Wastewater Treatment in Greenland

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PhD Thesis

Department of Civil Engineering

2012

DTU Civil Engineering Report R-265 (UK)
May 2012

DTU Civil Engineering
Department of Civil Engineering

Preface

This thesis is submitted for the degree of Philosophiae Doctor at the Technical University of Denmark. The focus of the thesis has been to increase the knowledge on wastewater treatment under Arctic conditions with focus on small communities.

The project was carried out from December 2007 until April 2012 and was funded by The Technical University of Denmark (DTU).

The work has been conducted at The Arctic Technology Centre (ARTEK), Department of Civil Engineering, DTU; Division of Epidemiology and Microbial Genomics, National Food Institute, DTU; Department of Chemical and Biochemical Engineering, Center for BioProcess Engineering, DTU; Department of Plant and Environmental Sciences, Norwegian University of Life Sciences (UMB), Ås, Norway. Field work was carried out in Qeqqata municipality in W-Greenland. The supervisors were professor Arne Villumsen (ARTEK), researcher Pernille Erland Jensen (ARTEK) and professor Petter Deinboll Jenssen (UMB).

Kongens Lyngby, April 2012

Ragnhildur Gunnarsdóttir

Tileinkað Hirti, Önnu Dúfu og Gunnari, og mömmu og pabba

Acknowledgements

Many people have supported and assisted me during my time as a PhD student. I would like to thank them all for their help and contribution. I would like to give special thanks to:

- My great team of supervisors, Arne Villumsen, Pernille Erland Jensen and Petter Deinboll Jenssen, from whom I have learned so much. Thank you for your consistent support and advice throughout the project.
- My colleagues at ARTEK for a very nice and inspiring working environment. Special thanks to my good friends at the office, Janne Fritt-Rasmussen and Louise Josefine Belmonte, as well as Gunvor Marie Kirkelund. It has been a privilege to work with you all.
- The laboratory technicians at ARTEK for their assistance, especially Malene Møller for her help with laboratory work.
- The employees at DTU-FOOD, the Division of Epidemiology and Microbial Genomics, National Food Institute, Technical University of Denmark. First of all I would like to thank Karoline Müller for her assistance, inspiring discussions and always good advice. I would also like to thank professor Frank Møller Aarestrup, research manager, for putting laboratory and lab-equipment at my disposal. Eliza Maria Bielak also deserves my acknowledgement. Finally, I would like to thank the laboratory technicians at DTU-FOOD for their assistance and good advice, especially Hanne Mordhorst, Jacob Dyring Jensen and Inge Marianne Hansen.
- Jens Ejbye Schmidt, Head of Programme, Center for BioProcess Engineering (BIOENG), DTU-Chemical Engineering, Technical University of Denmark, for providing access to laboratory facilities- and equipment.
- Stefan Heiske for an inspiring cooperation at Risø and many good discussions.
- Andreea Oarga for collaboration in laboratory experiments at UMB
- Jon Frederik Hanssen, microbiologist at the University of Life Sciences in Ås, Norway, for his good advice concerning laboratory work and supervision during one of my special courses.
- The employees at NILU in Tromsø, Norway, for an inspiring research stay. A special acknowledgement goes to Morten Moe.
- The participants of the NORA project, especially Karen Erla Erlingsdóttir and Þórhallur Þorsteinsson at the Tourist Association in West Iceland.
- The employees of Qeqqata municipality. Special thanks to director Laust Løgstrup, Lone Kristensen and Erik Lomholt-Bek.
- Rasmus Nielsen for assistance during field work in Qeqqata Kommunia
- Laarseeraq Skifte for guiding and assistance during field work in Qeqqata municipality
- The Technical University of Denmark for funding of the PhD project.
- My friends and family, especially my parents for always believing in me and being proud of me.
- My husband for his endless support and good advices - Ég hefði aldrei getað þetta án þín, Hjörtur minn.

- Last, but absolutely not least, my two wonderful children for unknowingly being my biggest support and joy at all times.

List of papers

This thesis consists of a synopsis and three scientific journal papers; one scientific review paper and two papers presenting results of laboratory experiments. A fourth research paper is being prepared for publication with the author of this thesis as a co-author. The manuscript is not included in appendix but instead some of the central results in the manuscript are incorporated into the thesis. In the synopsis the papers included in the thesis are referred to by their roman number, e.g. as "Paper II". Other papers and reports by the authors, which have not been included, are referred to by the Harvard method, e.g. “Johnson et al., 2011”.

I Gunnarsdóttir, R., Jenssen, P.D., Jensen, P.E., Villumsen, A., Kallenborn, R., 2012. A review of wastewater handling in the Arctic with special reference to Pharmaceuticals and Personal Care Products (PPCPs) and microbial pollution, 2012. Accepted for publication in Ecological Engineering. DOI: 10.1016/j.ecoleng.2012.04.025.

II Gunnarsdóttir, R., Müller, K., Jensen, P.E., Jenssen, P.D., Villumsen, A., 2012. Effect of long-term freezing and freeze/thaw-cycles on indigenous and inoculated microorganisms in dewatered blackwater. Submitted to Environmental Science and Technology.

III Gunnarsdóttir, R., Heiske, S., Jensen, P.E., Schmidt, J.E., Jenssen, P.D., Villumsen, A., 2012. Effect of anaerobiosis on indigenous microorganisms in blackwater with fish offal as co-substrate. Manuscript for Water Research.

Manuscript in progress which has not been included in the thesis

[A] Oarga A., Hanssen J.F., Jenssen P. D., Gunnarsdóttir R., Bulc G.T., 2012. Blackwater solids compost with selected (organic) bulk materials. Manuscript in progress.

Abstracts and papers by the author presented at international conferences which have not been included in the thesis

[B] Gunnarsdóttir, R., Jørgensen, M.W., 2008. Utilization possibilities of waste products from fishing and hunting to biogas and bio-oil production in Uummannaq County. A paper presented at the conference ARTEK Event, 2008, Sisimiut, Greenland, March 1-3 2008.

[C] Gunnarsdóttir, R., Ingeman-Nielsen, T., Jenssen, P.D., Villumsen, A., 2009. Tourist cottages in the Arctic: Possibilities of wastewater treatment. A paper presented at the conference ARTEK Event, 2009, Sisimiut, Greenland, August 11-13 2009.

[D] Gunnarsdóttir, R., Jenssen, P.D., Nyborg, I., Kallenborn, R., Villumsen, A., Jensen, P.E., 2010. Environmental and social benefits of improved handling and disposal of black wastewater in Greenland. An abstract presented at the conference Arctic Frontiers 2010: Living in the North, Tromsø, Norway, January 24-29 2010.

[E] Gunnarsdóttir, R., Jenssen, P.D., Hanssen, J-F, Villumsen, A., Jensen, P.E., Kallenborn, R., 2011. Sanitation in the Arctic-Challenges and solutions. An abstract presented at the conference SmallWat11: Wastewater in small communities, Sevilla, Spain, May 2-4 2011.

Reports by the author which have not been included in the thesis

[F] Gunnarsdóttir, R., Jørgensen, M.W., 2008. Affaldshåndtering i Grønland: Status på affaldshåndtering i 10 grønlandske kommuner, september 2008. Byg Rapport R-190. ISBN: 9788778772664.

