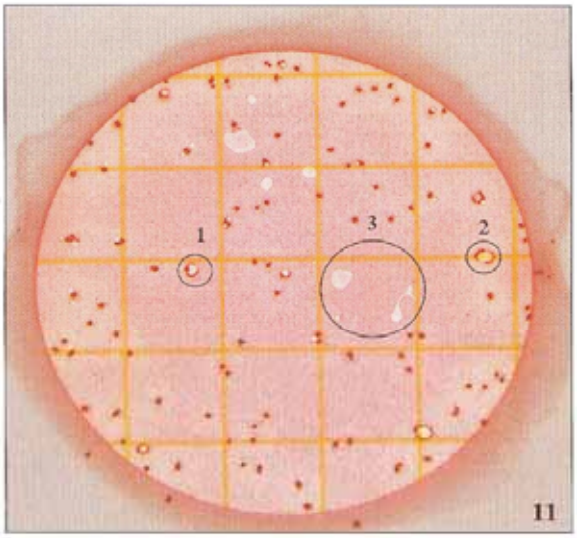
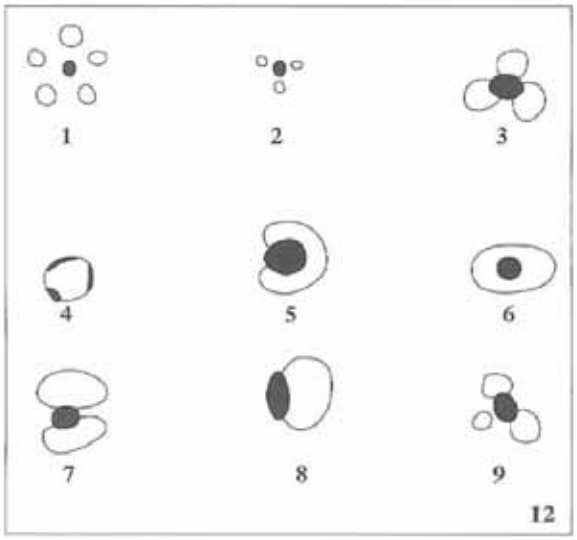


Figure 11 shows how bubble patterns may vary. Sometimes gas disrupts the colony so that the colony "outlines" the bubble. See Circles 1 and 2.

Artifact bubbles may result from improper inoculation of the Petrifilm EC plate or from trapped air within the sample. They are irregularly shaped and are not associated with a colony. See Circle 3.

Do not count colonies on the foam dam since they are removed from the selective influence of the medium.



The following are additional examples of various bubble patterns associated with a colony. All of them should be taken into account.

Test 3M™ Petrifilm™

E. coli and Coliform Count Plates

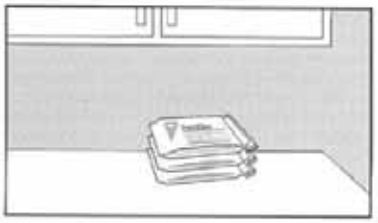
For detailed Warnings, Cautions, Limited Warranty, Limited Remedies, Storage and Disposal, Instructions for Use see product's package insert.

Reminders for use

Storage

1 **Store** unopened packages at ≤8°C (≤46°F). Use before expiration date on package.

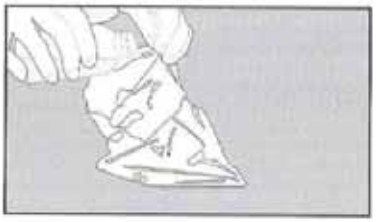
2 To seal opened package, fold end over and tape shut.



3 Keep resealed package at ≤25°C (≤77°F) and ≤50% RH. **Do not refrigerate opened packages.** Use Petrifilm plates within one month after opening.

Preparation

4 Weigh or pipette food product into an appropriate sterile container such as stomacher bag, dilution bottle, Whirl-Pak® bag, or other sterile container.

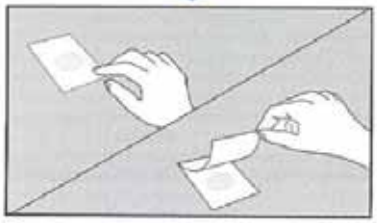


5 Add appropriate quantity of one of the following sterile diluents : Butterfield's phosphate buffer (IDF phosphate buffer, KH_2PO_4 at 0.0425g/L, adjust pH to 7.2), 0.1% peptone water, peptone salt diluent (ISO method 6887), saline solution (0.85 - 0.90%), or distilled water.

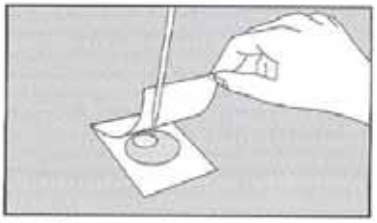
Do not use buffers containing citrate, bisulfite or thiosulfate.
Adjust pH of the diluted sample between 6.6 and 7.2 :
- for acidic products, use NaOH 1N,
- for alkaline products, use HCl 1N.

6 Blend or homogenize sample as per current procedure.

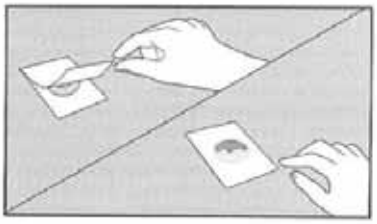
Inoculation



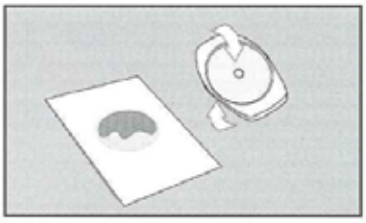
7 Place Petrifilm plate on **level** surface. Lift top film.



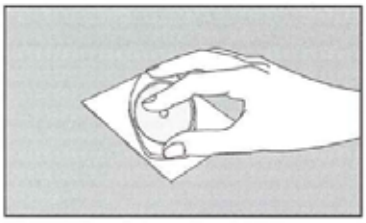
8 With pipette **perpendicular** to Petrifilm plate, place 1mL of sample onto center of bottom film.



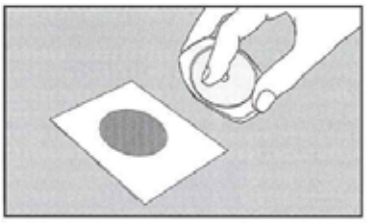
9 **Carefully roll** top film down to avoid trapping air bubbles. Do **not** let top film drop.



10 With **flat** side down, place spreader on top film over inoculum.

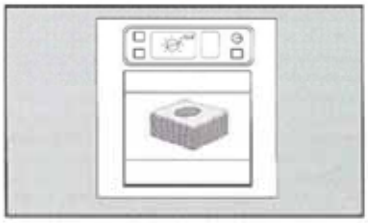


11 **Gently** apply pressure on spreader to distribute over circular area. Do not twist or slide the spreader.



12 Lift spreader. Wait at least one minute for gel to solidify.

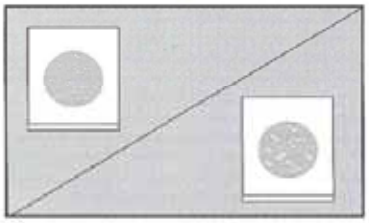
Incubation



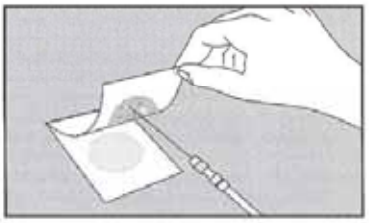
13 Incubate plates with clear side up in stacks of up to 20.
Incubation time and temperature vary by method*.
Most common approved methods :
• AOAC Official Method 991.04 :
For coliforms, incubate 24h ± 2h at 35°C ± 1°C ; for *E. coli*, incubate 48h ± 2h at 35°C ± 1°C.
• AFNOR validated method 01/4-09/92 :
For *E. coli*, incubate 24h ± 2h at 42°C ± 1°C.
It may be necessary to humidify incubator to minimize moisture loss during incubation.
• MNKL Method 147.1993 :
For Coliforms, incubate 24h ± 2h at 37°C ± 1°C ; for *E. coli*, incubate 48h ± 2h at 37°C ± 1°C.

