Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences
Institutionen för energi och teknik

Pathogen and Indicator Organisms Removal in Artificial Greywater Subjected to Aerobic Treatment

– Comparison of four filter media

Ramiyeh Molaei

Institutionen för energi och teknik
Department of Energy and Technology

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Ramiyeh Molaei

Supervisor: Cecilia Lalander, Swedish university of agriculture, Energy and technology

Examiner: Björn Vinnerås, Swedish university of agriculture, Energy and technology

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Energy and technology

Abstract

Treated greywater has a good potential to be used as a source of water. This study analyzes the removal of *Salmonella* spp., *Enterococcous faecalis*, bacteriophage ϕX174 and bacteriophage MS2 from greywater by filtration through biochar, bark, activated carbon and mixture of bark and activated carbon. Reduction of pathogen and indicator organisms were studied over a period of 63 days in column experiments (height 65 cm, diameter: 4.3 cm) supplying artificial greywater at a hydraulic loading rate of 0.032 m^3 m^{-2} day^{-1} and an organic loading rate of 76 g BOD_5 m^{-2} day^{-1} (240 g COD m^{-2} day^{-1}). Biochar filters were more effective than other filters in removal of *Salmonella* spp. (3 log_{10} reduction) and were less effective in removal of bacteriophages. Bark and mixture filters performed inefficiently in removal of pathogen and indicator organisms (1-2 log_{10} reduction). High reduction of pathogen and indicator organisms was detected in activated carbon filters within the first half of the experimental period. The reduction was 7 log_{10} for *Salmonella* spp., 5 log_{10} for *E. faecalis*, 6 log_{10} for bacteriophage ϕX174 and 3 log_{10} for MS2. The reduction of *Salmonella* spp. and ϕX174 correlated to the inflow concentration in all filter media.

Keywords: activated carbon, pine bark, biochar, BOD_5 reduction, *Enterococcous faecalis*, greywater reuse, bacteriophage MS2, bacteriophage ϕX174, *Salmonella* spp.

Author's address: Ramiyeh Molaei, SLU, Department of Energy and Technology,
P.O. Box 7032, 75007 Uppsala, Sweden
E-mail: mora0004@stud.slu.se

Foreword

This master thesis has been conducted at the Department of Energy and Technology at the Swedish University of Agricultural Science in Uppsala, Sweden as a part of a research project that focused on on-site greywater treatment in order to reuse for irrigation, service or recharge of surface and groundwater. It was realized by funding from the Swedish International Development Cooperation Agency (Sida) and the Swedish Research Council (Formas).

I would like to sincerely thank my supervisor, Cecilia Lalander (Department of Energy and Technology/SLU), for her cheerful continuous supervision. I wish to thank Björn Vinnerås (Department of Energy and Technology/SLU) for valuable advices and support. I also want to thank Sahar Dalahmeh (Department of Energy and Technology/SLU), Annika Nordin (Department of Energy and Technology/SLU) and Jörgen Fidjeland (Department of Energy and Technology/SLU) for offering their professional laboratory experiences. At last, I want to thank Yury Chaiko for his help and encouragement.

Filthy water cannot be washed.
West African Proverb

Contents

Abbreviations

AC	Activated carbon
BC	Biochar
B	Bark
BOD_5	Five day biochemical oxygen demand
COD	Chemical oxygen demand
CFU	Colony forming unit
EC	Electrical conductivity
E. coli	*Escherichia coli*
E. faecalis	*Enterococcous faecalis*
ISP	Isoelectric pH
M	Mixture of activated carbon and bark
PFU	Plaque forming unit

1 Introduction

Water is a finite precious resource (UNU-INWEH, 2011-2012, Anwar, 2011) which is essential for multidimensional development (FAO, 2007).

Low rainfall (Eriksson et al., 2002), population growth, increased urban, agricultural and industrial water demand (UNU-INWEH, 2011-2012, Rose et al., 1991) in combination with unbalanced water demand and availability, accentuate the water issue (FAO, 2007). Water stress is a serious challenge for many countries all over the world (Li et al., 2010, Pinto et al., 2010) specially in arid and semi-arid areas (Santos et al., 2012, FAO, 2010). Around one third of the world's population face water shortage and scarcity, but this number is estimated to increase to two-third by 2025 (FAO, 2007).

The highest demand for water, 70%, come from agriculture and it is estimated to double by 2050 (FAO, 2007, FAOWATER, 2008). Consequently the wise use of water is a key for sustainable development (FAO, 2007).

The consumption of fresh water can be decreased between 30% to 50% (Pinto et al., 2010, Jeppesen, 1996) by using greywater for toilet flushing, cleaning purposes, industrial cooling processes and agricultural irrigation (Sellner, 2009, Pinto et al., 2010). Greywater is the wastewater produced in kitchen sinks, bathtubs, showers, hand basins and laundry machines (Eriksson et al., 2002). Despite the fact that faeces, urine and toilet papers are absent in greywater, it requires pre-treatment before irrigation, for health and environmental reasons (Eriksson et al., 2002). Irrigation with untreated greywater can lead to spread of disease (Eriksson et al., 2002), dissolution of organic matter (Anwar, 2011), reduced yield (Chen et al., 2000, Morel and Diener, 2006), oxygen depletion (Eriksson et al., 2002), eutrophication (Morel and Diener, 2006) and separation of soil particles (Anwar, 2011).

1.1 Objective

The objective of this study was to compare the efficiency of four filter materials: biochar, bark, activated carbon and mixture of bark and activated carbon, in removal of pathogen and indicator organisms, *Salmonella* spp., *Enterococcous faecalis*, bacteriophage ϕX174 and bacteriophage MS2, from artificial greywater.

1.2 Hypothesis

Concentration of pathogens and indicator organisms in artificial greywater can be reduced through vertical biofilter columns.

Biochar filters can provide high removal of pathogens and indicator organisms due to their big effective size similar to bark, high retention time, surface area and porosity. Biochar material might be a practical alternative for activated carbon material due to its simple and low cost production comparing to activated carbon.

Bark filters can significantly remove the pathogens and indicator organisms as previous studies illustrated effective reduction in bark filters due to their chemical composition (rich in tannins, low pH) and surface charge.

Activated carbon particles have high specific surface area, porosity and specific surface which lead to high biofilm formation potential and adsorption capacity. Hence, it included in this experiment as a reference.

Mixture of bark and activated carbon can perform significant reduction of pathogens and indicator organisms since both filter materials are effective and also can decrease the installation cost due to reducing the use of expensive activated carbon.

2 Background

The use of greywater in agriculture has become an interesting option in order to reduce the use of freshwater, but it requires treatment in order to insure the human and environmental safety (FAO, 2010).

2.1 Greywater characteristic and analysis

The share of greywater in total volume of wastewater , in which toilet water is included, is around 50-80% (Al-Jayyousi, 2003, Li et al., 2010). The concentration of organic matter, nitrogen, phosphorous, salts, solids and pathogens is extensively different among households depending on type of the detergents, type of goods being washed, food diet and life style of residents (Pinto et al., 2010, Eriksson et al., 2002). The parameters that should be analyzed before irrigating greywater on land are pH, electric conductivity (EC), suspended solids, heavy metals, dissolved oxygen, biological and chemical oxygen demands (BOD and COD), pathogens and indicator organisms ; fecal coliforms, *E.coli*; total nitrogen and phosphorus, (Pinto et al., 2010, Negahban-Azar et al., 2012).

2.1.1 Physical characteristics

Suspended solids in high concentration can clog the filters and irrigation suppliers (Eriksson et al., 2002). Sources of solids in greywater are food material, hair and fibers (Eriksson et al., 2002). Eriksson et al. (2002) reported the solids concentration to be between 113 and 2410 mg L^{-1}.

2.1.2 Chemical characteristics

Alkalinity and pH

The alkalinity of the greywater is usually in a range of 20-340 mg $CaCO_3$ L^{-1} and is highly related to the pH of water supply. The pH in greywter originated from laundry is higher than other fractions of greywater, around 8-10, due to the presence of chemical products (Morel and Diener, 2006). Buffering capacity and pH of the soil can be effected by the alkalinity and pH of the greywater (Eriksson et al., 2002). High pH (>10) can cause dissolution of organic matter, plant suffer and soil particles separation (Anwar, 2011).

Organic load

The main organic substances found in greywater are proteins, carbohydrates, fats and surfactants (Morel and Diener, 2006). Organic load is measured by the chemical oxygen demand (COD) and/or biological oxygen demand (BOD) and vary between 13 and 8000 COD mg^{-1} L^{-1} or between 5 and 1460 BOD mg^{-1} L^{-1}. COD mainly originates from dishwashing and laundry detergents (Eriksson et al., 2002). The source of oil is kitchen and is related to the cooking habits. The BOD and COD shows the organic matter load in the water which in high load can lead to oxygen depletion and sulphide production due to activity of anaerobic bacteria (Eriksson et al., 2002). The fat can congeal and stop the irrigation or treatment process (Morel and Diener, 2006). It shows the particle content which can lead to clogging the filters used for greywater treatment or irrigation suppliers (Eriksson et al., 2002).

The main source of nitrogen in greywater is kitchen waste, 40-74 mg L^{-1}. Bathroom and laundry have the minimum share of nitrogen in greywater. The source of phosphates in greywater is mainly washing detergents, in the countries that are still using phosphorus-containing detergents, and the total phosphorus concentration is around 6-23 mg L^{-1}. In countries that have banned phosphorus-containing detergents, the concentration is about 4-14 mg L^{-1}, which mainly originate from bathroom greywater (Eriksson et al., 2002). Despite the fact that nitrogen and phosphorus are good fertilizers they have negative effects on aquatic environment. If spread into water bodies they can cause algae growth and eutrophication (Morel and Diener, 2006).