[G] Gunnarsdóttir, R., Jenssen, P.D., Villumsen, A., Jensen, P.E., Erlingsdóttir, E., Jóhannesson, H., and other participants in the NORA project Sanitet i turisthytter, 2011. Sanitet i turisthytter: Afsluttende rapport, 17. juni 2011. Available on NORAs homepage (April 27 2012): http://www.nora.fo/files/13/20120103140213454.pdf

Abstract

The Arctic nature is vulnerable to environmental contaminants because of low biological diversity, lack of nutrients and extreme seasonal variations in light. In Greenland neither industrial nor domestic wastewater is treated before it is discharged to the recipients, which in most cases is the sea. Wastewater contains a variety of substances, including anthropogenic pollutants, residues of pharmaceuticals and personal care products (PPCPs), pathogenic microorganisms and parasites as well as antibiotic resistant bacteria that can be harmful for the environment as well as human health. Due to the vulnerability of the Arctic nature, the direct release of untreated sewage may have severe consequences for the receiving aqueous environment. With increasing populations in the Arctic communities and an increased demand to the level of comfort, it becomes even more vital to improve the status of wastewater treatment in these regions. However, designing, constructing and operating wastewater collection systems in the Arctic is challenging because of e.g. permafrost conditions, hard rock surfaces, freezing, limited quantity of water and high costs of electricity, fuel and transportation, as well as a settlement pattern with limited accessibility, particularly in the rural parts of the Arctic. For those reasons bucket toilets are still used in parts of the towns and in almost all settlements in Greenland. This particular toilet solution has been considered a problem for many years with respect to uncontrolled spreading of nutrients, diseases and potential pollution issues. Due to the above mentioned challenges alternative treatment methods are needed, especially in small and remotely located communities. Decentralized solutions are well suited for Greenland. Ideal solutions should reduce the need for expensive collection systems, and be more economically and environmentally sustainable than traditional wastewater collection and treatment systems. Possible alternative wastewater treatment methods for Greenlandic communities are dry composting or anaerobic digestion of excreta, collected at household level using dry or water saving toilets. This opens up for co-treatment of organic waste fractions. Freezing and thawing has also been recognised as being a cost-effective wastewater treatment method in cold regions. Thus it was chosen to concentrate on the effect of the mentioned processes, namely freezing, anaerobic digestion and composting, in this PhD project, focusing on their hygienic effect.

Laboratory experiments were conducted to test the effect of the selected processes on inoculated and indigenous microorganisms in blackwater. In the first laboratory experiments the effect of long-term freezing and repeated freezing and thawing on inoculated and indigenous microorganisms in dewatered blackwater was analyzed. The results indicated that freezing has a lethal effect on some microbial groups, such as coliforms, and sublethal on others, e.g. *Salmonella.* Other microorganisms, like faecal streptococci and coliphages, showed a limited reduction during the long-term freezing. Repeated freezing and thawing did, however, have an enhancing effect on both coliphages and amoxicillin resistant enteric bacteria.

The effect of anaerobiosis on selected indigenous microorganisms and microbial groups in blackwater during mesophilic anaerobic digestion, using fish offal as co-substrate, was investigated. The selected microorganisms and microbial groups were all reduced substantially during the experiment, and the overall results indicated that the anaerobic environment might not be the main cause of reduction of some of the microorganisms, but rather competition with active methanogenic bacteria and carbon limitation.

The third laboratory experiments analyzed the effect of composting of dewatered blackwater with different bulking materials. Even though the temperature profiles of the different composting mixtures did not reach thermophilic conditions the reduction of both *Escherichia coli* and faecal streptococci was substantial.

None of the tested processes had the ability to completely hygienize the blackwater, but some of the microorganisms and microbial groups were reduced strongly during the laboratory experiments. Other factors also play a role when selecting a suitable treatment method, e.g. operational and maintenance cost. Combining the processes might enhance the microbial reduction. One recommendation for the settlements is to combine composting and natural freezing or alternating freezing and thawing. Another alternative could be to use small and simple biogas plants, followed by dewatering of the degassed biomass, either by utilizing possible surplus of energy from the biogas plant or natural freezing, which might be a more cost-effective way. After dewatering the liquid part can be treated by filtration and the fibre part can be composted. These combinations of relatively simple processes have the possibility of a good microbial reduction. In the non-seweraged parts of the towns, the same combination could be utilized, but more advanced biogas plants could also be used, for instance with additional heat treatment, even by utilizing waste heat from the waste incinerators. For the seweraged parts of the towns it might be most beneficial to maintain the flush toilet solutions, while introducing a treatment step prior to discharging to the recipient, such as simple mechanical treatment which might even be followed by further treatment, e.g. chemical precipitation or for smaller systems, sand filtration.

Resumé

Den arktiske natur er sårbar overfor miljømæssig forurening på grund af begrænset biologisk mangfoldighed, mangel på næringsstoffer og ekstreme årstidsbestemte variationer i lys. I Grønland bliver hverken industrielt spildevand eller husholdningsspildevand renset inden udledning til recipienterne, som i de fleste tilfælde er havet. Spildevand indeholder diverse miljøfremmede substanser, inklusiv medicinrester og kosmetiske produkter, samt patogener, parasitter og antibiotika resistente bakterier, som kan være skadelige for miljøet og menneskelig sundhed. På grund af den arktiske naturs sårbarhed kan udledning af urenset spildevand have alvorlige konsekvenser for det marine miljø. En voksende befolkning i de arktiske lande og et øget krav om komfort, gør det nødvendigt at forbedre spildevandsbehandling i disse lande. Det er dog udfordrende at designe, konstruere og operere spildevandssystemer i Arktis på grund af f.eks. permafrost, klippe undergrund, frysning, begrænsede mængder af ferskvand, høje priser på elektricitet, brændstof og transport, samt en spredt bebyggelse, især i de små samfund. På grund af dette bruges såkaldte "posetoiletter" (tørklosetter) stadigvæk i dele af byerne og næsten alle bygder i Grønland. Denne toiletløsning har i mange år været betragtet som et problem, både på grund af sygdomme samt eventuelle forureningsproblemer. Alternative behandlingsmetoder til spildevand er derfor nødvendige, især i de små bygder. Decentrale løsninger kan f.eks. være et passende alternativ for Grønland. Ideelle løsninger bør reducere behovet for dyre rørledninger, og være bæredygtige, både økonomisk og miljømæssigt. Mulige løsninger for de grønlandske samfund er at bruge tørre eller vandsparende toiletløsninger, og behandle det sorte spildevand ved tørkompostering eller bioforgasning. Begge metoder muliggør at inkludere andre organiske affaldsfraktioner i behandlingen. Frysning og tøning kan også være en omkostningseffektiv behandlingsmetode i lande med koldt klima. Det blev derfor besluttet at fokusere på effekten af disse processer, nemlig frysning, kompostering og bioforgasning, i det pågældende ph.d. projekt, med fokus på den hygiejniske effekt af processerne.

Effekten af de valgte processer på både inokulerede og naturligt tilstedeværende mikroorganismer i sort spildevand blev analyseret ved laboratorieforsøg. I de første forsøg blev effekten af langtidsfrysning og skiftevis frysning og optøning på inokulerede og naturligt tilstedeværende mikroorganismer i afvandet sortvand analyseret. Resultaterne indikerede en dødelig effekt af frysningen på nogle mikrobiologiske grupper, f.eks. koliforme, og begrænset dødelig effekt (Engelsk: Sublethal) på andre, f.eks. *Salmonella.* Andre mikroorganismer, såsom fækale streptokokker og fager, viste en begrænset reduktion under langtidsfrysning. Skiftevis frysning og optøning havde en ekstra effekt på både fager og amoxicillin resistente tarmbakterier.