*See product's package insert.

Interpretation



14 Petrifilm plates can be counted with a standard colony counter or other magnifier. Refer to the Interpretation Guide section when reading results.



15 Colonies may be isolated for further identification. Lift top film and pick the colony from the gel.

Additional comments

- **Remember to inoculate and spread each Petrifilm plate before going on to the next plate.**
- **Incubation time and temperature vary by method, see product's package insert.**

3M

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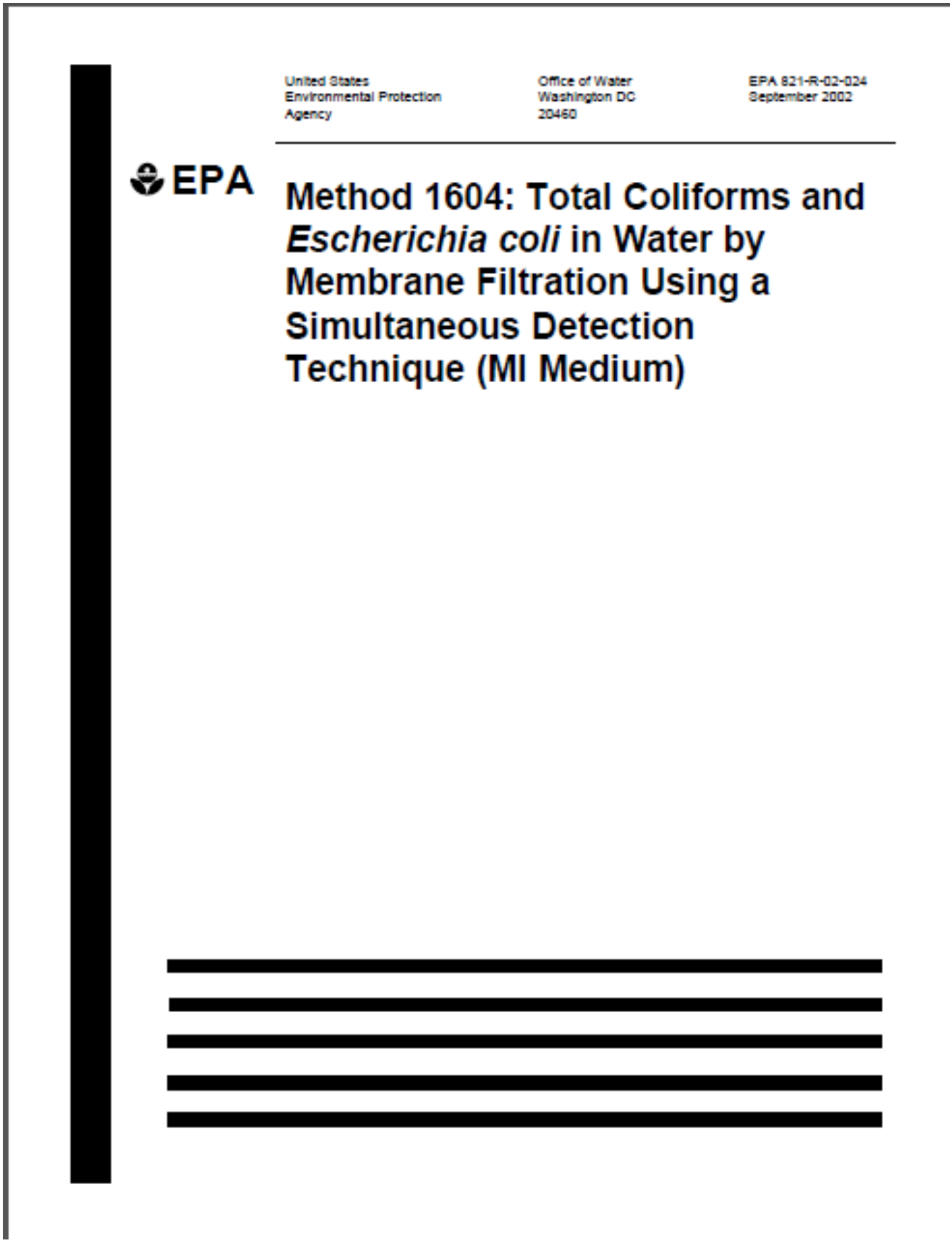

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EPA

Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)

U.S. Environmental Protection Agency
Office of Water (4303T)
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The content of this method version is identical to the February 2000 version of *Membrane Filter Method for the Simultaneous Detection of Total Coliforms and Escherichia coli in Drinking Water* (EPA-600-R-00-013) with one exception, the addition of MI broth. Since MI broth was approved on November 6, 2001, as a minor modification of the MI agar method, it has also been included in this document.

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Questions concerning this method or its application should be addressed to:

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Table of Contents

Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)

September 2002

1.0 Scope and Application

1.1 This test method describes a sensitive and differential membrane filter (MF) medium, using MI agar or MI broth, for the simultaneous detection and enumeration of both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 hours or less on the basis of their specific enzyme activities. Two enzyme substrates, the fluorogen 4-Methylumbelliferyl-β-D-galactopyranoside (MUGal) and a chromogen Indoxyl-β-D-glucuronide (IBDG), are included in the medium to detect the enzymes β-galactosidase and β-glucuronidase, respectively, produced by TC and *E. coli*, respectively.

1.2 Total coliforms include species that may inhabit the intestines of warm-blooded animals or occur naturally in soil, vegetation, and water. They are usually found in fecally-polluted water and are often associated with disease outbreaks. Although they are not usually pathogenic themselves, their presence in drinking water indicates the possible presence of pathogens. *E. coli*, one species of the coliform group, is always found in feces and is, therefore, a more direct indicator of fecal contamination and the possible presence of enteric pathogens. In addition, some strains of *E. coli* are pathogenic (Reference 16.12).

1.3 This method, which has been validated for use with drinking water in single-lab and multi-lab studies (References 16.8 - 16.10), will be used primarily by certified drinking water laboratories for microbial analysis of potable water. Other uses include recreational, surface or marine water, bottled water, groundwater, well water, treatment plant effluents, water from drinking water distribution lines, drinking water source water, and possibly foods, pharmaceuticals, clinical specimens (human or veterinary), other environmental samples (*e.g.*, aerosols, soil, runoff, or sludge) and/or isolation and separation of transformants though the use of *E. coli lac Z* or *gus A*/*uid* reporter genes (Reference 16.11).

1.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of *E. coli* and TC levels in water can be detected and enumerated.

2.0 Summary of Method

2.1 An appropriate volume of a water sample (100 mL for drinking water) is filtered through a 47-mm, 0.45-µm pore size cellulose ester membrane filter that retains the bacteria present in the sample. The filter is placed on a 5-mL plate of MI agar or on an absorbent pad saturated with 2-3 mL of MI broth, and the plate is incubated at 35°C for up to 24 hours. The bacterial colonies that grow on the plate are inspected for the presence of blue color from the breakdown of IBDG by the *E. coli* enzyme β-glucuronidase and fluorescence under longwave ultraviolet light (366 nm) from the breakdown of MUGal by the TC enzyme β-galactosidase (Reference 16.8).

3.0 Definitions

3.1 Total coliforms (TC) - In this method, TC are those bacteria that produce fluorescent colonies upon exposure to longwave ultraviolet light (366 nm) after primary culturing on MI agar or broth (See Figure 1.). The fluorescent colonies can be completely blue-white (TC other than *E. coli*) or blue-green (*E. coli*) in color or fluorescent halos may be observed around the edges of the blue-

green *E. coli* colonies. In addition, non-fluorescent blue colonies, which rarely occur, are added to the total count because the fluorescence is masked by the blue color from the breakdown of IBDG (Reference 16.8).

3.2 *Escherichia coli* - In this method, the *E. coli* are those bacteria that produce blue colonies under ambient light after primary culturing on MI agar or broth (See Figures 1 and 2.). These colonies can be fluorescent or non-fluorescent under longwave ultraviolet light (366 nm) (Reference 16.8).