Inorganic load

Inorganic components- Calcium carbonate ($CaCO_3$) and some other inorganic salts such as sodium chloride (NaCl) are present in greywater (Eriksson et al., 2002). The electric conductivity of greywater which shows the salinity of the greywater is between 300-1500 µS cm^{-1} and sometimes up to 2700. Irrigation with saline greywater in the first place can decrease the yield and in the long term can lead to topsoil salinization in arid regions with clay and loamy soils (Morel and Diener, 2006). The EC of irrigation water is usually about 1 ds m^{-1} (Anwar, 2011) and recommended range is 0-1500 µS cm^{-1} (Pinto et al., 2010).

Essential mineral elements- Manganese (Mn), iron (Fe), copper (Cu), nickel (Ni), zinc (Zn) can be found in greywater, for instance concentration of zinc is around 0.01-1.8 mg L^{-1} or less (Eriksson et al., 2002). Despite the living organism requirement to such elements, excessive levels can have damaging effects on them.

Non-essential mineral elements- Aluminum (Al) and heavy metals; cadmium (Cd), lead (Pb), mercury (Hg), chromium (Cr) can also be found in low concentrations in greywater (Eriksson et al., 2002). Human exposure to such elements can cause physical and mental problems. The high concentration of heavy metals can affect the quality and quantity of the yield as well as aquatic environment (Chen et al., 2000) and the risk of metal transportation to the groundwater is related to physical, chemical and biochemical properties of the soil (EPA, 2002). Accumulation of heavy metals in animals can cause serious illness in long term (EU, 2009).

The source of mineral elements (except P and N) can be plumbing materials, jewelry, cutlery, coins and home maintenance products (Eriksson and Donner, 2009).

2.1.3 Pathogenic characteristics

Greywater is less contaminated in term of pathogens compared to wastewater since the toilet water is excluded. Although it is available in higher volume with lower level of microbial load (Pinto et al., 2010). Pathogens such as bacteria, viruses, protozoa, parasites can be found in grewywater (Morel and Diener, 2006) . Several studies observed fecal contamination in greywater (Jeppesen, 1996, Ottoson and Stenström, 2003, O'Toole et al., 2012, Rose et al., 1991) which raise health concerns on irrigation with greywater (Negahban-

Azar et al., 2012). Fecal coliforms concentration in greywater varies from 0 to 10^6-10^7 CFU 100 mL^{-1} (Friedler et al., 2006).

Pathogenic viruses and bacteria can reach the greywater in different ways. Greywater originated from kitchen can be contaminated by uncooked vegetables and raw meat and it contains higher levels of micro-organism comparing to laundry and bathroom greywater. *Escherichia coli* concentration in the greywater was found in the range of 2.5×10^5-$1.3\times10^8$100 mL^{-1} arisen from kitchen. The laundry greywater contains total coliforms in range of 8.9×10^5-56 $\times10^5$100 mL^{-1}. The bathroom greywater contains total coliforms in range of 70-$2.4\times10^7$100mL^{-1} which can be introduced to greywater by hand washing after toilet visit, diaper changing, bathing children (Eriksson et al., 2002) and anal cleansing.

In this study *Salmonella* spp. were used as fecal pathogen model and indicator organisms *Enterococcous faecalis* and bacteriophages ϕX174 and MS2 were used as model organisms.

Bacteria divided into two main groups, gram-negative and gram-positive, based on the presence or absence of an outer lipid membrane. Outer lipid membrane is present in gram-negative bacteria which make the wall impenetrable and thus more resistant against antibodies (Murray et al., 2002).

Salmonella genus belongs to the family *Enterobacteriaceae* and comprises over 2600 serovars, which are divided into typhoidal and non typhoidal serovars. Typhoidal *Salmonella* cause systemic invasive disease (enteric fever) by the fecal-oral route and via contaminated food and water. Non typhoidal serovars cause less sever, but commonly occurring gastroenteritis (salmonellosis), mainly in animal production (poultry and eggs). Salmonellosis infection circle begins with the bacteria entering and localizing the host intestines within 12 to 72 hours and results in diarrhea, nausea, vomiting and fever (Rhen, 20007). *Salmonella* spp. are gram-negative, motile, rode-shaped (0.7-1.5 × 2-5 µm), facultative anaerobic, zoonotic intestinal (Rose et al., 1991) with 2-5 µm size.

Enterococcous faecalis genus is part of Enterococci family, gram-positive, spherical or ovoid shaped (0.6-2.0 × 0.6-2.5 µm), occurring in pairs or short chains, facultative anaerobe, non-spore forming with 0.5-1 µm size (Bergey and Holt, 1994). *E. faecalis* live in gastro intestinal of humans and other mammals (R.Eley, 1996) and its presence in water bodies and foods inferred to

fecal contamination (Riemann and Cliver, 2006) . This, together with tolerance to extreme conditions such as hyperosmolarity, heat (growing at 10°C and 45°C, surviving at 60°C for 30 minutes), ethanol, hydrogen peroxide, acidity, alkalinity (growing at pH 9.6) and salinity (6.5% NaCl broth) (Güven Kayaoglu and Ørstavik, 2004) makes *E. faecalis* good indicators.

Viruses pose serious health risk due to their relatively low infectious dose (Dixon et al., 1999). Constant presence of bacteriophages in treated sewage (Henze, 2008) in addition to theirs inexpensive, simple and quick analyze and being non-pathogenic to humans propose them as good fecal and viral indicators (Lalander et al., 2012, Ottoson, 2004). Bacteriophages have been wildly used to examine the effect of water and wastewater treatments on viruses (Henze, 2008).

Bacteriophage ϕX174 is an icosahedral shaped somatic coliphage (attach to the host cell wall), with 27 nm diameter and isoelectric pH between 6.6 and 6.8. Previous studies showed high rate of adsorption to filter media which makes it a reliable model organism for viruses (Collins et al., 2006).
Bacteriophage MS2 is an icosahedral shaped f-specific bacteriophage (infect the host cell appendage sex pili), 27 nm in size and with an isoelectric pH around 3.5. Even though previous studies showed low levels of MS2 adsorption to filter media it is considered as a convenient model organisms for viruses (Collins et al., 2006).

2.2 Greywater treatment

Greywater is considered a reliable source of irrigation in The World Health Organization Guidelines for safe use of wastewater, excreta and greywater (WHO, 2006a) due to the considerable level of plant nutrients and low concentration of pathogens comparing to wastewater. Common method for treating greywater is filtering system; various filter media have been explored. Sand filtering is the most common approach for treating the greywater (Dalahmeh et al., 2012). Despite the fact that sand filters can reduce the BOD_5 by 75% in vertical sand filters, investigations into alternative filter materials have started due to the clogging and accessing problems of sand in some regions, considering that the high bulk density of sand make it costly to transport (Dalahmeh et al., 2012).

Horizontal subsurface flow constructed wetland (using sand or oil-shale ash as a filter media) is another method which is reliable to remove P and N from

wastewater but efficiency of this system decreasing by time due to saturation, clogging and biofilm formation (Vohla et al., 2007).

In 1990s vertical flow systems became very popular in Europe (Stefanakis and Tsihrintzis, 2012) and several studied examine their efficiency in treating the greywater. Coetzee et al. (2010) studied aerobic treatment using PVC vertical columns (150 cm×15 cm) filled with stone. The best result shown in term of nitrogen removal from greywater at hydraulic load 35.7 L m^{-2} d^{-1} (Coetzee et al., 2010) whereas it was more effective in removal of nitrogen and phosphorus in higher organic load (up to 200 g COD m^{-2}). Zapater et al. (2011) showed that re-circulating vertical flow constructed wetland (RVFCW) is reliable in term of nitrogen, phosphorus, BOD and COD excepting *E. coli*. In another study by Stefanakis and Tsihrintzis (2012) carbonate and igneous rock, zeolite and bauxite used as filter material in vertical flow constructed wetlands and the results showed that this system is efficient in BOD_5 ,COD (up to 78%) and nitrogen removal but not efficient in removal of phosphorous.

Dalahmeh et al. (2012) compared both pine bark and activated carbon with sand using the vertical PVC columns. In this study, bark and charcoal filters performed higher BOD_5, P and N removal and fecal coliform reduction than sand filters. Lalander et al. (2012) study and Lewis et al. (1995) showed that bark material can efficiently remove the pathogens from greywater. Nonetheless the efficiency of bark, charcoal and similar carrier materials requires further investigation.

2.3 Greywater reuse guidelines

According to WHO guidelines for reuse of greywater in agriculture, acceptable values of *E. coli* and thermotolerant coliforms in irrigation water are 10^3-10^5 100 mL^{-1} and 10^4-10^6 100 mL^{-1} respectively. WHO suggests different combinations of greywater treatment, crops and irrigation system in order to achieve a total pathogen reduction of 7 $\log_{10}$. The required level of reduction by treatment varies between 1 to 4 $\log_{10}$ reduction (WHO, 2006b).

In restricted irrigation which 7 $\log_{10}$ reduction can be achieved in a number of scenarios: (I) treatment (4 $\log_{10}$ reduction) and labor intensive (3 $\log_{10}$ reduction); (II) treatment (3 $\log_{10}$ reduction) and highly mechanized system (4 $\log_{10}$ reduction); (III) treatment (1 $\log_{10}$ reduction) and subsurface irrigation (6 $\log_{10}$ reduction); (IV) cooking (6-7 $\log_{10}$ reduction). Peeling before consumption can provide 2 $\log_{10}$ reduction. Restricted irrigation includes non-

food crops (*e.g.* cotton and biodiesel crops), food crops that are processed before consumption (wheat) and all crops that have to be cooked before human consumption (potatoes, rice) (WHO, 2006b).