Formålet med de næste laboratorieforsøg var at analysere effekten af anaerobt miljø på udvalgte mikroorganismer og mikrobielle grupper i sortvand, under mesofil samudrådning med fiskeaffald. De

udvalgte mikroorganismer viste alle en betydelig reduktion under forsøgene. Overordnet set indikerede resultaterne at hovedårsagen til reduktionen af mikroorganismer ikke nødvendigvis var det anaerobe miljø, men snarere konkurrence med aktive metanogener og kulstof begrænsning.

Under de tredje laboratorieforsøg blev effekten af kompostering af afvandet sortvand med forskellige tilsætningsmaterialer analyseret. Selvom temperaturudviklingen under komposteringen ikke resulterede i termofile temperaturer blev *Escherichia coli* og fækale streptokokker reduceret betydeligt.

Ingen af de testede processer resulterede i en fuldstændig hygiejnisering, men nogle af mikroorganismerne blev dog kraftigt reduceret under laboratorieforsøgene. Andre faktorer spiller også en rolle når en passende behandlingsmetode skal vælges, f.eks. drifts- og vedligeholdelsesomkostninger. En anbefaling for bygderne er at kombinere kompostering og frysning eller skiftevis frysning og tøning. Et alternativ kunne være at bruge små og simple biogas anlæg, efterfulgt af afvanding af den afgassede biomasse, enten ved at udnytte overskudsenergi fra biogas anlægget eller naturlig frysning, som kunne være mere omkostningseffektivt. Efter afvanding kan væskedelen behandles ved filtration mens fiberdelen kan komposteres. Disse kombinationer af relativt simple processer har muligheden for en god mikrobiel reduktion. I de ikke-kloakerede dele af byerne kan samme kombinationer benyttes, eventuelt med mere avancerede biogas anlæg, hvor der for eksempel kan tilføjes yderligere varmebehandling, hvor spildvarme fra affaldsforbrændingsanlæggene endda kan udnyttes. For de kloakerede bydele kan det være mest hensigtsmæssigt at beholde de vandskyllende toiletter, men at behandle spildevandet inden udledning til recipienten. Dette kan f.eks. gøres ved simpel mekanisk rensning, eventuelt efterfulgt af videre behandling, såsom kemisk fældning, eller for mindre systemer, ved sandfiltrering.

Eqikkaaneq

Issittumi pinngortitamiittut uumassusillit kinguaassiorsinnaaneri inuussutissartaqarsinnaasullu killeqaqimmata, taamatuttaaq qaamarngup ukiup ingerlanerani nikerartarnerujussua peqqutaalluni pinngortitaq qajannartorujussuuvoq. Kalaallit Nunaanni suliffissuaqarfinnit illuniilluunniit imeq maanngaannaq amerlanerpaatigut imeqarfinnut, immamut salinneqaqqaarani kuutsiinnarneqartarpoq. Imeq maanngaannaq kuutsinneqartoq akuutissanik, nakorsaatit sinnikuinik, pinnersaatit sinnikuinik, napparsimalersitsisinnaasunik, bakterianik antibiotikamut akiuussinnaasunik pinngortitamut inunnullu peqqissutsikkut akornuteqalersitsisinnaasunik akoqartarpoq. Issittumi pinngortitap qajannartuunera peqqutaalluni imermik salinneqanngitsumik maanngaannaq kuutsitsineq immami uumassusilinnut annertuumik akornutaalersinnaavoq. Issittumi inuit amerliartortillugit atukkallu pitsaanerunissaanik piumasaqaataasartut annertusiartortillugit, erngup maanngaannaq kuutsinneqartarnerinut nunani pineqartuni pitsanngorsaasoqarnissaa pisariaqarpoq. Issittarnerujussua, qaarsoqarpallaarnera, nunap qerisarnera, imermik tarajoqanngitsumik soqarpiannginnera, kallerup innerata, ikummatissat assartuinerullu akisuallaarneri, taamatuttaaq inuiaqatigiinnguit ikittunnguit isorartoorujussuarmi najugaqartitertut eqqarsaatigalugit issittumi imeqarfinnut saliinermut atortorissaarutinik sanaartornissaq inerisaanissarlu unammillernartorujussuuvoq. Taamaammat Kalaallit Nunaanni ”anartarfiit igittakkat” (perusuersartarfiit kuutsittagaanngitsut) illoqarfinni ilaatigut nunaqarfimmiunillu tamangajanni suli atorneqartarput. Anartarfeqartitseriaaseq taamaattoq nappaatit mingutsitsinermut ajornartorsiutit peqqutaallutik ukiorpassuarni ajornartorsiutitut isiginiarneqartarsimapput. Taamaammat erngup maanngaannaq kuutsinneqartarneranut iluarsiissutaasinnaasunik, annermik nunaqarfeeqqani nassaartoqartariaqarpoq. Siamasissumik iluaqutissaasinnaasunik pilersitsiortorneq Kalaallit Nunaannut iluaqutaasinnaavoq. Isumassarsiat pitsaasut makkuusinnaasariaqarput, kuuffinnik akisuunik pisariaqartitsinerup annikillisinnissaa taamalu aningaasaqarniarnikkut pinngortitarlu eqqarsaatigalugu atorneqarsinnaasut. Kalaallit Nunaanni inuiaqatigiinnut sipaarutaasinnaasutut isikkoqartut makkuupput, anartarfiit kuutsittagaanngitsut imaluunniit imermik sipaarutitallit taamatuttaaq anartalinnik naggorissaatitut atugassanngortitsisarneq imaluunniit biogassi atorlugu ikummatissaliorneq. Periutsit marluk atornerini eqqagassat suliarinerinut atatillugu uumassusillit ilanngullugit atugassanngortinneqarsinnaanissaat ajornarunnaarsinneqarsinnaavoq. Nunani issittuni qerisitsisarneq panertunillu naggorissaatissanik atugassanngortitsineq aningaasaqarniarnerup pitsanngorsarniarnissaanut atorsinnaagaanni iluaqutaasinnaapput. Taamaammat atortussiat qanoq sunniuteqarsinnaanersut, tassa qerisitsisinnaaneq, naggorissaatissatut atugassanngortitsisinnaaneq taamalu biogassi atorlugu ikummatissaliorsinnaaneq, Ph.D-nngorniarnermut atatillugu projektiliornermi atueriaatsit taamaattut mingutsitsinnginnersut isiginiarneqarput.

Pinngortitaassutsimikkut uumasuaqqat tappiorannartut pioreersut imaluunniit tigusilluni ilanngussukkat qanoq sunnerneqarsinnaanersut paasiniarlugit, laboratoriani misissuinerit ingerlanneqarsimapput.

Misileraanermi siullermi tappiorannartut pioreersut aamma misissugassat tigooqqakkat imermit qernertumit tigoorariarlugit misissorneqarput. Misissukkat inernerisa ilaani malunnaarsivoq qerititsisarnerup sunniutai uumasuaqqat ilaannut soorlu koliformenut ulorianartortaqartoq. Samonellamut toqunartuusinnaanera killeqarneruvoq (Tuluttoorlugu: Sublethal). Uumasuaqqanik sivisuumik qerititsinermi soorlu bakteriat annaniittut inuussutissartaallu ikiliartorsinnaaneri killeqartut paasineqarpoq. Inuussutissartanut bakterianullu inaluarsuaniittunut amoxicillinimik nungusarneqarsinnaanngitsut nungusaaniarnermi atussallugit qerititsisaqattaarneq aatsitsisaqattaarnerlu pitsaalluinnartuupput. Laboratoriani tulliani misileraanissami uumasuaqqat uumasuaqqallu mikinerpaat aalajangersimasut, tassa aalisakkat igitassartaaniittunut imermiittumut qernertumiittumut ilagitillugit gassiliuutsinnaveersaarlugillu misissorneqarnissaat siunertaavoq. Taamatuttaaq uumasuaqqat immikkoortinneqarsimasut tamarmik misileraanerup nalaani malunnartumik ikileriaateqangaatsiarput. Ataatsimut isigalugu, uumasuaqqanik ikileriaateqarnermut peqqutaanerpaasoq tassaanngilaq silaannarmik pisariaqartitsineq kisiannili metanitalimmik kulstoffitalimmilluunniit silaannaliorsinnaasut killeqarneri peqqutaanerupput.