4.0 Interferences and Contamination

4.1 Water samples containing colloidal or suspended particulate material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies. However, the blue *E. coli* colonies can often be counted on plates with heavy particulates or high concentrations of total bacteria (See Figures 2 and 3.) (Reference 16.8).

4.2 The presence of some lateral diffusion of blue color away from the target *E. coli* colonies can affect enumeration and colony picking on plates with high concentrations of *E. coli*. This problem should not affect filters with low counts, such as those obtained with drinking water or properly diluted samples (Reference 16.8).

4.3 Tiny, flat or peaked pinpoint blue colonies ($\leq$ 0.5-mm in diameter on filters containing $\leq$ 200 colonies) may be due to species other than *E. coli*. These colonies occur occasionally in low numbers and should be excluded from the count of the *E. coli* colonies, which are usually much larger in size (1-3-mm in diameter). The small colonies have never been observed in the absence of typical *E. coli*, but, if such should occur, the sample should not be considered *E. coli*-positive unless at least one colony has been verified by another method [*e.g.*, EC medium with 4-Methylumbelliferyl-β-D-glucuronide (MUG) or API 20E strips] (Reference 16.8).

4.4 Bright green, fluorescent, non-blue colonies, observed along with the typical blue/white or blue-green fluorescent TC colonies, may be species other than coliforms. These colonies, which generally occur in low numbers ($\leq$ 5%) and can usually be distinguished from the TC, should be eliminated from the TC count. An increase in the number of bright green colonies may indicate an unusual sample population or a breakdown of the cefsulodin in the medium (Reference 16.8).

5.0 Safety

5.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

5.2 Mouth-pipetting is prohibited.

5.3 Avoid prolonged exposure to longwave or germicidal ultraviolet light.

5.4 Autoclave all contaminated plates and materials at the end of the analysis.

6.0 Equipment and Supplies

6.1 Incubator set at 35°C ± 0.5°C, with approximately 90% humidity if loose-lidded petri dishes are used.

6.2 Stereoscopic microscope, with magnification of 10-15x, wide-field type.

6.3 A microscope lamp producing diffuse light from cool, white fluorescent lamps adjusted to give maximum color.

6.4 Hand tally.

6.5 Pipet container of stainless steel, aluminum, or Pyrex glass, for pipets.

6.6 Graduated cylinders (100-mL for drinking water), covered with aluminum foil or kraft paper and sterilized.

6.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel. These are wrapped with aluminum foil or kraft paper and sterilized.

6.8 Germicidal ultraviolet (254 nm) light box for sanitizing the filter funnels is desirable, but optional.

6.9 Line vacuum, electric vacuum pump, or aspirator is used as a vacuum source. In an emergency, a hand pump or a syringe can be used. Such vacuum-producing devices should be equipped with a check valve to prevent the return flow of air.

6.10 Vacuum filter flask, usually 1 liter, with appropriate tubing. Filter manifolds to hold a number of filter bases are desirable, but optional.

6.11 Safety trap flask, placed between the filter flask and the vacuum source.

6.12 Forceps, straight (preferred) or curved, with smooth tips to permit easy handling of filters without damage.

6.13 Alcohol, 95% ethanol, in small wide-mouthed vials, for sterilizing forceps.

6.14 Bunsen or Fisher-type burner or electric incinerator unit.

6.15 Sterile T.D. (To Deliver) bacteriological or Mohr pipets, glass or plastic (1-mL and 10-mL volumes).

6.16 Membrane Filters (MF), white, grid-marked, cellulose ester, 47-mm diameter, 0.45 µm ± 0.02-µm pore size, presterile or sterilized for 10 minutes at 121°C (15-lb pressure).

6.17 Longwave ultraviolet lamp (366 nm), handheld 4-watt (preferred) or 6-watt, or microscope attachment.

6.18 *Dilution water*: Sterile phosphate-buffered dilution water, prepared in large volumes (*e.g.*, 1 liter) for wetting membranes before addition of the sample and for rinsing the funnel after sample filtration or in 99-mL dilution blanks [Section 9050C in *Standard Methods* (Reference 16.2)].

6.19 Indelible ink marker for labeling plates.

6.20 Thermometer, checked against a National Institute of Science and Technology (NIST)-certified thermometer, or one traceable to an NIST thermometer.

6.21 Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids, or 15 x 60 mm, glass or plastic, with loose-fitting lids; 15 x 100 mm dishes may also be used.

6.22 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1:100 dilutions (if needed). Dilution bottles marked at 90 mL, or tubes marked at 9 mL may be used for 1:10 dilutions.

6.23 Flasks, borosilicate glass, screw-cap, 250- to 2000-mL volume, for agar preparation.

6.24 Waterbath maintained at 50°C for tempering agar.

6.25 Syringe filter, sterile, disposable, 25-mm diameter, 0.22-µm pore size, to filter cefsulodin for MI agar.

6.26 Syringe, sterile, plastic, disposable, 20-cc capacity. Autoclaved glass syringes are also acceptable.

6.27 Test tubes, sterile, screw-cap, 20 x 150-mm, borosilicate glass or plastic, with lids.

6.28 Sterilization filter units, presterile, disposable, 500- or 1000-mL capacity, 0.2-µm pore size, to filter stock buffer solutions.

6.29 Sterile 47-mm diameter absorbent pads (used with MI broth).

Note: *Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

7.0 Reagents and Standards

7.1 *Purity of Reagents*: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 16.1). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 *Purity of Water*: Reagent-grade distilled water conforming to Specification D1193, Type II water or better, *ASTM Annual Book of Standards* (Reference 16.3).

7.4 Buffered Dilution Water (Reference 16.2)

7.4.1 *Stock Phosphate Buffer Solution* (Reference 16.2):

Potassium Dihydrogen Phosphate (KH_2PO_4)	34.0 g
Reagent-Grade Distilled Water	500 mL

7.4.2 *Preparation of Stock Buffer Solution*: Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring volume to 1000 mL with reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C (15-lb pressure).

7.4.3 *$MgCl_2$ Solution* (Reference 16.2): Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2 \cdot 6H_2O$) in one liter of reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C (15-lb pressure).

7.4.4 *Storage of Stock Buffer and $MgCl_2$ Solutions*: After sterilization of the stock solutions, store in the refrigerator until used. Handle aseptically. If evidence of mold or other

contamination appears in either stock, the solution should be discarded, and a fresh solution should be prepared.

7.4.5 *Working Solution* (Final pH 7.0 ± 0.2): Add 1.25 mL phosphate buffer stock (Section 7.4.2) and 5 mL $MgCl_2$ stock (Section 7.4.3) for each liter of reagent-grade distilled water prepared. Mix well, and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C (15-lb pressure) for 15 minutes. Longer sterilization times may be needed depending on the container and load size and the amount of time needed for the liquid to reach 121°C.

7.5 MI Agar (Reference 16.8)

7.5.1 *Composition:*

Proteose Peptone #3	5.0	g
Yeast Extract	3.0	g
β-D-Lactose	1.0	g
4-Methylumbelliferyl-β-D-Galactopyranoside (MUGal) (Final concentration 100µg/mL)	0.1	g
Indoxyl-β-D-Glucuronide (IBDG) (Final concentration 320 µg/mL)	0.32	g
NaCl	7.5	g
K_2HPO_4	3.3	g
KH_2PO_4	1.0	g
Sodium Lauryl Sulfate	0.2	g
Sodium Desoxycholate	0.1	g
Agar	15.0	g
Reagent-Grade Distilled Water	1000	mL

7.5.2 *Cefsulodin Solution* (1 mg / 1 mL): Add 0.02 g of cefsulodin to 20 mL reagent-grade distilled water, sterilize using a 0.22-µm syringe filter, and store in a sterile tube at 4°C until needed. Prepare fresh solution each time. Do not save the unused portion.

7.5.3 *Preparation:* Autoclave the medium for 15 minutes at 121°C (15-lb pressure), and add 5 mL of the freshly-prepared solution of Cefsulodin (5 µg/mL final concentration) per liter of tempered agar medium. Pipet the medium into 9 x 50-mm Petri dishes (5 mL/plate). Store plates at 4°C for up to 2 weeks. The final pH should be 6.95 ± 0.2.