In unrestricted irrigation, which includes crops that are eaten uncooked by humans, leaf crops (lettuce) and root crops (onion) require a total reduction of (6 and 7 $\log_{10}$ reduction), which can be achieve by various scenarios: (I) treatment (3 and 4 $\log_{10}$ reduction), pathogen die-off on crop surfaces (2 $\log_{10}$ reduction) and washing of with clean water prior to consumption (1 $\log_{10}$ reduction). High growing crops and low growing crops require 6 $\log_{10}$ reduction, which can be achieved by: (II) treatment (2 $\log_{10}$) and drip irrigation (4 $\log_{10}$) for high crops (such as tomato) and (III) treatment (4 $\log_{10}$) and drip irrigation (2 $\log_{10}$) for low crops (WHO, 2006b).

Among different irrigation systems, flood and furrow irrigation are the lowest cost methods but highest risk to the fieldworkers. Spray and sprinkler method have highest potential to spread the contamination on to crops and field workers (just 1 $\log_{10}$ reduction). Drip irrigation considered as the most health secure method for fieldworkers and the most expensive one. However, reducing the greywater consumption and increasing the crop yield may recover the expenses (WHO, 2006b).

3 Material and Methods

3.1 Experimental design

The experiment took place over a period of 78 days. For filter materials prepared and a total of twelve vertical columns were installed with triplicates of each filter material. The bulk density, particle density, total porosity, gravimetric water content, residence time and tracer recovery of each filter material was determined before feeding the columns with greywater. The columns were fed with artificial greywater over a period of 63 days and duplicate microbial analysis performed for each column.

3.2 Filter materials

Four filter materials; biochar, bark, activated carbon and mixture of bark and activated carbon were sieved to meet particle size distribution correlated to previous studies by Dalahmeh et al. (2012) and Berger (2012) .

The biochar originated from salix wood in Germany were sieved through 7, 5, 2.8, 1.4 and 1 mm screens. Biochar retained on the 2.8 and 1 mm screens was mixed a ratio 3:2 by weight. The bark originated from an undefined air-dried pine bark and sieved through 7, 5, 3.15 and 1 mm screens. Bark retained on the 3.15 and 1 mm screens was mixed in a ratio 3:2 by weight. The activated carbon obtained from Merck with two different sizes; 1.5 and 3-5 mm length and 1.5 mm diameter. The material was mixed in a ratio 2:3 by weight. The mixture material was prepared by mixing the bark and activated carbon in a ratio 1:1 by weight.

The vertical columns that were used in the experiment were made of transparent acrylic plastic with a height of 65 cm, diameter of 4.3 cm and bottom outlet with 0.5 cm diameter. The columns were covered by aluminum foil in order to inhibit algae growth. Perforated aluminum foil was used as lid for the columns.

A total of twelve columns were installed and filled up to a depth of 50 cm with biochar, bark, activated carbon and mixture, with three columns for each material. The filter material was added spoon by spoon and shaken manually in order to have higher density (Figure1). Two layers of 10-25 mm gravel were placed under and over the filter material in order to facilitate drainage (Figure1).

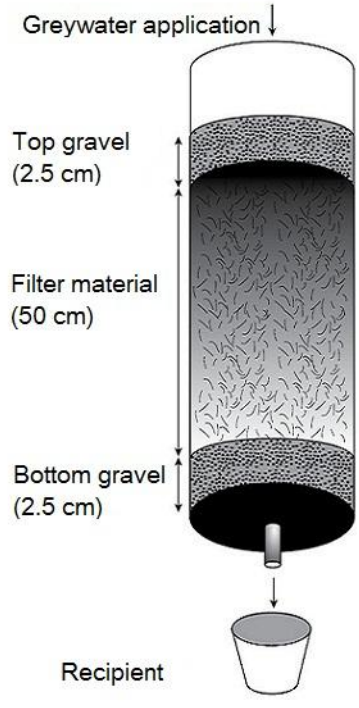

Figure 1.Installation of experimental column with filter material, top and bottom gravel and greywater application.

The bulk density, particle density, total porosity, gravimetric water content and residence time of the filter material were determined after the column installation (Table 1).

Bulk density was calculated by dividing the dry weight of the filter material by the total volume of cylinder occupied by the material (50 cm depth and 4.3 cm diameter).

Particle density of filter material was determined by dividing the dry weight of material by the volume of solids excluding the pores.

The liquid immersion method was used in order to measure the volume of deionized water displaced by particles. The mixture of dry material and deionized water was boiled gently in a volumetric flask in order to determine the air-filled pores and cover flask remained in lab for 24 hours to saturate and they filled up with water. The weight of the flask was recorded in all the steps.

Porosity was calculated by:

$$p = 100 \times \left(\frac{\rho_B}{\rho_p}\right), \quad (1)$$

where ρ_B is the bulk density and ρ_p is the particle density.

Gravimetric water content was determined by dividing the mass of water by the weight of oven dried material. The mass of water was calculated by subtracting the weight of the oven dried material from the weight of the air dried material.

Residence time was determined after saturating the filter material by immersing the columns in tap water. The process started by adding a 0.03 L pulse of NaCl tracer solution (10 g L^{-1}) to the columns. Over the period of 11 days columns were fed with 45 mL of tap water (30 mL at 9:00 a.m. and 15 mL at 16:00 p.m.) and the electrical conductivity (EC) of the effluent was measured an hour after each feeding incident. Mean and longest residence times of tracer were determined. The mean residence time is the time required to recover 50% of the tracer and the longest residence time is the time when no tracer is recovered.

Table 1.Characteristics of the filter materials

Filter material	Particle size (mm)	Effective size (mm)	Bulk density (g m^{-3})	Particle density (g m^{-3})	Total porosity (%)	Water content (%)
Biochar	1-1.4 2.8-5	1.4	0.27	0.74	63.3	0.06
Bark	1-3.15 3.15-5	1.4	0.36	1.3	73	7.6
Activated carbon	1.5 3-5	1.2	0.56	1.89	70.6	1.9
Mixture	1-1.4, 2.8-5 1-3.15, 3.15-5	1.5	0.15	1.42	99.9	6.8

3.3 Artificial greywater

Artificial greywater was prepared by mixing 1.25 g standard nutrient broth (OXIOD, Sollentuna, Sweden), 0.16 g washing powder (Ariel, Germany), 0.16 g dishwashing gel (YES Original, Procter and Gamble, Stockholm, Sweden), 0.16 g hair shampoo (VO5, Uplands Väsby, Sweden) and 0.1 g corn oil (El Nada, Al-Asher for products, 10th Ramadan City, Egypt) in 0.25 L tap water. The total volume of 3 L greywater was prepared every week and kept in 5℃ during the week. The temperature of greywater that columns were fed with was 25℃.

3.4 Pathogen and indicator organims

Salmonella enterica subspecies~1 serovar Typhimurium phage type~178 (isolated from sewage sludge) (Sahlstrom et al., 2004) and *Enterococcous faecalis* (ATCC 29212) were grown in nutrient broth (Oxiod AB, Sweden) at 37 ℃ over 24 and 48 hours. Bacteriophages ϕX174 and MS2 were used as model organisms. Inoculate solution had 4% volumetric share of the greywater and was added to 25℃ greywater every morning.

3.5 Pathogenic and indicator organisms analysis

Every week a duplicate analysis was performed for inflow and effluent. *Salmonella* spp. were grown on Xylose lysine deoxycholate (XLD) plates and incubated at 37 ℃ for 24 hours. *E. faecalis* were grown on Slanetz & Bartley agar (SlaBa) and incubated at 44 °C for 48 hours. Analysis of ϕX174 was performed using *E. coli* (ATCC 13706) as host bacteria while *Salmonella* Typhimurium (WG49, ATTC 700730) host bacteria were used for *MS2*. The analysis was conducted by double layer agar method using blood agar base (BAB) plates as a first layer and soft agar as a second layer. All BAB plates were incubated at 37 ℃ over night. Triple sugar iron-agar method used to validate typical *Salmonella* spp. colonies.

3.6 Statistics

The One-way ANOVA test and a post-hoc Tukey's test were used to analyze the data using the statistical software Minitab version 10.

One-way ANOVA can show whether there are statistically significant differences somewhere in the data but cannot show just where those differences lie. Multiple comparisons test like Tukey's test in order to point out just where the real differences lie. The null Hypothesis (H_0) was that there is no significant difference between the means and alternative Hypothesis (H_1) was that there is a significant difference between the means. P-value below 0.05 rejects the null Hypothesis, which means there is a significant difference between filters.

4 Results

4.1 Residence time and tracer recovery

The mean residence time for biochar filter was 90 hours and the longest residence time was 170 hours (Figure 2, Figure 3) (Table 2).

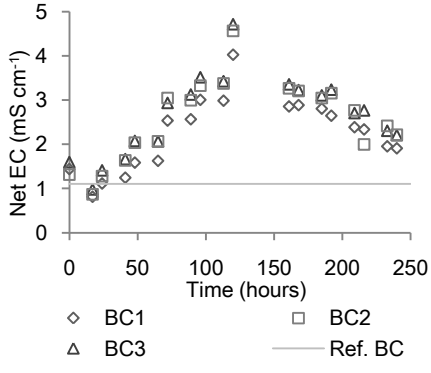

Figure 2. Net EC vs. time for biochar 1,2 and 3after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

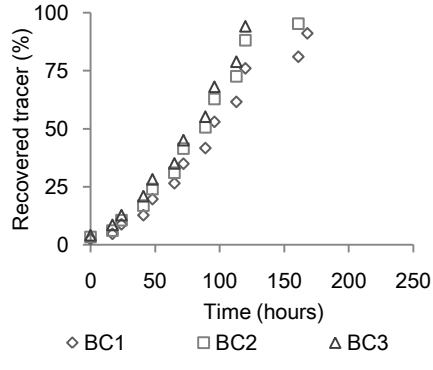

Figure 3. Percentage recovery of tracer vs. time for biochar 1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

In case of bark filters, the mean and the longest residence time were 90 and190 hours respectively (Figure 4, Figure 5) (Table 2).