Laboratoriani pingajussaanik misileraanerup nalaanni akuutissat assigiinngitsut imermit qernertumeersut naggorissaatissatut suliarigaanni qanoq sunniuteqarsinnaanersut misissorneqarput. Naak naggorissaasiornermi kissassuseq naleqqussarneqanngikkaluartoq, *Escherichia coli* taamatuttaaq annani bakteriat (streptokokker) malunnartumik ikileriaateqarput.

Misileraanerit tamarmik tamakkiisumik eqqiluisaartitsinissamut periusissanik atorneqarsinnaasunik nassaarfiunatik taamaattorli laboratoriani misileraanerit nalaanni uumasuaqqat ikileriangaatsiarput. Eqqortumik suliarinninnissamut pissutsit allat apeqqutaalluinnarsinnaapput, soorlu ingerlatsinermut-aserfallatsaaliuiniarnermullu aningaasaqarniarneq. Nunaqarfinnut innersuussutigineqarsinnaasoq unaavoq, paarlakaajaattumik qerititsisarlunilu naggorissaasiortarnissaq imaluunniit qerititsisarneq aatsitsisarnerlu. Aaqqiissutaasinnaasoq allaanerusoq tassaasinnaavoq biogassiliuuteerannguanik atoruminartunik atuineq. Nukiit biogassiliuutinit sinneruttut imaluunniit issittumi qerititsisarnerit atorluarnerisigut aningaasartuuterpassuarnut sipaarutaasinnaapput. Erngup kuutsinneqareernerata kingorna sorujuiaasoqassaaq kingornalu ipat naggorissaatissanngorlugit suliarineqarsinnaalissapput. Periutsit ajornanngitsunnguit ataqatigiisinnerisigut uumasuaqqat ikileriartorluartinneqarsinnaapput. Illoqarfinni illuni kuuffilersugaanngitsuni ataqatigiisitsinerit taamaattut atorneqarsinnaallutillu biogassiliuutit atortorinnerusunik tassa kissassuseq atorlugu atortorissaarutinik suliarinninnissamut atorneqarsinnaasunik pilersorneqarsinnaapput. Taamatuttaaq eqqakkanit ikuallaavinnit kissarneq maanngaannartartoq atorluarneqarsinnaavoq. Illoqarfinnili kuuffilersorneqarsimasuni anartarfiit kuutsittakkat atatiinnarneqarunik taamatuttaaq imeq maanngaannartitassap immamut anngutinnginnerini salinneqaqqaartarnissaa tulluarnerpaavoq. Tamanna maskiinat atorlugit kingornalu suliareqqiineq soorlu akuutissat atorlugit katagartitsisarnikkut imaluunniit minnerusunik soorlu sioqqanut sukuiaatit atornerisigut anguneqarsinnaavoq.

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Chapter 1 Introduction

1.1 Background

Engineering challenges in the Arctic

The Arctic nature is vulnerable to environmental contaminants because of low biological diversity, lack of nutrients and extreme seasonal variations in light (AMAP, 2009). There are different ways of defining the Arctic boundaries, one of them being that the Arctic represents the Nordic polar areas, demarcated in the South by the Polar Circle (66.32°N). Another definition is that a region belongs to the Arctic if the mean temperature in the year's warmest month, July, is below 10°C (figure 1.1). Regardless of the definition used, the area is characterized by a cold climate with extreme seasonal variations in both light and temperature. In the summer the average temperature is approximately 0 to 2°C and it is light day and night, while during winter the average temperature falls to -30 to -35°C, with no sunlight in the middle of the winter (AMAP, 1997) .

Figure 1.1. The Arctic regions defined by an average temperature in July below 10°C (Arctic Studies, 2012).

Arctic engineering is in many ways challenging and solution possibilities limited by the cold climate and other factors that are different from more temperate zones. In many Arctic regions, wastewater treatment is inadequate or even completely lacking. Wastewater contains a variety of substances, including anthropogenic pollutants (Eriksson et al., 2002), metals (Palmquist and Hanæus, 2005), residues of pharmaceuticals and personal care products (PPCPs) (Kallenborn et al., 2008; Vasskog et al., 2009), pathogenic microorganisms and parasites (Bitton, 2005) as well as antibiotic resistant bacteria (Batt and Aga, 2005), that can be harmful for the environment as well as human health. Due to the vulnerability of the Arctic nature, the direct release of untreated sewage may have severe consequences for the receiving aqueous environment (Kallenborn et al., 2008, Bergheim et al., 2010; Bach et al., 2009 and 2010). With increasing populations in the Arctic communities it becomes even more vital to improve the status of wastewater treatment in these regions. However, designing, constructing and operating wastewater collection systems in the Arctic is challenging because of permafrost conditions, hard rock surfaces, freezing, flooding in the spring, limited quantity of water and high costs of electricity, fuel and transportation, as well as a settlement pattern with limited accessibility, particularly in the rural parts of the Arctic (Paper I).

Wastewater handling in Greenland

Greenland is the world's largest non-continental island (2 166 086 km^2), located on the North American continent between the Arctic Ocean and the North Atlantic Ocean, north east of Canada (Statistics Greenland, 2012). Cape Morris Jesup is the northernmost point of Greenland, only 740 km from the North Pole, while the southernmost point is Cape Farewell which is at about the same latitude as Oslo in Norway (Statistics Greenland, 2012). The climate is Arctic to subArctic with cold winters and cool summers, in which the mean temperature normally does not exceed 10°C (Statistics Greenland, 2012). Because of the milder climate in South Greenland agriculture is practiced in this part of the country. Fishery is still the main industry in Greenland, shell fishery the largest one and Greenlandic halibut fishery the second largest (Statistics Greenland, 2012). In 2012 the population of Greenland was 56 749 (Statistics Greenland, 2012) whereof 8 517 lived in small settlements (Statistics Greenland, 2012). There are long distances between the towns and settlements in Greenland, which is shown in figure 1.2, but no roads to connect the communities. Passengers and supplies of goods are therefore transported by sea or by air.