7.6 MI Broth: The composition of MI broth is the same as MI agar, but without the agar. The final pH of MI broth should be 7.05 ± 0.2. The broth is prepared and sterilized by the same methods described for MI agar in Sections 7.5.1, 7.5.2, and 7.5.3, except that absorbent pads are placed in 9 x 50 mm Petri dishes and saturated with 2-3 mL of MI broth containing 5 µg/mL final concentration of Cefsulodin. Alternately, the broth can be filter-sterilized. Excess broth is poured off before using the plates. Plates should be stored in the refrigerator and discarded after 96 hours (Reference 16.15).

7.7 Tryptic Soy Agar/Trypticase Soy Agar (Difco 0369-17-6, BD 4311043, Oxoid CM 0129B, or equivalent) (TSA)

7.7.1 *Composition:*

Tryptone	15.0 g
Soytone	5.0 g
NaCl	5.0 g
Agar	15.0 g

7.7.2 *Preparation:* Add the dry ingredients listed above to 1000 mL of reagent-grade distilled water, and heat to boiling to dissolve the agar completely. Autoclave at 121°C (15-lb pressure) for 15 minutes. Dispense the agar into 9 x 50-mm petri dishes (5 mL/plate). Incubate the plates for 24 - 48 hours at 35°C to check for contamination. Discard any plates with growth. If > 5% of the plates show contamination, discard all plates, and make new medium. Store at 4°C until needed. The final pH should be 7.3 ± 0.2.

8.0 Sample Collection, Preservation, and Storage

8.1 Water samples are collected in sterile polypropylene sample containers with leakproof lids.

8.2 Sampling procedures are described in detail in Sections 9060A and 9060B of the 18th edition of *Standard Methods for the Examination of Water and Wastewater* (Reference 16.2) or in the *USEPA Microbiology Methods Manual*, Section II, A (Reference 16.6). Residual chlorine in drinking water (or chlorinated effluent) samples should be neutralized with sodium thiosulfate (1 mL of a 10% solution per liter of water) at the time of collection. Adherence to sample preservation procedures and holding time limits are critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

8.2.1 *Storage Temperature and Handling Conditions:* Ice or refrigerate water samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water from melted ice during transit or storage.

8.2.2 *Holding Time Limitations:* Analyze samples as soon as possible after collection. Drinking water samples should be analyzed within 30 h of collection (Reference 16.13). Do not hold source water samples longer than 6 h between collection and initiation of analyses, and the analyses should be complete within 8 h of sample collection.

9.0 Calibration and Standardization

9.1 Check temperatures in incubators twice daily to ensure operation within stated limits (Reference 16.14).

9.2 Check thermometers at least annually against an NIST-certified thermometer or one traceable to NIST. Check mercury columns for breaks.

10.0 Quality Control (QC)

10.1 Pretest each batch of MI agar or broth for performance (*i.e.*, correct enzyme reactions) with known cultures (*E. coli*, TC, and a non-coliform).

10.2 Test new lots of membrane filters against an acceptable reference lot using the method of Brenner and Rankin (Reference 16.7).

10.3 Perform specific filtration control tests each time samples are analyzed, and record the results.

10.3.1 *Filter Control*: Place one or more membrane filters on TSA plates, and incubate the plates for 24 hours at 35°C. Absence of growth indicates sterility of the filter(s).

10.3.2 *Phosphate-Buffered Dilution Water Controls*: Filter a 50-mL volume of sterile dilution water before beginning the sample filtrations and a 50-mL volume of dilution water after completing the filtrations. Place the filters on TSA plates, and incubate the plates for 24 hours at 35°C. Absence of growth indicates sterility of the dilution water.

10.3.3 *Agar or Broth Controls*: Place one or more TSA plates and one or more MI agar plates or MI broth pad plates in the incubator for 24 hours at 35°C. Broth pad plates should be incubated *grid-side up*, not inverted like the agar plates. Absence of growth indicates sterility of the plates.

10.4 See recommendations on quality control for microbiological analyses in the "*Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures; Quality Assurance*" (Reference 16.15) and the *USEPA Microbiology Methods Manual*, part IV, C (Reference 16.6).

11.0 Procedure

11.1 Prepare MI agar or MI broth and TSA as described in Sections 7.5, 7.6, and 7.7. If plates are made ahead of time and stored in the refrigerator, remove them and allow them to warm to room temperature. The crystals that form on MI agar after refrigeration will disappear as the plates warm up (Reference 16.8).

11.2 Label the bottom of the MI agar or MI broth plates with the sample number/identification and the volume of sample to be analyzed. Label QC TSA plates and the MI agar or MI broth sterility control plate(s).

11.3 Using a flamed forceps, place a membrane filter, grid-side up, on the porous plate of the filter base. If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum. The separation paper will curl up, allowing easier removal.

11.4 Attach the funnel to the base of the filter unit, taking care not to damage or dislodge the filter. The membrane filter is now located between the funnel and the base.

11.5 Put approximately 30 mL of sterile dilution water in the bottom of the funnel.

11.6 Shake the sample container vigorously 25 times.

11.7 Measure an appropriate volume (100 mL for drinking water) or dilution of the sample with a sterile pipette or graduated cylinder, and pour it into the funnel. Turn on the vacuum, and leave it on while rinsing the funnel twice with about 30 mL sterile dilution water.

11.8 Remove the funnel from the base of the filter unit. A germicidal ultraviolet (254 nm) light box can be used to hold and sanitize the funnel between filtrations. At least 2 minutes of exposure time is required for funnel decontamination. Protect eyes from UV irradiation with glasses, goggles, or an enclosed UV chamber.

11.9 Holding the membrane filter at its edge with a flamed forceps, gently lift and place the filter grid-side up on the MI agar plate or MI broth pad plate. Slide the filter onto the agar or pad, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying agar or absorbent pad. Run the tip of the forceps around the outside edge of the filter to be sure the filter makes contact with the agar or pad. Reseat the membrane if non-wetted areas occur due to air bubbles.

11.10 Invert the agar petri dish, and incubate the plate at 35°C for 24 hours. Pad plates used with MI broth should be incubated *grid-side up* at 35°C for 24 hours. If loose-lidded plates are used for MI agar or broth, the plates should be placed in a humid chamber.

11.11 Count all blue colonies on each MI plate under normal/ambient light, and record the results (See Figures 1 and 2.). This is the *E. coli* count. Positive results that occur in less than 24 hours are valid, but the results cannot be recorded as negative until the 24-hour incubation period is complete (Reference 16.14).

11.12 Expose each MI plate to longwave ultraviolet light (366 nm), and count all fluorescent colonies [blue/green fluorescent *E. coli*, blue/white fluorescent TC other than *E. coli*, and blue/green with fluorescent edges (also *E. coli*)] (See Figure 1.). Record the data.

11.13 Add any blue, non-fluorescent colonies (if any) found on the same plate to the TC count (Reference 16.8).

12.0 Data Analysis and Calculations

12.1 Use the following general rules to calculate the *E. coli* or TC per 100 mL of sample:

12.1.1 Select and count filters with ≤ 200 total colonies per plate.

12.1.2 Select and count filter with ≤ 100 target colonies (ideally, 20-80).

12.1.3 If the total number of colonies or TC on a filter are too-numerous-to-count or confluent, record the results as "TC$^+$ (TNTC)" and count the number of *E. coli*. If both target organisms are ≥ 200, record the results as "TC$^+$ EC$^+$ (TNTC)".

12.1.4 Calculate the final values using the formula:

$$\textit{E. coli}/100\text{ mL} = \frac{\text{Number of blue colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

$$\text{TC}/100\text{ mL} = \frac{\text{Number of fluorescent colonies + Number of blue, non-fluorescent colonies (if any)}}{\text{Volume of sample filtered (mL)}} \times 100$$

12.2 See the *USEPA Microbiology Manual*, Part II, Section C, 3.5, for general counting rules (Reference 16.6).

12.3 Report results as *E. coli* or TC per 100 mL of drinking water.

13.0 Method Performance

13.1 The detection limits of this method are one *E. coli* and/or one total coliform per sample volume or dilution tested (Reference 16.8).