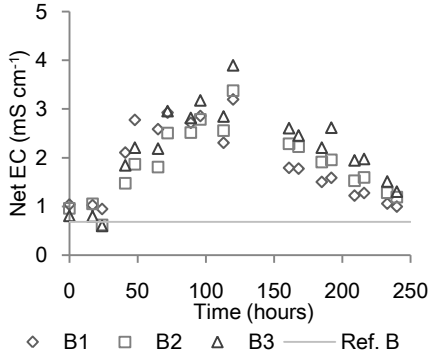

Figure 4. Net EC vs. time for bark1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

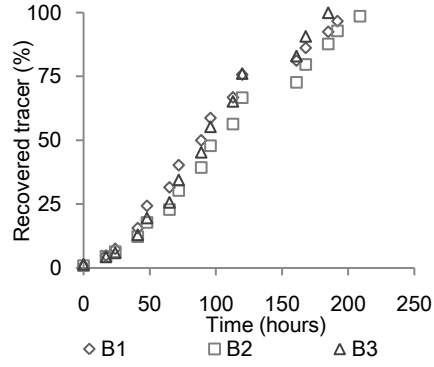

Figure 5. Percentage recovery of tracer vs. time for bark1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

The mean and longest residence time for activated carbon were 180 and 250 hours respectively (Figure 6, Figure 7) (Table 2).

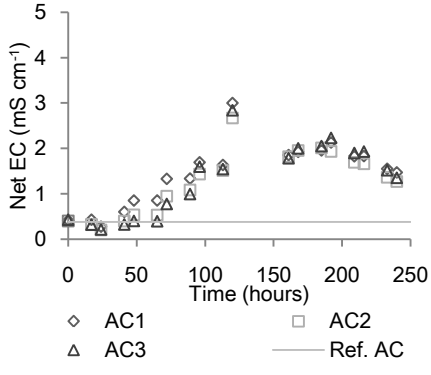

Figure 6. Net EC vs. time for Activated carbon 1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

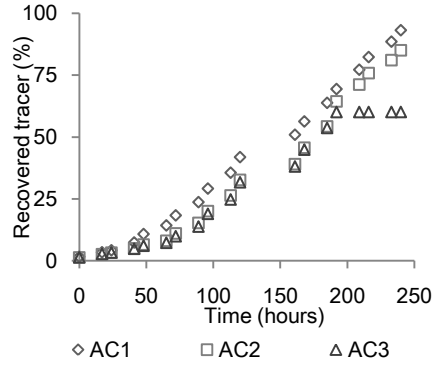

Figure 7. Percentage recovery of tracer vs. time for Activated carbon 1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

Mixture filters showed no tracer recovery within 11 days after adding the NaCl pulse (Figure 8, Figure 9) (Table 2).

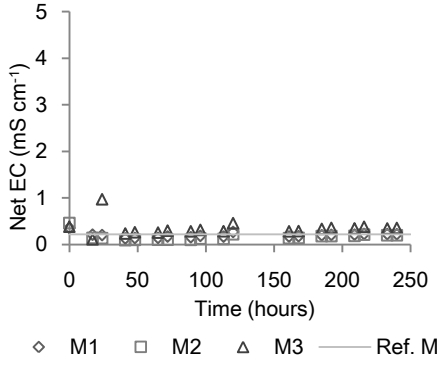

Figure 8. Net EC vs. time for Mixture 1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

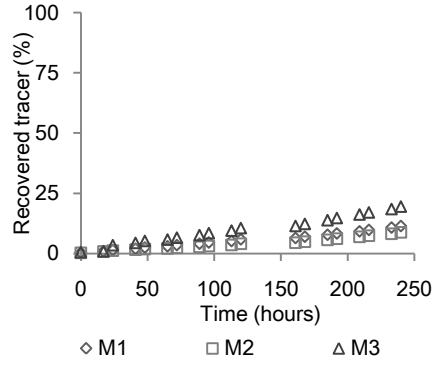

Figure 9. Percentage recovery of tracer vs. time for Mixture 1, 2 and 3 after adding a pulse of NaCl to influent tap water, loaded at a rate of 0.032 m^3 m^{-2} day^{-1}.

In comparison with other filter materials biochar achieved complete tracer recovery in shortest period of time and together with bark reached the mean residence time much earlier (half), compared to activated carbon. Activated carbon was slower than the other filter materials in term of mean residence time and full recovery (twice in comparison to biochar and 1.5 times in comparison to bark) (Table 2).

Table 2.The mean and longest residence time for biochar, bark, activated carbon and mixture.

Filter material	Mean residence time (hour)	Longest residence time (hour)
Biochar	90	170
Bark	90	190
Activated carbon	180	250
Mixture	no recovery	no recovery

4.2 pH values

In biochar, activated carbon and mixture filters, pH was fairly constant during the experiment. In biochar filter pH was slightly below 9, in activated carbon filters was around 9 and in mixture filters was around 8. Bark filters showed high variation of pH between 4 to up to 7 (Figure 10).

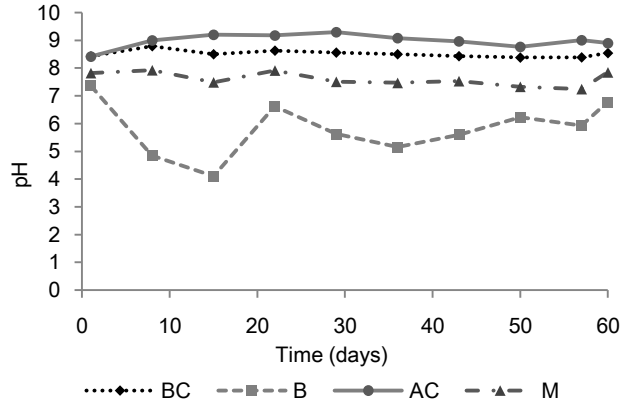

Figure 10. pH of biochar, bark, activated carbon and mixture filters during the experiment.

4.3 Reduction of pathogen and indicator organims

4.3.1 The pathogenic and indicator organisms concentration in the inflow

The concentration of *Salmonella* spp. and bacteriophages was not constant during the experimental period and was varying from week to week. However *E. faecalis* obtained almost the same concentration over the 60 days of the operation (Figure 11).

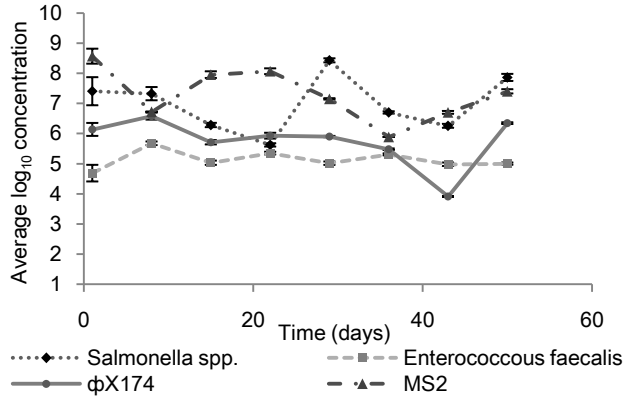

Figure 11. Concentration of pathogens and indicator organims in the inflow during the experiment. Unit is CFU mL^{-1} for Salmonella spp. and E. faecalis and PFU mL^{-1} for bacteriofaghes.

4.3.2 *Salmonella* spp.

The reduction in *Salmonella* spp. was around 2 $\log_{10}$ or higher in the biochar filter. In activated carbon filter the reduction was very high at the start, specially during the first week, around 7 $\log_{10}$, but decreased to around 1 $\log_{10}$ after fifteen days. Mixture showed around 1 $\log_{10}$ reduction in the first 30 days but then increased to 3 $\log_{10}$. Bark filter showed the smallest reduction, 1 $\log_{10}$, in comparison with three other filter materials over time (Figure 12, Figure 13).

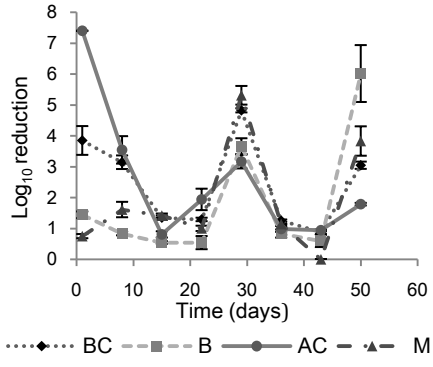

Figure 12. Log_{10} reduction of Salmonella spp. in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

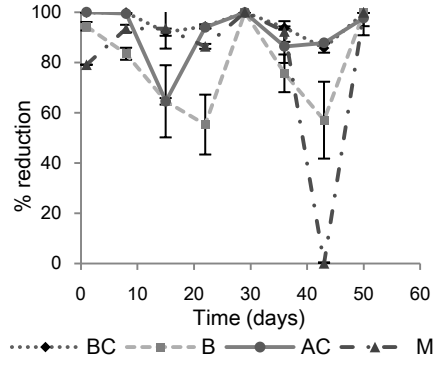

Figure 13. Percentage reduction of Salmonella spp. in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

4.3.3 *Enterococcous faecalis*

The effect of biochar filter on *E. faecalis* reduction was high in the first week, (4 $\log_{10}$), but decreased to 2 $\log_{10}$ in the second week and less than 1 $\log_{10}$ in the six following weeks. Bark filter performed low reduction, 1 $\log_{10}$ or less through entire nine weeks of experience. In activated carbon filter 4 $\log_{10}$ reduction achieved at the start but it decreased to 1 or 2 $\log_{10}$ from day fifteen. Mixture filters reduction was varying between 0 and 1 $\log_{10}$ (Figure 14, Figure 15).