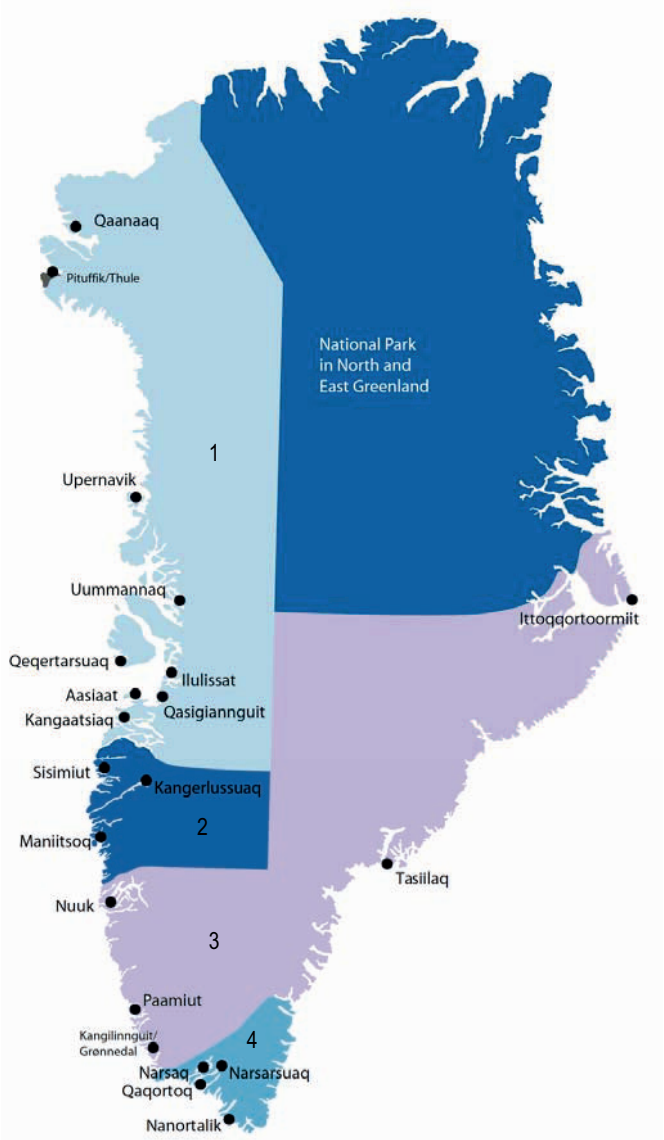


Figure 1.2. Greenland (Greenlandic name: Kalaallit Nunaat), and the largest towns and settlements (Statistics Greenland, 2012). The numbers indicate the four municipalities: 1) Qaasuitsup, 2) Qeqqata, 3) Sermersooq, 4) Kujalleq.

In Greenland neither industrial nor domestic wastewater is treated before it is discharged to the recipients, which in most cases is the sea (Paper I). The volume of wastewater from each dwelling depends on the water supply system. The main options for drinking water systems in Greenland are self-haul systems, vehicle-haul systems and piped systems. Households with self-haul drinking water

systems produce much less wastewater than those on piped water (Smith and Low, 1996). In the towns the residents have pressurized in-home drinking water. The dwellings have either traditional water flush toilets or bucket toilets. Those who have water flush toilets in the larger towns are either connected to a sewer or the blackwater is stored in a holding tank outside the residence while the greywater is discharged directly to the terrain. In the small settlements of Greenland some dwellings have pressurized in-home drinking water, in some only during the summer, while other residents have self-hauled water, typically obtained from a community water point. Bucket toilets are still used in parts of the towns in Greenland and in almost all settlements. This particular toilet solution has been considered a problem for many years with respect to uncontrolled spreading of nutrients, diseases and potential pollution issues. It has been reported that the potential for natives in Alaska to contract hepatitis A and other diseases is unacceptably high in villages where bucket toilets are still used and water supply for excrement processing as well as for human consumption is limited (U.S. Congress, 1994). Furthermore the relationship between inadequate sanitation and higher rates of respiratory tract infections (U.S. Department of Health and Human Services, 2006; Gessner, 2008; Hennessy et al., 2008), skin, and gastrointestinal tract infections (Hennessy et al., 2008) has been documented among rural Alaska natives. Outbreak of epidemics of other diseases, such as impetigo, bronchitis, serious ear infection, meningitis as well as hepatitis A and B in remote Alaskan communities is often ascribed to poor sanitary facilities (U.S. Congress, 1994). Since the sanitary situation in the settlements of Greenland and parts of the towns is similar to the remote Alaskan communities, it is not unreasonable to presume that this might be valid for Greenland as well. Routine collection of the bags from the bucket toilets and pumping of the holding tanks is organized by the municipalities or local companies. In some towns and settlements there are automatic machines for emptying of the toilet bags but technical problems with those have occurred in several places, making it necessary for the workers to empty the bags manually (Gunnarsdóttir and Jørgensen, 2008). Individual hauling is also done in some settlements, in some cases to the ground, resulting in e.g. odour problems (Gunnarsdóttir and Jørgensen, 2008). This kind of contact to blackwater can pose a health risk for the population, especially children and weakened individuals, and the health and convenience level is in general considered being low when hauling is done individually due to limited water usage and varying individual disposal practices (Smith and Low, 1996). Those factors are improved when the hauling is done collectively by municipal or private organized operators. However, the conditions for emptying the toilet bags, whether done individually or by municipal or private organized operators, have in general been seen as being unhygienic in many localities in Greenland (Gunnarsdóttir and Jørgensen, 2008). The present sanitation solutions in Greenland are shown in figure 1.3.

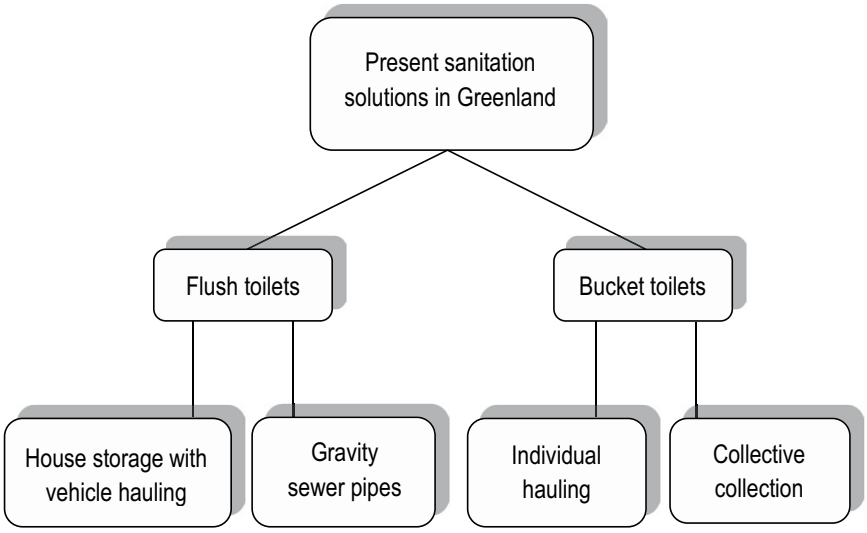


Figure 1.3. Present sanitation solutions in Greenland.

In 2004-2005 a cooperation project was carried out between the Danish Environmental Protection Agency (Danish EPA), the Greenlandic environment and nature directorate, KANUKOKA (the national association of Greenlandic municipalities), three Greenlandic municipalities and the consultative engineering firm COWI. The aim of the project was to investigate the effects of blackwater discharging on the recipients in Greenlandic towns and settlements. The main results were that the disposal of organic matter and nutrients (nitrogen and phosphorus) was not of great concern in Greenland when discharged directly to the sea due to low population density and large receiving water bodies. However, if the water exchange in the recipient is poor those substances can deteriorate the quality of the marine environment and even cause eutrophication (Danish EPA, 2005). It has been observed that visual contamination caused by discharging of wastewater in Greenland, such as turbidity in the water phase and larger particles, occurs by some outlets (Danish EPA, 2005). Other substances in the wastewater, such as heavy metals and xenobiotic substances might pollute the recipients but since no regular measurements of the concentrations of those substances in the Greenlandic wastewater streams are done it cannot be evaluated whether or not the discharging of them is harmful for the environment (Danish EPA, 2005). Bach et al. (2010) investigated whether anthropogenic contamination in an Arctic area affects the fecundity (i.e. reproductive potential) and reproductive success of the benthic amphipod, *Orchomenella pinguis*. The test site was an open fjord adjacent to the town Sisimiut in West Greenland (approx. 5 500 inhabitants), which serves as the town's wastewater recipient, receiving untreated domestic wastewater, as well as wastewater from the fishing industry and the local hospital (Bach et al., 2009). *O. pinguis* collected at contaminated sites in the fjord had significantly higher fecundity but also higher frequency of