13.2 The false-positive and false-negative rates for *E. coli* are both reported to be 4.3% (Reference 16.8).

13.3 The single lab recovery of *E. coli* is reported (Reference 16.8) to be 97.9% of the Heterotrophic Plate Count (pour plate) (Reference 16.2) and 115% of the R2A spread plate (Reference 16.2). For *Klebsiella pneumoniae* and *Enterobacter aerogenes*, two total coliforms, the recoveries are 87.5% and 85.7% of the HPC (Reference 16.8), respectively, and 89.3% and 85.8% of the R2A spread plate, respectively.

13.4 The specificities for *E. coli* and total coliforms are reported to be 95.7% and 93.1% (Reference 16.8), respectively.

13.5 The single lab coefficients of variation for *E. coli* and total coliforms are reported to be 25.1% and 17.6% (Reference 16.8), respectively, for a variety of water types.

13.6 In a collaborative study (References 16.4, 16.5, and 16.9), 19 laboratories concurrently analyzed six wastewater-spiked Cincinnati tap water samples, containing 3 different concentrations of *E. coli* ($\leq$ 10, 11-30, and > 30 per 100 mL).

13.6.1 The single laboratory precision (coefficient of variation), a measure of the repeatability, ranged from 3.3% to 27.3% for *E. coli* and from 2.5% to 5.1% for TC for the six samples tested, while the overall precision (coefficient of variation), a measure of reproducibility, ranged from 8.6% to 40.5% and from 6.9% to 27.7%, respectively. These values are based on $\log_{10}$-transformed data (Reference 16.5).

13.6.2 Table 1 contains the statistical summary of the collaborative study (Reference 16.9) results.

14.0 Pollution Prevention

14.1 Pollution prevention is any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. It is the environmental management tool preferred over waste disposal or recycling. When feasible, laboratory staff should use a pollution prevention technique, such as preparation of the smallest practical volumes of reagents, standards, and media or downsizing of the test units in a method.

14.2 The laboratory staff should also review the procurement and use of equipment and supplies for other ways to reduce waste and prevent pollution. Recycling should be considered whenever practical.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling releases from hoods and bench operations, complying with the letter and spirit of sewer discharge permits and regulations and by complying with solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. All infectious wastes should be autoclaved before disposal.

16.0 References

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16.2 American Public Health Association. 1992. Standard Methods for the Examination of Water and Wastewater, 18th edition. American Public Health Association, Washington, D.C.

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16.5 Association of Official Analytical Chemists. 1989. Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis. Journal of the Association of Official Analytical Chemists 72 (4): 694-704.

16.6 Bordner, R., J. Winter, and P. Scarpino (ed). 1978. Microbiological Methods for Monitoring the Environment: Water and Wastes. EPA-600/8-78-017, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

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17.0 Tables and Figures

Table 1. Statistical Summary of the Collaborative Study Results[1]

Target Organism	Sample Number	*E. coli* Count Category (Range)[2]	Initial n[3]	Final n[4]	S_r[5]	RSD_r[6] (%)	$\bar{\chi}$[7]	S_R[8]	RSD_R[9] (%)	$\frac{RSD_R}{RSD_r}$ Ratio
Escherichia coli	1	Low (≤ 10)	63	63	0.17	27.3	0.64	0.26	40.5	1.49
	2		63	63	0.21	25.0	0.84	0.33	39.0	1.56
	3	Medium (11-30)	63	63	0.10	7.9	1.27	0.15	12.1	1.52
	4		63	60	0.07	5.6	1.32	0.12	9.2	1.65
	5	High (> 30)	63	60	0.06	3.3	1.87	0.16	8.6	2.62
	6		63	63	0.09	4.3	1.99	0.25	12.6	2.91
Total Coliforms	1	Low (≤ 10)	63	63	0.10	4.3	2.35	0.62	26.4	6.11
	2		63	63	0.09	3.8	2.31	0.64	27.7	7.25
	3	Medium (11-30)	63	63	0.11	5.1	2.17	0.47	21.8	4.28
	4		63	57	0.10	3.3	3.07	0.21	6.9	2.08
	5	High (> 30)	63	63	0.15	4.8	3.10	0.43	14.0	2.96
	6		63	63	0.08	2.5	3.14	0.46	14.7	5.97

[1] The values are based on $\log_{10}$ transformed data (Reference 16.5).

[2] The samples were grouped by their *E. coli* count on MI agar into the following categories:

Low (≤ 10 *E. coli* / 100 mL, samples 1 and 2),

Medium (11-30 *E. coli* / 100 mL, samples 3 and 4), and

High (> 30 *E. coli* / 100 mL, samples 5 and 6).

[3] These values are based on triplicate analyses by each laboratory. The reference laboratory analyzed three sets of samples: the initial and final samples prepared and a sample shipped along with the other 18 lab samples.

[4] These values were obtained after removing outliers by the AOAC procedure (Reference 16.5).

[5] S_r, Single Operator Standard Deviation, a measure of repeatability.

[6] RSD_r, Single Operator Relative Standard Deviation (Coefficient of Variance), a measure of repeatability.

[7] $\bar{\chi}$, The mean of the replicate analyses for all laboratories.

[8] S_R, Overall Standard Deviation, a measure of reproducibility.

[9] RSD_R, Overall Relative Standard Deviation (Coefficient of Variation), a measure of reproducibility.

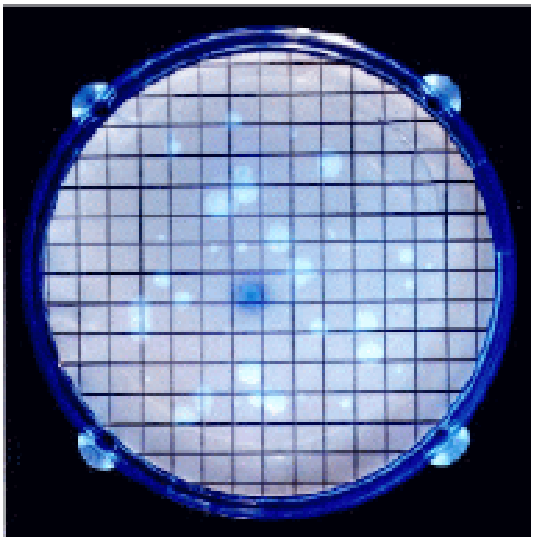

Figure 1. This photograph shows *Escherichia coli* (blue/green fluorescence) and total coliforms other than *E. coli* (blue/white fluorescence) on MI agar under longwave UV light (366 nm). The sample used was a wastewater-spiked Cincinnati, Ohio tap water.

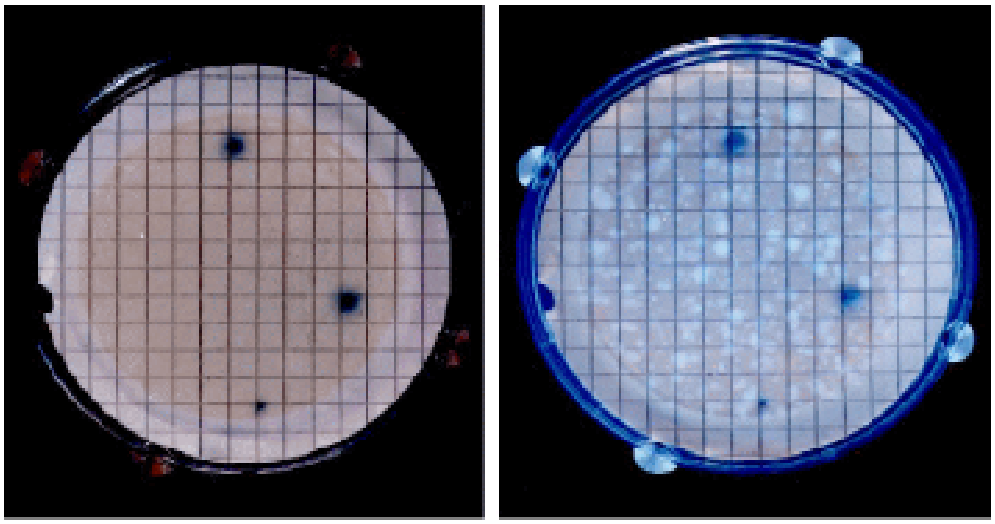

Figure 2. These photographs show *Escherichia coli* and total coliforms from cistern water on MI agar. The confluent plate was photographed under different lighting: ambient light on the left, and longwave UV light (366 nm) on the right. Under ambient light, *E. coli* are blue, and total coliforms other than *E. coli* and non-coliforms are their natural color. Under longwave UV light, all total coliforms, including *E. coli*, are fluorescent, and non-coliforms are non-fluorescent (*i.e.*, they are not visible).