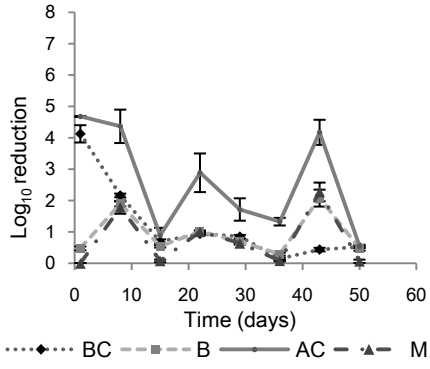

Figure 14. Log_{10} reduction of E. faecalis. in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

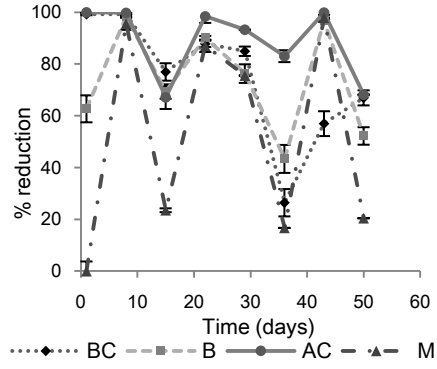

Figure 15. Percentage reduction of E. faecalis. in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

4.3.4 Bacteriophage ϕX174

The removal of the bacteriophage ϕX174 was the highest in the activated carbon filter. During the first week it was around 6 log_{10} but it decreased to 4 log_{10} for three weeks and 2 log_{10} in the end. The reduction in bark filter was around 1 log_{10} or less for the duration of experiment. Mixture filters performed similar to bark filters. In the biochar filter the reduction was 4 log_{10} and 2 log10 in the first and the second week but for the continuing seven weeks of experiment it was fairly constant, just under 1 log_{10} (Figure 16, Figure 17).

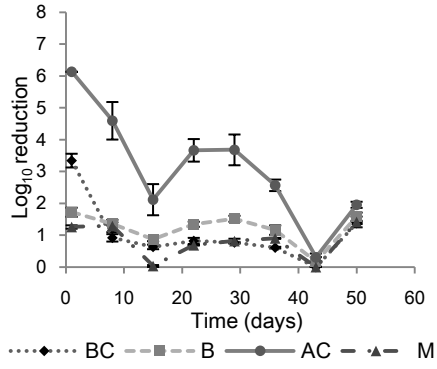

Figure 16. Log_{10} reduction of ϕX174 in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

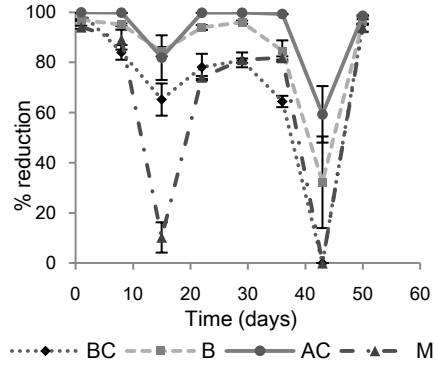

Figure 17. Percentage reduction of ϕX174 in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

4.3.5 Bacteriophage MS2

The reduction of bacteriophage MS2 was similar in both biochar and bark filter. In the first week the reduction was around 3 log_{10} in biochar filter and 2 log_{10} in bark and mixture filters but in all three filters the reduction was less than 1 log_{10} during the following nine weeks. Biochar, bark and mixture filters had highest reduction in the second week. Activated carbon filter had around 3 log_{10} reduction at the start, but from the day twenty nine it decreased to under 1 log_{10} (Figure 17, Figure 18).

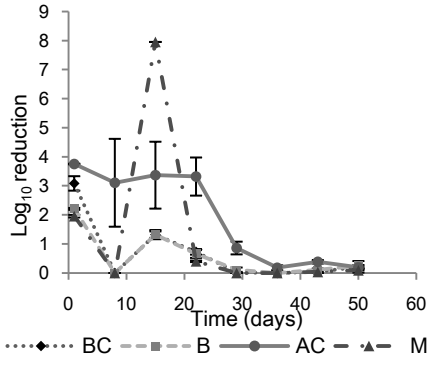

Figure 17. Log_{10} reduction of MS2 in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

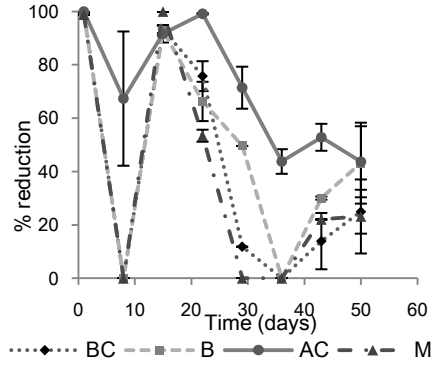

Figure 18. Percentage reduction of MS2 in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1}.

4.4 Log reduction and inflow concentration

The concentration of four microorganisms in the inflow was not constant during the experiment. The correlation between the inflow concentration and reduction of pathogen and indicator organisms, was determined for each organism by excel regression for the second half of experience since the filters were more stable from the middle of experiment.

R-square shows how good the regression line approximates the real data and ideally 0.6 is the least value for R-square. P-value shows the probability of real results. The lower the P-value, the higher the likelihood that results did not appear by chance.

4.4.1 Salmonella spp.

Reduction of *Salmonella* spp. in all filters is highly related to concentration of *Salmonella* spp. in the inflow, the higher the concentration the higher the $\log_{10}$ reduction (Figure 19).

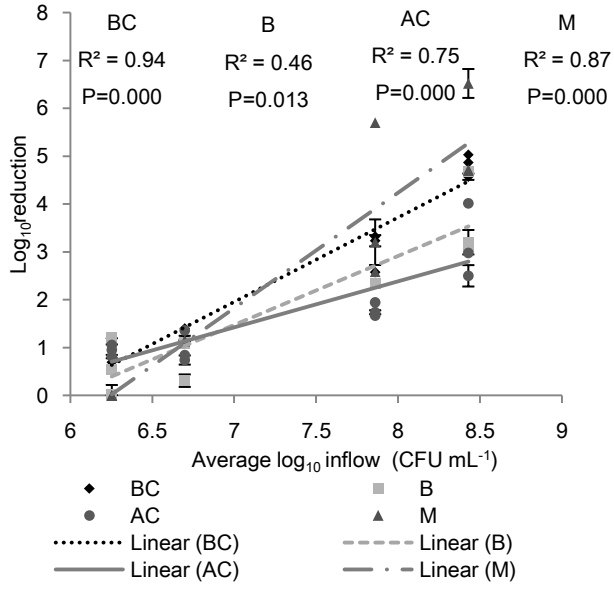

Figure 19. Log_{10} reduction of Salmonella spp. in biochar, bark, activated carbon and mixture at 240 g COD m^{-2} day^{-1} VS. inflow concentration.

4.4.2 Enterococcous faecalis

E. faecalis inflow concentration was fairly constant in opposite of the other microorganisms. The reduction of *E. faecalis* in relation to inflow concentration did not show any significant result (Figure 20).

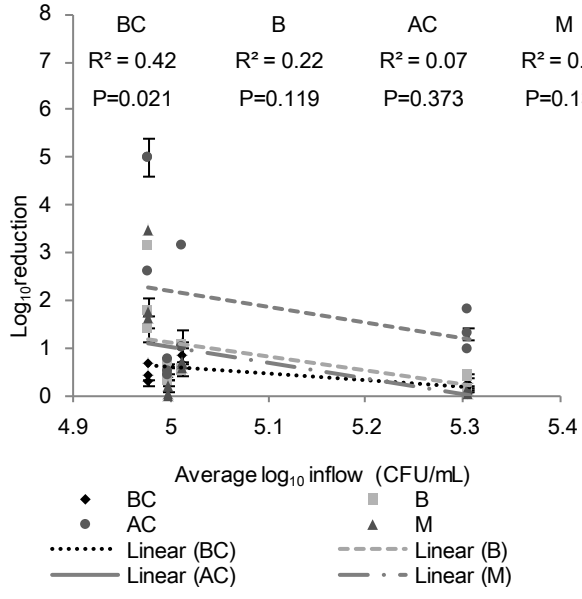

Figure 20. Log_{10} reduction of E. faecalis in biochar, bark, activated carbon and mixture at 240g COD m^{-2} day^{-1} VS. inflow concentration.

4.4.3 Bacteriophage ϕX174

Reduction of bacteriophage ϕX174 was highly related to ϕX174 inflow concentration in all filters (Figure 21).

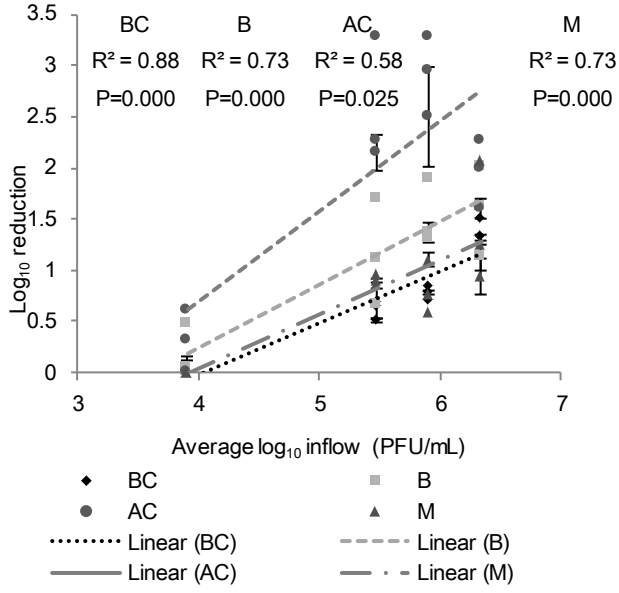

Figure 21.Log_{10} reduction of ϕX174 in biochar, bark, activated carbon and mixture at 240g COD m^{-2} day^{-1} VS. inflow concentration.