embryo aberrations resulting in lower fertility (i.e. actual reproductive success) compared to the individuals collected at clean sites outside the fjord (Bach et al., 2010). The results indicate ecotoxicological consequences for this marine system in terms of reduced reproductive success in highly contaminated environments (Bach et al., 2010). Analyses of heavy metals in sediment samples from Sisimiut Harbour revealed that the upper 0-1 cm of the sediment contained higher levels of heavy metals than in 1-3 cm depth, the highest levels being found close to wastewater outlets and in the waterway into the industrial harbour (Villumsen and Ottosen, 2006). Analyses of the condition of the same recipient, conducted by students at DTU, indicated a local pollution of the recipient, measured by elevated levels of nutrients and heavy metals (Ruggiera and Villemoes, 2005). Analyses of the currents in the recipient indicates that the water exchange is limited (Thomsen et al., 2003; Brogaard og Jørgensen, 2006), causing nutrients and heavy metals to deposit in the sediment (Ruggiera and Villemoes, 2006). The content of organic matter, nutrients and sulfur analyzed in sediment samples from the same recipient also indicated frequent occurrence of oxygen depletion (Brogaard and Jørgensen, 2006). Comparison of this recipient with Søndre Strømfjord (located approx. 180 km west of Sisimiut), which is not subjected to any major pollution, has further indicated a nutrient and heavy metal pollution of Sisimiut's wastewater recipient (Ruggiera and Villemoes, 2005).

Concerning the bacteriological risk of discharging untreated wastewater to the recipients in Greenland, it has been pointed out that special care should be taken with wastewater discharge in localities where food is being processed, either on or near the sea, which for instance is the case in some of the settlements where handling of seal products often takes place near the coast (Danish EPA, 2005). Spreading of antibiotic resistance is of global concern (Kunin, 1993), also in the vulnerable Arctic environment. The occurrence and distribution of antibiotic resistant bacteria (AR bacteria) in the wastewater recipient of Sisimiut has also been analyzed by students at DTU. Kabat (2010) isolated ciprofloxacin resistant *Escherichia coli* (*E. coli*) from an area close to the hospital outlet. *E. coli* is normally susceptible to quinolones, suggesting an acquired resistance (Kabat, 2010). Analyses in the same recipient of AR bacteria from blue mussels' hepatopancreas, gut bacteria from sculpins and sediment bacteria showed an increase in number of AR bacteria from the hospital outlet towards a reference site outside the fjord, indicating a connection between the occurrence of bacteria and presence of antibiotics in the recipient (Pedersen and Vilsgaard, 2010). Pedersen (2008) isolated four bacterial strains from water samples from the recipient, whereof three were isolated from water samples taken by the hospital outlet. All four strains were identified as being human pathogens within the bacterial family Enterobacteriaceae. Furthermore, *E. coli* resistant to ampicillin and amoxicillin, as well as ciprofloxacin resistant bacteria, have been found in both sculpin and mussel samples in the recipient (Lozano and Saltini, 2009). The ciprofloxacin resistant bacteria were not found at a reference site outside the recipient, indicating an acquired resistance against ciprofloxacin within the recipient (Lozano and Saltini, 2009). An ampicillin resistant bacterium identified as either *Aeromonas hydrophila* or *Vibrio fluvialis*, which are both potential human pathogens, was also isolated from the recipient (Lozano and Saltini, 2009). Analyses of AR bacteria in raw wastewater samples from different outlets in Sisimiut resulted in concentrations of ampicillin,

ceftriaxone and tetracycline resistant bacteria in the approximate ranges of $2 \cdot 10^5$-$4 \cdot 10^6$, $1 \cdot 10^5$-$9 \cdot 10^5$ and $2 \cdot 10^4$-$2.5 \cdot 10^5$ CFU/mL, respectively (Martinsen and Nicolajsen, 2011).

Handling of wastewater from tourist huts in Greenland is another challenge since they do not have running water supply and are often remotely located. A part of the PhD work was to participate in a project which focused on these problems (Gunnarsdóttir et al., 2011). The project was funded by NORA (Nordic Atlantic Cooperation) and was done in cooperation with tourist associations in Iceland and Norway, the University of Life Sciences in Norway, Bioforsk Tingvalla in Norway, and Qeqqata municipality in West Greenland. The project and its results are described further in sections 2.4.1 and 3.3.1.

Future projections show that the continuous loss of permafrost expected in the next two decades will add additional challenges to technological solutions chosen for sewage treatment (AMAP, 2011). An evaluation of the sewage pipeline system in Greenland in 2004-2005 revealed a general bad condition, with a risk of collapse of pipelines in some localities (Danish EPA, 2005). Maintenance of traditional wastewater collection and transport systems is expensive under Arctic conditions. Additionally the cold climate influences the efficiency of biological treatment processes in particular (Smith and Low, 1996), limiting the choices of wastewater treatment techniques. This is one of the reasons why bucket toilets are still being used in Greenland, mainly in the rural parts. The out phasing of this particular toilet solution is considered being an important task in order to improve the in-door health of the inhabitants where this toilet solution is in use (Heinke and Prasad, 1979; U.S. Congress, 1994). Due to the above mentioned challenges alternative treatment methods are needed, especially in small and remotely located communities. Decentralized treatment solutions are well suited for Greenland. Ideal solutions should reduce the need for expensive collection systems, and be more economically and environmentally sustainable than traditional wastewater collection and treatment systems. Possible alternative wastewater treatment methods for Greenlandic communities are dry composting or anaerobic digestion of excreta, collected at household level using dry or water saving toilets. This opens up for co-treatment with other organic waste fractions. Freezing and thawing may also be a cost-effective wastewater treatment method in cold regions, e.g. for dewatering of sludge (Vesilind and Martel, 1990; Sanin et al., 1994; Martel et al., 1998; Hedström and Hanæus, 1999) and reduction of microbial levels (Paper II). Thus it was chosen to concentrate on the effect of these processes in this PhD project, focusing on their hygienic effect.

1.2 Objective

Based on the above described challenges concerning today's wastewater handling in Greenland and limitations when it comes to choosing well suited wastewater treatment methods for Greenlandic conditions, the objective of the PhD project was to:

- Address the general challenges regarding wastewater treatment in cold climate through a literature study (Paper I)

- Make a feasibility study of already tested treatment methods in cold climate (Paper I)
- Test different wastewater treatment methods/unit processes that could be suited for Arctic climate and Greenlandic conditions, in both towns, settlements and tourist huts (Paper II, III and section 2.3.1 in synopsis)
- Evaluate the advantages and disadvantages of the different treatment methods, separately and in combination with each other.
- Support the Greenlandic municipalities in choices and testing of different toilet solutions.

1.3 Structure of thesis

This thesis starts with a methodology chapter (Chapter 2), giving a short theoretical background about the tested processes and the selected microorganisms. Since the manuscript of the fourth paper, which describes the results of small scale composting experiments, has not been included in the thesis, a description of the composting experimental setup and analytical methods of the experiments has been included in this chapter. Following the methodology chapter is a results chapter (Chapter 3), comparing selected microbial results from the different laboratory experiments. Subsequently there is a discussion chapter (Chapter 4) where the laboratory results are discussed, and possibilities to utilize the tested processes for wastewater treatment in Greenland, as well as possible advantages and disadvantages of the processes, are discussed. In this chapter future study needs are also addressed. Finally, an overall conclusion of the PhD project will be given in a concluding chapter (Chapter 5).