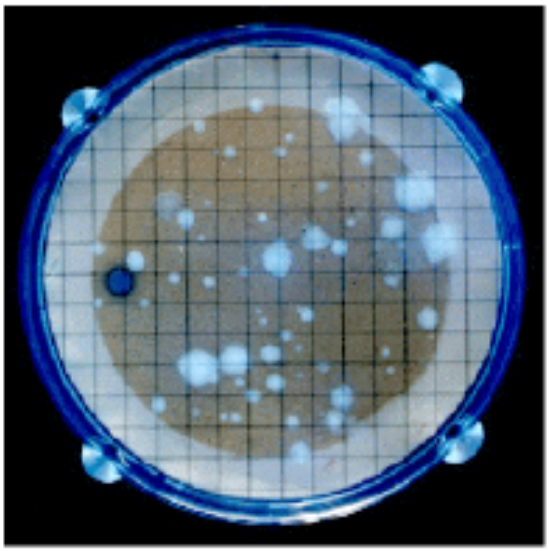

Figure 3. This photograph shows that *Escherichia coli* (blue/green fluorescence) and total coliforms other than *E. coli* (blue/white fluorescence) can easily be detected on MI agar plates from samples with high turbidity levels. The sample used was surface water-spiked Cincinnati, Ohio tap water.

III MDBW Sneek characterization including nutrient content

Table 14: The influent and effluent data of a 25˚ C Mesophilic digester

Parameter	Influent		Effluent	
	average	stdev.	average	stdev.
pH	8.51	-	7.72	-
CODtotal (gCOD/l)	8.78	0.76	1.38	0.03
CODsuspended (gCOD/l)	6.04	0.78	0.18	0.03
CODcolloidal (gCOD/l)	0.99	0.06	0.57	0.006
CODsoluble (gCOD/l)	1.75	0.01	0.64	0.006
VFA (gCOD/l)	0.68[a]	0.007	0.03 [b]	0.004
TS	7.5	0.2	2.7	0.03
VS	5.4	0.1	0.9	0.04
VS/TS	0.72	-	0.34	-
TSS	4.4	0.2	0.2	0.03
VSS	3.9	0.2	0.1	0.04
VSS/TSS	0.88		0.51	
Alkalinity (g CaCO3/l)	4.46	0.78	4.59	0.008
NH_4^+-N (g/l)	0.91	0.01	1.18	0.01
NH_4^+-N (g/l) after autoclave			0.76	0.01
PO_4-P (g/l)	0.080	0.001	0.084	0.004
PO_4-P (g/l) after autoclave			0.061	0.002
TN (g/l)	1.18	0.01	1.22	0.004
TP (g/l)	0.15	0.02	0.09	0.003

IV Additional results

IV.I BW Characterization

	Black water		10^-5 dilution			
			TCFU	E-coli	TCFU/1 ml	E-coli/1ml
Black water	Sample date: 17-12'13	1e experiment	353	268	3.922.222	2.977.778
	Plating date: 18-12-13		308	238	3.422.222	2.644.444
Medium 1604	Medium:1604		288	226	3.200.000	2.511.111
	Sample date: 17-12-13	2e experiment	197	176	2.188.889	1.955.556
	Plating date: 9-1'14		227	203	2.522.222	2.255.556
Medium 1604			204	182	2.266.667	2.022.222
3M petrifilm	Sample date: 17-12-13	A	51	38	5.100.000	3.800.000
	Plating date 18-12-13	B	53	40	5.300.000	4.000.000
		C	56	38	5.600.000	3.800.000
		Avarage 1e exp	316	244	3.514.815	2.711.111
		Avarage 2e exp	209	187	2.325.926	2.077.778
		3M average	53	39	5.333.333	3.866.667
		Total avarage	263	216	2.920.370	2.394.444
			10^-6 dilution			
			TCFU	E-coli	TCFU/1 ml	E-coli/1ml
Black water	Sample date: 17-12'13	1e experiment	34	27	3.777.778	3.000.000
	Plating date: 18-12-13		35	31	3.888.889	3.444.444
Medium 1604			39	32	4.333.333	3.555.556
	Sample date: 17-12-13	2e experiment	28	25	3.111.111	2.777.778
	Plating date: 9-1'14		38	32	4.222.222	3.555.556
Medium 1604			36	25	4.000.000	2.777.778
3M petrifilm	Sample date: 17-12-13	A			0	0
	Plating date 18-12-13	B			0	0
		C			0	0
		Avarage 1e exp	36	30	4.000.000	3.333.333
		Avarage 2e exp	34	27	3.777.778	3.037.037
		3M average				
		Total avarage	35	29	3.888.889	3.185.185
			10^-7 dilution			
			TCFU	E-coli	TCFU/1 ml	E-coli/1ml
Black water	Sample date: 17-12'13	1e experiment	4	3	4.000.000	3.000.000
	Plating date: 18-12-13		4	3	4.000.000	3.000.000
	Medium:1604		4	3	4.000.000	3.000.000
	Sample date: 17-12-13	2e experiment	9	7	9.000.000	7.000.000
	Plating date: 9-1'14		10	5	10.000.000	5.000.000
			5	4	5.000.000	4.000.000
3M petrifilm	Sample date: 17-12-13	A			0	0
	Plating date 18-12-13	B			0	0
		C			0	0
		Avarage 1e exp	4	3	4.000.000	3.000.000
		Avarage 2e exp	8	5	8.000.000	5.333.333
		3M average				
		Total avarage	6	4	6.000.000	4.166.667

IV.II MDBW Characterization

Mesophilic Digested Black Water

			10^-1 dilution TCFU	E. coli	Coliform/1 ml	E-coli/1ml
MDBW	Sample name: 18-2	1e experiment	204	204	227	227
			217	217	241	241
method 1604	plating date : 26-2		198	198	220	220
Digested BW	Sample name: 18-2	2e experiment	128	128	142	142
Mesophilic	Plating date: 10-3		115	115	128	128
Method 1604			123	123	137	137
3M petrifilm	Sample name: 18-2	A	25	2	250	20
	plating date 7-4	B	44	2	440	20
		C	38	1	380	10
		Avarage A 1e exp	206	206	229	229
		Avarage B 2e exp	122	122	136	136
			36	2	357	17
		Total avarage	164	164	182	182

			10^-2 dilution TCFU	E. coli	Coliform/1 ml	E-coli/1ml
MDBW	Sample name: 18-2	1e experiment	19	19	211	211
	Mesophilic 18-2		14	14	156	156
method 1604	plating date : 26-2		20	20	222	222
	Sample name: 18-2	2e experiment	6	6	67	67
	Plating date: 10-3		14	14	156	156
Method 1604			6	6	67	67
3M petrifilm		A	1	0	100	0
		B	1	0	100	0
		C	2	1	200	100
		Avarage A 1e exp	18	18	196	196
		Avarage B 2e exp	9	9	96	96
		petrifilm	1	0	133	33
		Total avarage	13	13	146	146

			10^-3 dilution TCFU	E. Coli	Coliform/1 ml	E-coli/1ml
MDBW	Sample name: 18-2	1e experiment	0	0	0	0
	Mesophilic 18-2		1	1	111	111
method 1604	plating date : 26-2		1	1	111	111
	Sample name: 18-2	2e experiment	2	2	200	200
	Plating date: 10-3		0	0	0	0
Method 1604			1	1	100	100
3M petrifilm		A				
		B				
		C				
		Avarage A 1e exp	1	1	74	74
		Avarage B 2e exp	1	1	100	100
		Total avarage	1	1	87	87