4.4.4 Bacteriophage MS2

Reduction of MS2 was not related to MS2 inflow concentration in any of filters (Figure 22).

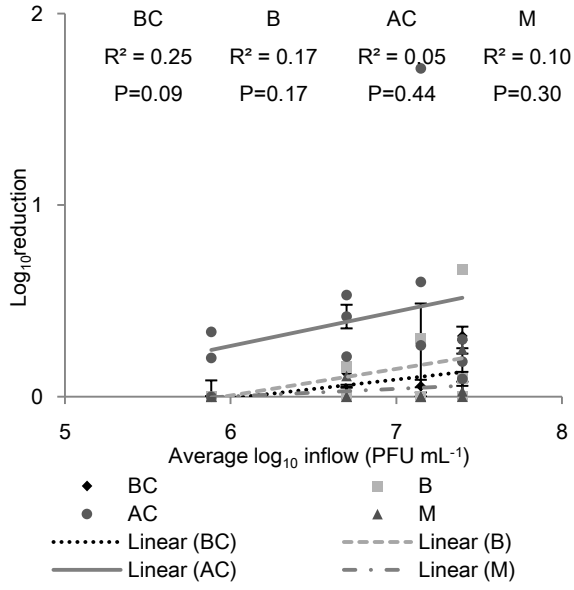

Figure 22. Log_{10} reduction of MS2 in biochar, bark, activated carbon and mixture at 240g COD m^{-2} day^{-1} VS. inflow concentration.

4.5 Statistic

The result of one-way ANOVA test for 60 days of experiment showed that there was a significant difference between all filters in reduction of *E.faecalis* (P-value=0.009), ϕX174 (P-value=0.003) and MS2 (P-value=0.020), but not in removal of *Salmonella* spp. (P-value=0.736). Tukey's test showed that activated carbon is significantly different from other filters in *E. faecalis* and ϕX174 removal. There was only a significant difference between activated carbon and mixture in removal of MS2 (Table 3).

Table3. Tuckey's test for the entire experimental period

Organisms	Filter material*	Filter material	P-value
E. faecalis	Activated carbon	Biochar	0.0030
		Bark	0.0002
		Mixture	0.0000
ϕX174	Activated carbon	Biochar	0.0000
		Bark	0.0000
		Mixture	0.0000
MS2	Activated carbon	Mixture	0.02

*filter material which is significantly different from the other filter materials

The concentration of four microorganisms in the inflow was not constant during the experiment and $\log_{10}$ reduction of organisms was compared at the highest and lowest inflow concentration during the second half of the experiment since filters were more stable. Concentration of *Salmonella* spp. in the inflow was the highest on the 5^{th} week and the lowest on 7^{th} week. Inflow concentration of *E.faecalis* was fairly constant comparing to *Salmonella* spp. and bacteriophages, however week five and seven were used for analysis. Concentration of ϕX174 was the highest on week 8 and lowest on week 7, while the highest concentration of MS2 was observed on week 8 and lowest on week 6.

The results of one-way ANOVA test showed that there was a significant difference in reduction of *Salmonella* spp. (P-value=0.000) (w5, w7), *E.faecalis* (P-value=0.000) (w6, w7) and ϕX174 (P-value=0.000) (w7, w8) between the weeks of highest and lowest inflow concentration for respective organism, while no difference was established in the case of MS2 (P-value=0.054) (w6, w8). Tukey's test showed that reduction of *Salmonella* spp. was significant at the highest and lowest inflow concentration for all filters. The same result was established for the reduction of ϕX174. The removal of *E. faecalis* was significantly different between week 7 and 8 in all filters except biochar filters (Table 4).

Table4. Tukey's test comparing the log_{10} reduction of each pathogen and indicator organims between each filter in the highest and lowest inflow concentration

Organisms	Comparing weeks	Filter material	P-value
Salmonella spp.	5 & 7	Biochar	*
		Bark	*
		Activated carbon	*
		Mixture	*
E. faecalis	6 & 7	Biochar	0.998
		Bark	0.019
		Activated carbon	*
		Mixture	0.002
ϕX174	7 & 8	Biochar	*
		Bark	*
		Activated carbon	*
		Mixture	*

*P-value= 0.000

At the high inflow concentration of *Salmonella* spp. (Week 5) biochar performed similar to bark and mixture filters and different from activated carbon. Bark filters performance was similar to biochar and activated carbon. At low inflow concentration of *Salmonella* spp. (Week 7) all filters performed the same.

Reduction of *E. faecalis* at higher inflow concentration (Week 6) was similar in all filters but at the lower inflow concentration (Week 7) biochar and activated carbon were different from the other filters while bark and mixture were similar.

The concentration of ϕX174 was higher in week 8 and the reduction in biochar and bark filters were similar to each other and also similar to activated carbon and mixture while activated carbon and mixture were different from each other. In the week 7 where the inflow concentration was lower all filters performed similar to each other.

4.6 Pathogen and indicator organisms residence time

Over a period of 56 days filters were fed with mixture of artificial greywater spiked with high concentration of model pathogen and indicator organisms. In order to examine the pathogen and indicator organisms resident time, filters were fed for a week with only artificial greywater.

4.6.1 Salmonella spp.

No *Salmonella* spp. was found in the effluent in the final week. The average *Salmonella* spp inflow concentration in week 8 was around 7×10^{7} CFU mL^{-1}.

4.6.2 Enterococcous faecalis

The average *E. faecalis* inflow concentration from week 8 was around 10^{5} and the concentration of *E. faecalis* decreased a lot in all filters. The biochar effluent concentration was around 10 CFU mL^{-1}, except the second day which was 22 CFU mL^{-1}.The effluent from bark contained high concentration of *E. faecalis* in the first day, 342 CFUmL^{-1}, but decreased to 20 in second day and 10 for the last three days. In activated carbon filters the effluent concentration was around 10 CFU mL^{-1} in the first day but increased to 100 in the second day and then decreased to 43, 20 and 18 in the following days. In mixture filters the concentration in the first day was around 10 CFU mL^{-1} and decreased to half by time. In general the concentration of *E. faecalis* was

around 4 log_{10} lower than the average inflow concentration in week 8 (Figure 22).

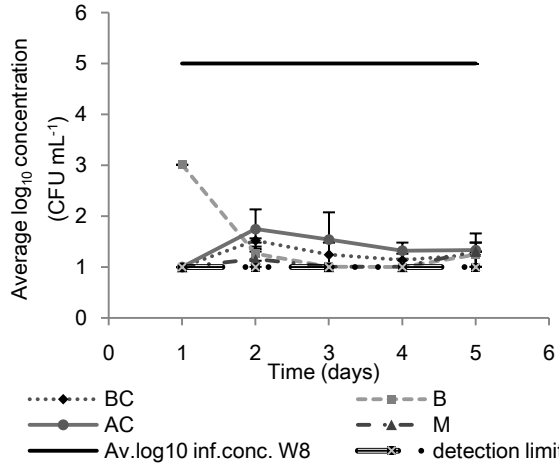

Figure 22. Average Log_{10} concentration of E. faecalis. in biochar, bark, activated carbon and mixture over the last six days of experience.

4.6.3 Bacteriophage ϕX174

The average ϕX174 inflow concentration in week 8 was around 2×10^6 PFU mL^{-1}. In effluent from biochar, the concentration of ϕX174 was around 3000 PFU mL^{-1} in the beginning of week 9 and decreased by time to 1500 PFU mL^{-1}. Concentration of ϕX174 in bark effluent was around 12000 PFU mL^{-1} in the beginning and decreased to 9000 PFU mL^{-1} in the last day. Activated carbon filters showed the lowest concentration, around 300 PFU mL^{-1} in the first day, but it increased to around 1000 PFU mL^{-1} for few days and 400 PFU mL^{-1} on the last day. In mixture filters the concentration was changing between 5000 and 10000 PFU mL^{-1}. The concentration of ϕX174 was generally between 2 and 3 log_{10} lower than average inflow concentration in week 8 (Figure 23).

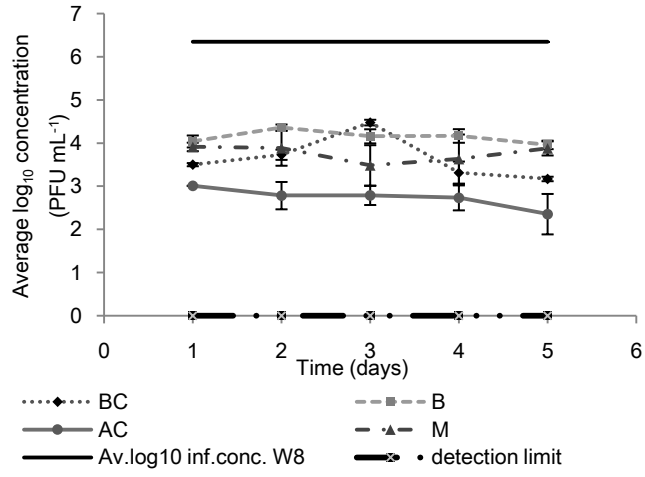

Figure 23. Average Log_{10} concentration of ϕX174 in biochar, bark, activated carbon and mixture over the last six days of experience.

4.6.4 Bacteriophage MS2

The average MS2 inflow concentration in week 8 was around 3.5×10^{7} PFU mL^{-1}. Concentration of MS2 in effluent from biochar and activated carbon was around 10^{5} in the first day and it did not decrease by time. In bark and mixture filters the concentration was around 10^{6} all the time except for the second day on which no MS2 was found in the effluent. In general the concentration of MS2 was around 2 log_{10} lower than the average inflow concentration in week 8 (Figure 24).