Chapter 2 Methodology

In the present chapter a short description of the basic theory behind the processes tested in the laboratory experiments as well as the selected microorganisms and microbial groups will be provided. Since the manuscript for the fourth paper, describing results from small scale composting experiments, has not been included in the thesis, the setup of the composting experiments will be described in section 2.3.1.

2.1 Treatment methods tested in laboratory experiments

The processes that were tested in laboratory experiments were the following:

- Long-term freezing and repeated cycles of freezing and thawing (Paper II)
- Anaerobic digestion and aerobic storage (Paper III)
- Composting (section 2.3.1)

Additionally, recovery treatment was carried out by the end of the long-term freezing and the anaerobic digestion/aerobic storage experiments.

2.1.1 Freezing and thawing

Damage of cells by freezing is different at slow and fast freezing rates. Under fast freezing damage of the cells can be caused by intracellular ice formation because the temperature is reduced at a faster rate than water can flow through the cell membrane (Gao et al., 2009). Under fast freezing, the size of the ice crystals is smaller, which causes less mechanical disruption (Davies and Obafemi, 1985). Under slow freezing ice forms extracellularly, causing the cells to dehydrate and shrink (Gao et al., 2009). Lethal effects of slow freezing result from buildup of high-solute concentration inside the cells (Davies and Obafemi, 1985). Strong evidence have been provided that salt concentration rather than ice is the cause of freezing injury to cells (Lovelock, 1953a,b). Slow freezing usually causes greater loss of cell viability and release of more outer-membrane materials (Davies and Obafemi, 1985). The rate at which thawing occurs also influences the cells, since slow warming allows the ice crystals to recrystallize and coalesce and grow, which damages the cells in which it occurs, whereas during rapid warming there is insufficient time for this to happen and the ice simply melts (Pegg, 2007). Both lethally and nonlethally injured cells of frozen bacteria manifest some characteristics that are quite different from those of unfrozen cells, e.g. extended lag phase, increased nutritional need, increased sensitivity to surfactants and other compounds, leakage of cellular material, loss of the ability to multiply, and others (Ray and Speck, 1973).

2.1.2 Anaerobic digestion

Anaerobic digestion involves a series of biological processes where a number of microorganisms break down biodegradable material under anaerobic circumstances (Wang, 2010). During the process, methane (CH_4)- and carbon dioxide (CO_2)-rich biogas is produced which is suitable for energy production. The main degradation routes during anaerobic digestion are fermentation and acidogenesis where complex organic substances are converted into volatile fatty adics (VFAs) (Gustavsson, 2005), which are essential for the microorganisms involved in the biochemical pathways responsible for biological removal of nitrogen (N) and phosphorus (P) (Banerjee et al., 1998). The end product of fermentation and acidogenesis is removed by sulphate reducers or methanogenic bacteria (Gustavsson, 2005). The methanogens utilize acetate for CH_4 production but are also capable of utilizing hydrogen for the CH_4 production (Christensen, 1998). After the digestion the nutrient-rich digestate can be used as fertilizer. Anaerobic digestion has been used for industrial and domestic purposes to treat waste and produce energy (Wang, 2010). The biogas process is influenced by many parameters, such as content of macro- and micro nutrients, temperature, pH, ammonium, possible substrate inhibition and hydraulic or organic overload, as well as possible toxic components (in the substrate) and sudden variations, e.g. in substrate feeding of the reactor, temperature variations, etc. (Christensen, 1998).

2.1.3 Composting

Composting is an aerobic, biological process whereby organic waste is degraded by various microorganisms, such as bacteria, actinomycetes and fungus, resulting in a material which can be used as a fertilizer in agriculture but also in construction work (Christensen, 1998). The composting process consists of three phases. Phase 1 is a mesophilic lag period with an increased microbial population, characterized by low pH caused by fatty acids. Phase 2 is thermophilic with high microbial activity, resulting in an increased heat production. At the end of this phase the pH increases due to degradation of fatty acids. In phase 3 the microbial activity decreases due to limited release of nutrients from the particle surfaces into the surrounding media (Smårs, 2002).

The degradation of the organic material (polymers of carbohydrates, proteins and fat) is mainly aerobic, meaning that the main elements in the organic material are aimed to be converted into their most oxidized form (Christensen, 1998). The process can be described as follows (Christensen, 1998):

$$\text{CHONS (organic material)} + O_2 \rightarrow CO_2 + H_2O + NO_3^- + OS_4^{2-} + \text{energy}$$

As the formula shows energy is released under the aerobic degradation of the organic material. The release of energy results in elevated temperatures in the composting material, leading to a faster biological degradation, which again leads to a rise in the temperature (Christensen, 1998). The maximum temperature that can be expected in composting material is 75-80°C because only a few species can function at such high temperature (Finstein and Morris, 1975). The temperature in the composting material will though usually be lower because it reflects a certain balance between the amount of heat

generated during the aerobic degradation and the amount of heat released to the surroundings (Christensen, 1998). There are several parameters that affect the composting process, such as the water content (ideally 35-60%), pH, C/N-ratio, temperature and addition of structure material (Christensen, 1998).

2.2 Experimental microorganisms

2.2.1 Definition of bacterial stress

Bacterial populations exposed to rapidly changing and sometimes hostile environments constantly switch between growth, survival and death (Aertsen and Michiels, 2004). Stress occurs when the bacteria experience sudden changes in the environment (Rychlik and Barrow, 2005). Different terms have been used to describe injury, death, and survival. Ray and Speck (1973) defined dead cells as nonviable, lethally or irreversibly injured or damaged. After e.g. freezing, these cells lose their ability to multiply and form colonies on a nonselective agar medium. Surviving cells were defined as those that retain their ability, e.g. after freezing, to multiply and form colonies on a nonselective complete agar medium (Ray and Speck, 1973). Nonlethally injured cells are defined as those that are reversibly injured, debilitated, or damaged, and they form two categories: Metabolically injured and structurally injured (Ray and Speck, 1973). Finally, uninjured cells are the survivors who are unharmed, undamaged and normal. They are able to multiply and form colonies equally well on complete and minimal or on nonselective and selective agar media (Ray and Speck, 1973). Sublethal injury is a consequence of exposure to a chemical or physical process that damages but does not kill a microorganism (Hurst et al., 1977).

2.2.2 Selection of microorganisms

Members of the coliform group were analyzed in all three laboratory experiments. In the long-term experiment (Paper II) total and faecal coliforms as well as *E. coli* were determined while only *E. coli* was analyzed in the anaerobic digestion/aerobic storage experiments (Paper III) and the composting experiment (section 2.3.1). The coliform group was selected to represent gram-negative bacteria. The total coliform group includes all aerobic and facultatively anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation in prescribed culture media within 48 hours at 35°C (Maier et al., 2000). The group includes *Escherichia, Citrobacter, Enterobacter,* and *Klebsiella* species. They are discharged in high numbers ($2 \cdot 10^9$ coliforms/(day·capita)) in human and animal feces, but not all of them are of faecal origin (Bitton, 2005). They have been used as the standard for assessing faecal contamination of recreational and drinking water for the past century but it has been recognised that a number of deficiencies in the use of this indicator exist, such as; regrowth in aquatic environments and distribution systems and suppression by high background bacterial growth (Maier et al, 2000). Besides it is not an indicator of a health threat, and there is no relationship between enteric protozoan and viral concentration (Maier et al, 2000). Faecal coliforms are, as the name indicates, of faecal origin, opposite to members of the total coliform group which are not limited to faecal sources.