IV.III TDBW Characterization

Thermophilic	Digested Black Water		no dilution TCFU	E. coli	Coliform/1 ml	E-coli/1ml
TDBW	sample date: 12-3-14	1e experiment	1	1	1	1
	date Plated: 13-3-14		11	11	11	11
Method 1604			89	89	89	89
	Sample 28-3-14 2e	2e experiment	1	0	1	0
	Date plated 7-4-14		2	0	2	0
Method 1604			1	0	1	0
3M Petrifilm		A	400		4.000	
		B	320		3.200	
		C	480		4.800	
3M Petrifilm		a	500	0	5.000	0
		b	700	0	7.000	0
		c	580	0	5.800	0
		Avarage A	34	34	34	34
		Avarage B	1	0	1	0
		3M average	497	0	4.967	0
		Total avarage	18	17	18	17
			10*-1 dilution TCFU	E. coli	Coliform/1 ml	E-coli/1ml
TDBW	sample date: 12-3-14	1e experiment				
Method 1604	date Plated: 13-3-14					
	Method 1604					
	Sample 28-3-14 2e	2e experiment	1	0	1	0
	Date plated 7-4-14		1	0	1	0
Method 1604			0	0	0	0
3M Petrifilm		a	400	0	40.000	0
		b	360	0	36.000	0
		c	360	0	36.000	0
		Avarage A	0	0	0	0
		Avarage B	1	0	1	0
		3M average	373	0	37.333	0
		Total avarage	1	0	1	0
			10^-2 dilution TCFU	E. coli	Coliform/1 ml	E-coli/1ml
TDBW	sample date: 12-3-14	1e experiment				
Method 1604	date Plated: 13-3-14					
	Method 1604					
	Sample 28-3-14 2e	2e experiment	0	0	0	0
	Date plated 7-4-14		0	0	0	0
Method 1604			0	0	0	0
3M Petrifilm		A	0	0	0	0
		B	0	0	0	0
		C	0	0	0	0
3M Petrifilm		a				
		b				
		c				
		Avarage A	0	0	0	0
		Avarage B	0	0	0	0
		3M average	0	0	0	0
		Total avarage	0	0	0	0

IV. IV PBR 12 hours HRT start time samples

Algae HRT 12 begin			10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample: Hrt 12 tT0 3-12-13	1e experiment	68	0	85	0
HRT 12 1054	Plated: 8-4-14		65	0	81	0
Method 1604			61	0	76	0
	Sample: Hrt 12 T0 3-12-13	2e experiment	58	0	64	0
	Plated: 10-4		44	0	49	0
Method 1604			47	0	52	0
petrifilm	Sample HRT 12T0 3-13-13	A	4	0	40	0
	Plated: 26-3	B	5	0	50	0
		C	6	0	60	0
3M petrifilm	Sample: Hrt 12 T0 3-12-13	A	2	0	20	0
	Plated: 8-4-14	B	6	0	60	0
		C	0	0	0	0
		Avarage A	65	0	81	0
		Avarage B	50	0	55	0
		Average 3M	4	0	38	0
		Total avarage	57	0	68	0

			10^-2 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample: Hrt 12 tT0 3-12-13	1e experiment	0	0	0	0
HRT 121054	Plated: 8-4-14		2	0	25	0
Method 1604			3	0	38	0
	Sample: Hrt 12 T0 3-12-13	2e experiment	3	0	33	0
	Plated: 10-4		6	0	67	0
Method 1604			4	0	44	0
petrifilm	Sample HRT 12T0 3-13-13	A	0	0	0	0
	Plated: 26-3	B	0	0	0	0
		C	1	0	100	0
3M petrifilm	Sample: Hrt 12 T0 3-12-13	A				
	Plated: 8-4-14	B				
		C				
		Avarage A	2	0	21	0
		Avarage B	4	0	48	0
		Average 3M	0	0	0	0
		Total avarage	3	0	34	0

			10^-3 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample: Hrt 12 tT0 3-12-13	1e experiment	0	0	0	0
HRT 121054	Plated: 8-4-14		0	0	0	0
Method 1604			1	0	100	0
	Sample: Hrt 12 T0 3-12-13	2e experiment	0	0	0	0
	Plated: 10-4		0	0	0	0
Method 1604			0	0	0	0
petrifilm	Sample HRT 12T0 3-13-13	A	1	0	100	0
	Plated: 26-3	B	0	0	0	0
		C	0	0	0	0
3M petrifilm	Sample: Hrt 12 T0 3-12-13	A				
	Plated: 8-4-14	B				
		C				
		Avarage A	0	0	33	0
		Avarage B	0	0	0	0
		Average 3M	0	0	0	0
		Total avarage	0	0	17	0

IV.V PBR 12 hours HRT end time samples

	Algae HRT 12 end		10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae HRT 12	Sample:9-12-13	1e experiment	1	0	1	0
End	Date Plated: 8-4-14		5	0	6	0
Medium 1604			5	0	6	0
	Sample:9-12-13	2e experiment	3	0	3	0
	Date Plated: 10-4		3	0	3	0
Medium 1604			5	0	6	0
	Sample: 2-3-14	A	0	0	0	0
	Date Plated: 8-4-14	B	1	0	10	0
3M Petrifilm		C	1	0	10	0
		Avarage A	4	0	5	1
		Avarage B	4	0	4	0
		Average 3M	1	0	7	0
		Total avarage	4	0	4	0
			10^-2 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae HRT 12	Sample name:HR 12T er	1e experiment	1	0	13	0
End	Date Plated: 8-4-14		1	0	13	0
Medium 1604			1	0	13	0
	Sample name:HR 12T er	2e experiment	0	0	0	0
	Date Plated: 10-4		1	0	11	0
Medium 1604			0	0	0	0
	Sample: HRT12 2-3-14	A	0	0	0	0
	Date Plated: 8-4-14	B	0	0	0	0
3M Petrifilm		C	0	0	0	0
		Avarage A	1	0	13	0
		Avarage B	0	0	4	0
		Average 3M	0	0	0	0
		Total avarage	1	0	8	0
			10^-3 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae HRT 12	Sample name:HR 12T er	1e experiment	0	0	0	0
End	Date Plated: 8-4-14		0	0	0	0
Medium 1604			0	0	0	0
	Sample name:HR 12T er	2e experiment	0	0	0	0
	Date Plated: 10-4		0	0	0	0
Medium 1604			0	0	0	0
	Sample: HRT12 2-3-14	A				
	Date Plated: 8-4-14	B				
3M Petrifilm		C				
		Avarage A	0	0	0	0
		Avarage B	0	0	0	0
		Average 3M				
		Total avarage	0	0	0	0

IV.VI PBR 21 hours HRT start time samples

			10^-3 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample: Hrt 12 tT0 3-12-13	1e experiment	0	0	0	0
HRT 121054	Plated: 8-4-14		0	0	0	0
Method 1604			1	0	100	0
	Sample: Hrt 12 T0 3-12-13	2e experiment	0	0	0	0
	Plated: 10-4		0	0	0	0
Method 1604			0	0	0	0
petrifilm	Sample HRT 12T0 3-13-13	A	1	0	100	0
	Plated: 26-3	B	0	0	0	0
		C	0	0	0	0
3M petrifilm	Sample: Hrt 12 T0 3-12-13	A				
	Plated: 8-4-14	B				
		C				
		Avarage A	0	0	33	0
		Avarage B	0	0	0	0
		Average 3M	0	0	0	0
		Total avarage	0	0	17	0

			10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample name: 20-2 hrt 21	1e experiment	300	0	375	0
HRT 21 1056	Date plated: 13-3-14		300	0	375	0
Method 1604			300	0	375	0
	sample name: 20-2 -14	2e experiment	28	0	35	0
	plated: 8-4'14		300	0	375	0
Method 1604			300	0	375	0
Petrifilm	Sample name 20-2 hrt 121	A	29	0	290	0
	Date plated: 13-3	B	30	0	300	0
		C	23	0	230	0
petrifilm	Sample name: HRT 21 T0 2	A	54	0	540	0
	Date sample: 8-4	B	60	0	600	0
		C	55	0	550	0
		Avarage A	300	0	375	0
		Avarage B	209	0	262	0
		Average 3M	42	0	418	0
		Total avarage	255	0	318	0