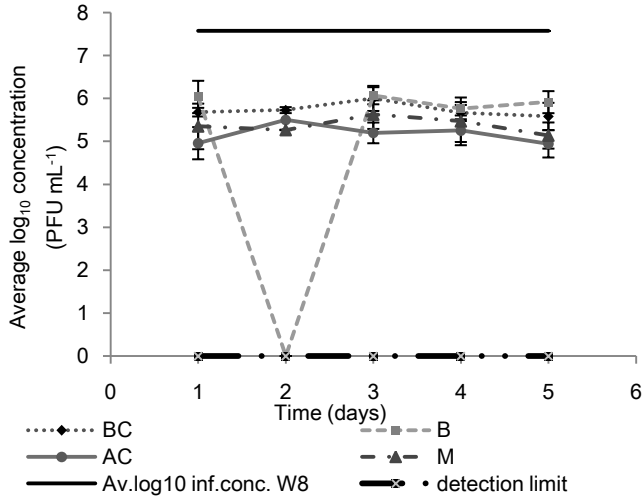

Figure 24. Average Log_{10} concentration of MS2 in biochar, bark, activated carbon and mixture over the last six days of experience.

5 Discussion

5.1 Organic loading of greywater

The organic load can affect the reduction of pathogens and indicator organisms. Adsorption of bacteria can decrease at high organic load due to the occupation of adsorption sites by organic matter (Lalander et al., 2012).

In this study, the organic load of artificial greywater was 76 g BOD_5 m^{-2} day^{-1} (240 g COD m^{-2} day^{-1} or 5 g L^{-1}). This is much higher than the organic load in Dalahmeh et al. (2012) study which was 14 g BOD_5 m^{-2} day^{-1} (890 mg L^{-1}), corresponding to what has been reported for greywater in Palestine, Jordan and Israel (Dalahmeh et al., 2012). The reduction of thermotolarant fecal coliforms in Dalahmeh et al. (2012) was higher in comparison to this study. Lens et al. (1994) study demonstrates similar results in removal of fecal coliforms and fecal streptococci at 312-410 mg COD L^{-1}. The results of Lalander et al. (2012) study revealed that reduction of *E. coli* at higher organic loads reduces in biochar and sand filters but increases in bark filters.

5.2 Filter material

Physical characteristics of filter material determine its microbial removal efficiency (Dalahmeh et al., 2012) and the physical characteristic of filter material is based on the starting organic material and also the process of carbonization or pyrolysis in case of biochar and activated carbon (Downie et al., 2009). Biochar and bark were originated from biomass (salix and pine) and activated carbon from mineral (black coal).

In comparison to sand, the most common filter material, filter materials examined in this study had lower bulk density and thus higher specific surface area which leads to higher removal efficiency. The particle density and thus bulk density, specific surface area and porosity of biochar were lower than bark and activated carbon. The water content was also very low compared to bark and activated carbon and mixture. In mixture of bark and activated carbon, bulk density was lower than the other filters, water content was high but porosity was unrealistically high (Table 1). The surface charge of the filter material particles can affect the adsorption of pathogens and indicator organisms. Biochar and bark are negatively charged material and activated carbon is positively charged material.

The effective particles size in small values provides bigger specific surface area which can lead to biofilm formation and thus higher reduction of bacteria (Dalahmeh et al., 2012). The lowest effective particles size observed in activated carbon (1.2 mm) and the highest in mixture (1.5 mm) while it was the same in biochar and bark (1.4 mm) (Table 1). Dalahmeh et al. (2012) and Lalander et al. (2012) demonstrated that bark filters efficiently remove the pathogens and indicator organisms contrariwise to this study. Bark filters studied by Lewis et al. (1995), also showed very good result in removal of MS2 and *E. faecalis*. Weak performance of bark filters studied in this project can be a result of physical parameter such as porosity, strain capacity and retention time. Lewis et al. (1995) show that the bark filters with more moist and compact material are considerably more efficient than the less compact one.

5.3 Residence time and tracer recovery

The tracer recovery and residence time show the characteristics of filter material in term of adsorption and degradation of organic matter, pathogens and indicator organisms. In longer residence time pathogens and indicators are in contact with the filter material for longer time, which can lead to bacterial biofilm formation and more bacterial reduction. The tracer recovery shows the adsorption capacity of the filter material, the lower the recovery the higher is the binding capacity (Dalahmeh et al., 2012). Tracer recovery is related to the specific surface area and surface activity of the filter media.

Biochar, bark and activated carbon achieved 100% recovery while mixture showed 25% of tracer recovery. It can be explained by the lower effective size and higher specific surface area of the filter material. Bark and biochar have

the same effective size and also the same mean residence time, activated carbon has the lower effective size among all the biofilters, while mixture has the highest effective size. In the study done by Dalahmeh et al. (2012) biochar and B bark showed 50% tracer recovery and only sand reached the 95% NaCl recovery. Biochar and bark were similarly weak in tracer recovery and it was explained by big surface area of biochar and the active surface of bark.

Activated carbon had the highest mean residence time, twice as longer as in biochar and bark. It was 90 hours for both biochar and bark at hydraulic load of 0.032 m^3 m^{-2} day^{-1}. Lens et al. (1994) reported the same results (92 h) for 0.5-m tall bark filter at different Hydraulic load (0.01 m^3 m^{-2} day^{-1}). Dalahmeh et al. (2012) reported 43 and 16 hours for 0.6-m tall bark and biochar filters in the same hydraulic load as in this study. Dalahmeh et al. (2012) and (Lens et al., 1994) demonstrated higher reduction of pathogens and indicator organims.

5.4 Pathogen and indicator organisms reduction

The mechanisms of bacterial removal is combination of biofilm formation, straining and adsorption in the filters (Dalahmeh et al., 2012, Stevik et al., 1999) while virus removal maintains by adsorption to the filter material similar to small particles (Lalander et al., 2012). A biofilm is a population of microorganisms adhered to a surface (Stepanović et al., 2004) in response to environmental signals such as nutritional condition and consist of initiation, maturation, maintenance and dissolution stages (O'Toole et al., 2000). Biofilm continue to develop as long as nutrients are available and then start to detach (O'Toole et al., 2000) but the detachment is not a well-known stage and the effect of nutrient shortage have not been investigated. Attachment of microorganisms to the media surface and formation of biofilm is driven by various parameters.

Porous media characteristics

Porous media with smaller particle size can lead to higher straining and also can offer more adsorption sites, which leads to higher pathogenic reduction (Stevik et al., 2004). Difference between the media charge and bacterial charge is another parameter to consider. However this electrostatic and van der waals forces are week and function at short distances (between the bacteria and media) and at high retention times. Presence of macropores decrease the hydraulic retention time which leads to lower adsorption (Stevik et al., 2004).

Shape of the particles can affect the attachment of bacteria to the surface, for instance Stevik et al. (2004) mentioned that small platy shape particles have higher adsorption capacity comparing to other particles.

Bacterial biofilm

Biofilm itself can increase the adsorption of bacteria by offering more adsorption sites in the biofilm (Stevik et al., 2004).

Bacterial cell surface characteristics

Presence of surface appendages *e.g.* flagella, fimbriae and pilli leads to bacterial motility, which enhances the chance of bacteria to meet a possible adhesion surface. The surface charge of the bacteria is influenced by size and pH and can also affect the adsorption onto the filter media. Number of electrostatic charge sites increase by increasing the size and may lead to higher level of adsorption. Bacterial surface of most species has negative net charge potential at pH around 7, which is higher than their isoelectric point (ISP). ISP is the pH at which surface charge changes. ISP for most bacterial species is around 1.5-4. At pH values higher than ISP, surface charge becomes more negative whereas at pH values lower than ISP surface charge becomes positive (Stevik et al., 2004). However, effect of ISP and electrostatic charges in adsorption becomes more significant at size smaller than 60 nm (ϕX174 and MS2 but not *Salmonella* spp. and *E. faecalis*) (Scot E. Dowd, 1998) .

Gram staining can influence the adsorption. Polysacharids present on the cell wall of gram positive bacteria can form hydrogen bonds and dipole-dipole interactions with media surface, which are stronger than van der Waals forces between gram negative bacteria and surface media.

Size and shape of the microorganism

Bigger surface area offers more interaction sites which leads to higher adsorption. Moreover, distance between microorganism and media decrease when the size of microorganism is bigger than the media particle size. Stevik et al. (2004) have illustrated better removal of long rod-shaped cells present in wastewater through porous media.

5.4.1 Bacterial reduction

Although both *Salmonella* spp. and *E. faecalis* are negatively charged and facultative anaerobic bacteria that form biofilm on the surfaces, reduction of *Salmonella* spp. was slightly better than *E. faecalis.*

Salmonella spp. is long rode-shaped and motile bacteria (fimbriae and pilli) which have higher chance to meet available adsorption sites whereas *E. faecalis* is cocci shaped and non-motile. On the other hand *E. faecalis* can tolerance very extreme conditions, such as starvation but *Salmonella* spp. prefer inert medium reach nutrient media for attachment (Stepanović et al., 2004). Moreover, *E. faecalis* produce extracellular antimicrobial peptides, bacteriocin, which results in efficient use of energy and inhibiting competitive bacteria (Fisher and Phillips, 2009).

In Dalahmeh et al. (2012) study $\log_{10}$ reduction of fecal coliforms was around 1.3 in biochar, similar to this study, while the $\log_{10}$ reduction in bark was 2.4, higher than this study

Salmonella spp. *reduction*

Reduction of *Salmonella* spp. in biochar filters was slightly better than other filters, 2 $\log_{10}$ reduction or higher, due to the presence of smaller particles (weight share of 1-1.4 mm particles= 50%).

Activated carbon was expected to perform much better than the other filters in term of bacterial reduction due to its highest specific area and positively charged particles. In fact, within the first week 7 $\log_{10}$ reduction of *Salmonella spp.* was observed in activated carbon, but from the third week it decreased to 1 $\log_{10}$ reduction. This is not likely to be the result of big pores since the rapid flow was not observed during the experiment. The high organic load engaging the adsorption sites is a more likely cause. Appearance of anaerobic conditions is another parameter to be considered. Filter material pores were not constantly filled with water, since the hydraulic load in this experiment was not enough to saturate the filters completely and filters were drained by gravity. Aerobic conditions in the filters prepare appropriate environment for biodegradation and nitrification, which release energy for microbial activity. Anaerobic condition leads to denitrification, which slows down the biodegradation process (Inglett et al., 2005). *Salmonella* spp. is facultative anaerobic organism which is capable to switch from aerobic

respiration to fermentation to derive energy, which means that can tolerate possible anaerobic condition.

Mixture filters, with lowest specific surface area and lower level of small particles (weight share of 1-1.4 mm particles= 50%) performed 1 $\log_{10}$ reduction within the first 30 days, similar to the bark, and 3$\log_{10}$ reduction in the following days similar to activated carbon.

Bark showed the least effect on *Salmonella spp.* removal. Although the effective size of bark was the same as biochar and porosity was similar to activated carbon, it seems that longer retention time is required in bark filters in order to efficiently remove pathogens.

E. faecalis reduction

Although *E. faecalis* are big size bacteria, their removal was similar to removal of bacteriophages.

Bark and mixture performed very weak reduction, 1 $\log_{10}$ reduction or less, which can refer to the fact that retention time was not sufficient.
In biochar and activated carbon the reduction was 4 $\log_{10}$ in the beginning but decreased to 1-2 $\log_{10}$, which can be the result of adsorption sites occupation over the time.

Apparently, *E. faecalis* biofilm formation was weak in bark filters and it was more likely removed by adsorbing similar to bacteriophages. *E. faecalis* re-growth in filters can be another considerable reason in lower reduction of *E. faecalis* (Dalahmeh et al., 2012). Although *Entrococcus* spp. concentration in the inflow was below the detection limit in Dalahmeh et al. (2012), it was detected in the effluent from biochar filters in the concentration around 100 CFU mL^{-1} due to bacterial re- growth in filters similar to this study. No *Entrococcus* spp. was found in effluent from bark filters, while performance of bark filters was weak in this study.

5.4.2 Bacteriophage reduction

Results showed lower reduction of bacteriophages in comparison to bacteria due to their small size, ϕX174: 27 nm and MS2: 27 nm (Collins et al., 2006). It means that in competition with organic particles and bacteria, bacteriophages have smaller chance to meet the adsorption sites.

Electrostatic interactions between the virus particles and the media strongly control the fate of bacteriophages in the porous media (Lalander et al., 2012). Number of electrostatic charges on the particles decrease when the size is reduced, lowering the affinity between particles and filter media. Moreover, ISP influence the attachment of small microorganisms (<60 nm) to the surface more than bigger ones (Pelleïeux et al., 2012). Bacteriophages behave as negatively charged particles at pH values higher than ISP, but become positively charged particles at pH values lower than ISP.

Bacteriphage ϕX174 ISP (6.6-6.8) is higher than MS2 ISP (3.5) (Langlet et al., 2008, Collins et al., 2006). It means that MS2 have more negative charge sites on the surface in all the filters (activated carbon> biochar> mixture> bark) while ϕX174 have less negative charge sites in three filters (activated carbon> biochar> mixture> bark). In bark filters pH was lower than ϕX174 ISP, which resulted in a change in surface charge from negative to positive, which resulted in low adsorption. Biochar, activated carbon and mixture had much higher pH values than the ISP of the bacteriophages studied in this project. In bark filters pH was close to ISP of ϕX174 but higher than ISP of the MS2. Activated carbon has positive charge and can absorb negatively charged particles. Ever since the bacteriophages could not meet their ISP they continued behaving as negatively charged particles and could adsorb better to activated carbon comparing to biochar and bark which have negatively charged surface.

Bacteriophage ϕX174 *reduction*

Reduction of ϕX174 was low in bark and mixture filters, 1 $\log_{10}$ reduction or less which can be an effect of low retention time and big distance to adsorption sites (Collins et al., 2006). Although the pH values in bark filters were lower than ϕX174 ISP which, it could not overcome the retention time limitation.

Activated carbon showed 6 $\log_{10}$ reduction in the first week, which decreased to 4 $\log_{10}$ reduction in following three weeks and 2 $\log_{10}$ reduction during the remaining experimental period. Activated carbon had positively charged particles, the highest specific surface area and more adsorption free sites, which resulted in high reduction in the beginning, but after the occupation of free adsorption sites the reduction decreased over time.

Biochar also performed higher reduction in the first week, 4 $\log_{10}$ reduction, which decreased to 2 $\log_{10}$ reduction in the second week and to 1 $\log_{10}$ reduction in the rest of experience.

Bacteriophage MS2

Low reduction of MS2 agree with other research (*e.g* Collins et al. (2006) Pelleïeux et al. (2012)). Although the reduction of MS2 was lower than ϕX174, filters followed the pattern similar to ϕX174.

Bark and mixture showed 2 $\log_{10}$ reduction and biochar and activated carbon showed 3 $\log_{10}$ reduction in the first week, which decreased by time to less than 1 $\log_{10}$ reduction in the last week.

5.5 Log reduction and inflow concentration

Statistical results showed that the concentration of the studied organisms in the inflow significantly affects the reduction of *Salmonella* spp., *E. faecalis* and ϕX174 in all filters, with one exception: reduction of *E. faecalis* in biochar filters. Higher concentration of *Salmonella* spp. and ϕX174 in the inflow led to higher reduction. Similar results were found by Bengtsson and Lindqvist (1995), Stevik et al. (2004) and Fletcher (1977). One possible reason is that higher concentration of bacteria and bacteriophage can increase the collisions between bacteria/bacteriophage and media surface result in higher adsorption. Furthermore, biofilm expanding increase the number of adsorption sites.

The reduction of *E. faecalis* decreased under higher inflow concentration. One possible reason is that biofilm formation was not the main removal mechanism in *E. faecalis* and the reduction was similar to particle adsorption on the media surface. On the other hand, *E. faecalis* enter the stationary phase (non-growth phase) and decrease the cell size under extreme condition such as lack of nutrients due to high concentration of bacteria (Portenier et al., 2003), which also could have decreased the adsorption rate.

5.6 Pathogen and indicator organisms residence time

Filters were fed with artificial greywater without any addition of pathogen and indicator organisms for the period of five days to examine the presence and survival of pathogens and indicator organisms in the filters.

No *Salmonella* spp. was detected in the effluent of any of the biofilters, which illustrates that biofilm did not reach the detachment stage within the experimental time. Nonetheless, the pathway of bacterial detachment from the surface is not known and requires further investigation.

Concentration of *E. faecalis* was very low in effluent form all filters; especially bark filters mainly due to the trapping than microbial or chemical processes.

Bacteriophages found in higher concentration than bacteria and among the bacteriophages, MS2 detected in higher concentration, especially in bark filters. At current hydraulic load bacteriophages appeared to detach from the surface, most likely due to their weak bonds with the media.

5.7 Reuse possibilities of filtered water

Activated carbon filters could provide the requirements for restricted irrigation while effluent from other biofilters can be used only for subsurface irrigation.
Activated carbon filters could reach the requirements for unrestricted irrigation considering bacteria and ϕX174 removal. However, since the reduction of MS2 was lower than standards for root crops and low growing crops, it can be applied for leaf crops and high growing crops.

Considering the installation and maintenance, aeration system should be provided to avoid anaerobic conditions. Furthermore, the results of feeding the columns with tap water illustrated that under rainy conditions filters would not release considerable concentration of bacteria. However, concentration of ϕX174 can increase close to the limit values and concentration of MS2 could exceed the limits. Preparing protection wall around the system can help to avoid the release of pathogens to the water bodies.

6 Conclusion

This study contributes with further knowledge of the aerobic treatment's efficiency in removal of pathogen and indicator organisms from greywater.

The study demonstrated that physical and chemical characteristics of the filters affect the reduction of studied microorganisms. It was found that biochar and activated carbon filters were slightly better than bark and mixture filters in removing the studied microorganisms. However, the characteristics of the pathogen and indicator organisms appeared to influence the reduction to a greater extends than the filter media properties. Little to no reduction of bacteriophage MS2 (0-0.5 $\log_{10}$) was observed in all four filter media, while *Salmonella* spp., *E. faecalis* and ϕX174 were reduced by 1-2 $\log_{10}$. It was also observed that the reduction of *Salmonella* spp. and ϕX174 correlated to the inflow concentration in all filter media.
It was also found that viruses were released from the filters for several days after the last feeding, while bacteria were not. There is thus a risk that viruses are released from this type of filters long after a virus related outbreak.
Although the treatment proved to reduce the pathogen and indicator organisms to certain extend at the given hydraulic and organic loading rate, the intended use of the treated greywater is the key factor when considering the minimal reduction required in the treatment.
The four filter materials studied would provide water suitable for subsurface irrigation, while only water treated in activated carbon filters would meet the WHO requirements for restricted irrigation.

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SLU
Institutionen för energi och teknik
Box 7032
750 07 UPPSALA
Tel. 018-67 10 00
www.slu.se/energyandtechnology

SLU
Department of Energy and Technology
Box 7032
S-750 07 UPPSALA
SWEDEN
Phone +46 18 671000