			10^-2 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample name: 20-2 hrt 21	1e experiment	2	0	25	0
HRT 21 1056	Date plated: 13-3-14		26	0	325	0
Method 1604			1	0	13	0
	sample name: 20-2 -14	2e experiment	55	0	688	0
	plated: 8-4'14		53	0	663	0
Method 1604			47	0	588	0
Petrifilm	Sample name 20-2 hrt 121	A	2	0	200	0
	Date plated: 13-3	B	3	0	300	0
		C	2	0	200	0
petrifilm	Sample name: HRT 21 T0 2	A	6	0	600	0
	Date sample: 8-4	B	5	0	500	0
		C	2	0	200	0
		Avarage A	10	0	121	0
		Avarage B	52	0	646	0
		Average 3M	3	0	333	0
		Total avarage	31	0	383	0

IV.VII PBR 21 hours HRT end time sample

Algae HRT 21 end			10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample HRT 21 Tend	1e experiment	300	0	375	0
HRT 21 1056	Plated: 13-3-14		300	0	375	0
Method 1604			300	0	375	0
	Sample: HRT 21 Tend	2e experiment	300	0	375	0
	Dateplated: 9-4		300	0	375	0
Method 1604			300	0	375	0
	Algae 3-2-14 HRT 21 t	A	83	0	830	0
	Date plated: 13-3-14	B	77	0	770	0
3M petrifilm		C	54	0	540	0
	Algae HRT 21 T end 2-	A	31	0	310	0
	Date Plated: 9-4-14	B	35	0	350	0
3M petrifilm		C	29	0	290	0
		Avarage A	300	0	375	0
		Avarage B	300	0	375	0
		3M average	52	0	515	0
		Total avarage	300	0	375	0

			10^-2 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample HRT 21 Tend	1e experiment	63	0	788	0
HRT 21 1056	Plated: 13-3-14		50	0	625	0
Method 1604			65	0	813	0
	Sample: HRT 21 Tend	2e experiment	33	0	367	0
	Dateplated: 9-4		42	0	467	0
Method 1604			39	0	433	0
Petrifilm	Algae 3-2-14 HRT 21 t	A	10	0	1.000	0
	Date plated: 13-3-14	B	4	0	400	0
		C	11	0	1.100	0
	Algae HRT 21 T end 2-	A				
	Date Plated: 9-4-14	B				
Petrifilm		C				
		Avarage A	59	0	742	0
		Avarage B	38	0	422	0
		3M average	4	0	417	0
		Total avarage	49	0	582	0

			10^-3 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample HRT 21 Tend	1e experiment	8	0	889	0
HRT 21 1056	Plated: 13-3-14		6	0	667	0
Method 1604			5	0	556	0
	Sample: HRT 21 Tend	2e experiment	4	0	400	0
	Dateplated: 9-4		7	0	700	0
Method 1604			4	0	400	0
Petrifilm	Algae 3-2-14 HRT 21 t	A	2	0	2.000	0
	Date plated: 13-3-14	B	0	0	0	0
		C	1	0	1.000	0
	Algae HRT 21 T end 2-	A				
	Date Plated: 9-4-14	B				
Petrifilm		C				
		Avarage A	6	0	704	0
		Avarage B	5	0	500	0
		3M average	1	0	500	0
		Total avarage	6	0	602	0

IV.VIII PBR 30 hours HRT start time samples

			10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Date sample: 13-12	1e experiment	300	0	375	0
HRT 30 1055	Plated: 3-2		300	0	375	0
Method 1604	Plated18-3-14		300	0	375	0
	Sampledate: 13-12-13	2e experiment	300	0	375	0
	DatePlated: 9-4-13		300	0	375	0
Method 1604			300	0	375	0
Petrifilm	Date sample 13-12-13	A	300	0	30	0
	Date plated: 9-4-14	B	300	0	30	0
		C	300	0	30	0
Petrifilm		A	19	0	190	0
		B	26	0	260	0
		C	31	0	310	0
		Avarage A	300	0	375	0
		Avarage B	300	0	375	0
		Average 3M	163	0	142	0
		Total avarage	300	0	375	0
			10^-2dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Date sample: 13-12	1e experiment	300	0	3.750	0
HRT 30 1055	Plated: 3-2		300	0	3.750	0
Method 1604	Plated18-3		300	0	3.750	0
	Sampledate: 13-12-13	2e experiment	225	0	2.500	0
	DatePlated: 9-4-13		235	0	2.611	0
Method 1604			236	0	2.622	0
	Date sample 13-12-13					
	Date plated: 9-4-14					
Petrifilm						
		A	12	0	1.200	0
		B	3	0	300	0
Petrifilm		C	8	0	800	0
		Avarage A	300	0	3.750	0
		Avarage B	232	0	2.578	0
		Average 3M	4	0	767	0
		Total avarage	266	0	3.164	0
			10^-3dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Date sample: 13-12	1e experiment	49	0	5.444	0
HRT 30 1055	Plated: 3-2		41	0	4.556	0
Method 1604	Plated18-3 -14		58	0	6.444	0
	Sampledate: 13-12-13	2e experiment	32	0	3.200	0
	DatePlated: 9-4-13		21	0	2.100	0
Method 1604			20	0	2.000	0
Petrifilm						
	Date sample 13-12-13	A	4	0	4.000	0
	Date plated: 9-4-14	B	7	0	7.000	0
Petrifilm		C	6	0	6.000	0
		Avarage A	49	0	5.481	0
		Avarage B	24	0	2.433	0
		Average 3M	3	0	5.667	0
		Total avarage	37	0	3.957	0

IV.IX PBR 30 hours HRT end time samples

			10^-1 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample date: HRT 30 19-12	1e experiment	300	0	375	0
HRT 30 1055	Date plated: 19-3		300	0	375	0
Method 1604			300	0	375	0
	Date sample: HRT 30 Tend	2e experiment	300	0	375	0
	Date Plated: 9-4-14		300	0	375	0
Method 1604			300	0	375	0
Petrifilm	Sample date : HRT 30 19-12	A	89	0	890	0
	Date plated: 19-3	B	63	0	630	0
		C	56	0	560	0
Petrifilm		A	17	0	170	0
		B	20	0	200	0
		C	27	0	270	0
		Avarage A	300	0	375	0
		Avarage B	300	0	375	0
		Average 3M	45	0	453	0
		Total avarage	300	0	375	0

			10^-2 dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample date: HRT 30 19-12	1e experiment	76	0	950	0
HRT 30 1055	Date plated: 19-3		67	0	838	0
End			88	0	1.100	0
	Date sample: HRT 30 Tend	2e experiment	53	0	589	0
Method 1604	Date Plated: 9-4-14		59	0	656	0
			3	0	33	0
Petrifilm	Sample date : HRT 30 19-12	A	11		1.100	0
	Date plated: 19-3	B	5		500	0
		C	16		1.600	0
Petrifilm						
		Avarage A	77	0	963	0
		Avarage B	38	0	426	0
		Average 3M	5	0	1.067	0
		Total avarage	58	0	694	0

			10^-3dilution TCFU	E-coli	Coliform/1 ml	E-coli/1ml
Algae	Sample date: HRT 30 19-12	1e experiment	4	0	500	0
HRT 30 1055	Date plated: 19-3		9	0	1.125	0
Methotd 1604			7	0	875	0
	Date sample: HRT 30 Tend	2e experiment	4	0	500	0
	Date Plated: 9-4-14		4	0	500	0
Methotd 1604			4	0	500	0
	Sample date : HRT 30 19-12	A	1		1.000	0
	Date plated: 19-3	B	2		2.000	0
Petrifilm		C	0		0	0
Petrifilm						
		Avarage A	7	0	833	0
		Avarage B	4	0	500	0
		Average 3M	1	0	1.000	0
		Total avarage	5	0	667	0