Presence of faecal coliforms indicates the presence of faecal material from warm-blooded animals. It cannot, however, be differentiated if the source of contamination is of human or animal origin. (Bitton, 2005) The organisms in this group, including the genera *Escherichia* and *Klebsiella,* are differentiated in the laboratory by their ability to ferment lactose with the production of acid and gas at 44.5°C within 24 hours (Maier et al, 2000).

Faecal streptococci were analyzed in all three laboratory experiments (Paper II, III and section 2.3.1), and furthermore it was investigated whether or not the streptococci belonged to the enterococcus group in the long-term experiment (Paper II) and the anaerobic digestion/aerobic storage experiments (Paper III). Faecal streptococci were selected to represent gram-positive bacteria. Faecal streptococci are a group of gram-positive Lancefield group D streptococci. Faecal streptococci belong to the genera *Enterococcus* and *Streptococcus*. They are differentiated from other streptococci by the ability to grow in 6.5% NaCl at pH 9.6 and 45°C (Maier et al., 2000). They are considered having certain advantages over the coliform and faecal coliforms bacteria, for instance that they rarely multiply in water, they are more resistant to environmental stress than coliforms and generally persist longer in the environement (Maier et al., 2000). The enterococci (*S. faecalis* and *S. faecium*), which is a subgroup of the faecal streptococci group, has been suggested as useful indicators of the presence of viruses in the environment, particularly in biosolids and seawater (Bitton, 2005).

Somatic coliphages which infect and replicate in *E. coli* were used as an indicator for viruses (Eaton et al., 2005) in the long-term freezing and freeze/thaw experiments (Paper II) and anaerobic digestion/aerobic storage experiments (Paper III). Bacteriophages are viruses of bacteria and there are three general types of them, classified on the basis of their mode of infection of the host (Maier et al., 2000). The first one is the F-specific or appendage phage that infect the host through the sex pili or flagella, the second is the capsule phage that recognize the outer host layer such as the polysaccharide capsule while the third one, the somatic phage, enter the host via the cell wall (Maier et al, 2000). Bacteriophages have been suggested as indicators of viral pollution because of their structure, morphology, size as well as behaviour in the aquatic environment which closely resemble those of enteric viruses. Pathogenic human viruses in waters and wastewaters are of public health concern and bacterial monitoring of wastewater may not indicate sufficiently the presence of viruses (Eaton et al., 2005). It is however beyond the capabilities of most water laboratories to perform analysis of human enteric viruses in water and wastewaters since these techniques have been time- and labour-intensive, are expensive and require skilled personnel (Eaton et al., 2005). Coliphage assays are easier to perform and yield overnight results.

Indigenous antibiotic resistant bacteria belonging to the bacterial family Enterobacteriaceae were analyzed in the long term-freezing and freeze/thaw experiment (Paper II) and the anaerobic digestion/aerobic storage experiments (Paper III). They were selected to determine antibiotic resistant bacteria's tolerance against the tested processes. The four antibiotics (amoxicillin, ciprofloxacin, tetracycline and vancomycin) used in the experiments were selected from a list of antibiotics being most

used in Greenland in 2007/2008. The selection was based on their different mechanisms of action. Amoxicillin is bactericidal against susceptible organisms through the inhibition of biosynthesis of cell wall mucopeptide during the stage of bacterial multiplication, used for the treatment of adults with mild/moderate, susceptible gram-positive and gram-negative bacterial infections of the ear, nose, and throat (U.S. FDA, 2008). Ciprofloxacin is a fluoroquinolone, and most strains of frequently encountered Enterobacteriaceae are highly susceptible to it whereas it is not as active against gram-positive organisms (Sanders, 1988). Tetracyclines were discovered in the 1940s and are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra and Roberts, 2001). Tetracyclines are broad-spectrum agents that exhibit activity against a wide range of gram-positive and gram-negative bacteria as well as atypical organisms such as chlamydiae, mycoplasmas,and rickettsiae, and protozoan parasites (Chopra and Roberts, 2001). Vancomycin was the first glycopeptide antibiotic, developed in the 1950s, and it is effective at low concentrations against the majority of gram-positive bacteria (Reynolds, 1989).

Salmonella enterica ssp. enterica serovar Enteritidis (*S.* Enteritidis) was included in microbial analyses in the long-term freezing experiment (Paper II). *Salmonella* is as a typical pathogenic bacterium, more frequently identified than any other pathogenic bacteria in wastewater sludge and is known to cause severe illness (Sanin et al., 1999).

2.2.3 Enumeration methods

The enumeration methods used in the long-term and freeze/thaw experiments as well as the anaerobic digestion/aerobic storage experiments are described in detail in the methodology chapters in Papers II and III, respectively. The microbial parameters analyzed in the composting experiment (section 2.3.1) were faecal streptococci and *E. coli.* The multiple tube fermentation (MTF) technique was used for enumeration of *E. coli* in the composting experiment according to Eaton et al. (2005). Triplicate experiments were performed and most probable number (MPN) values were calculated from the number of positive tubes as described in Blodgett (2010). Triplicate analyses of faecal streptococci in the compost material were determined by direct plate count. Before inoculation into the different growth media 10-fold dilution series of the compost samples were prepared with physiological salt water (0.9% NaCl) by manual shaking and with a mini shaker at medium speed. Table 2.1 summarizes the enumeration methods and incubation conditions used for the selected microorganisms in all three experiments.

Microbial group	Method from paper nr.	Growth media	Enumeration method	Incubation temp. (°C)	Incubation time (h)
Total coliforms (presumptive phase)	II, III	Lauryl sulphate broth	MTF	37	48 ± 3 h
Total coliforms (confirmed phase)	II	Brilliant Green Lactose Bile broth	MTF	37	48 ± 3 h
Faecal coliforms	II	EC-medium	MTF	45	24 ±2 h
Escherichia coli	II, III	Tryptone water + Kovac's reagent (indole test)	MTF	45	24 ±2 h
Escherichia coli	Section 2.2.3 (composting)	Tryptone water + Ehlrich reagent (indole test)	MTF	44	24 ±2 h
Faecal streptococci (presumptive phase)	II, III	Azide dextrose broth	MTF	37	48 ± 3 h
Faecal streptococci (confirmed phase)	II, III	Pfizer selective enterococcus agar	Brownish-black colonies w/brown halos confirm presence of faecal streptococci	37	24 ±2 h
Faecal streptococci	Section 2.2.3 (composting)	Slanetz-Bartley agar	CFU determined by direct plate count	37	44 ± 4 h
Enterococcus group	II, III	Brain heart infusion (BHI) broth + 6.5% NaCl	MTF	45	Overnight
Antibiotic resistant bacteria belonging to Enterobacteriaceae	II, III	MacConkey agar (selected antibiotics added separately to agar)	CFU determined by direct plate count	37	24-72 h
Somatic coliphages	II, III	Tryptone top and bottom agar	PFU determined by a variant of double-agar-layer method	37	Overnight
Salmonella Enteriditis	II	BHI and Xylose lysine deoxicholate agar	CFU determined by direct plate count	37	Overnight

Table 2.1. Microorganisms and microbial groups selected for analyses in the laboratory experiments as well as the methods used for enumeration. Abbreviations: MTF: Multiple Tube Fermentation, CFU: Colony Forming Units, PFU: Plaque Forming Units.

2.3 Experimental setup

Experimental setup of the long-term freezing and repeated freezing and thawing experiments is described in details in Paper II, and of the anaerobic digestion/aerobic storage experiments in Paper III. The setup of the small scale composting experiments will be described in the following section.

2.3.1 Experimental setup of composting experiments

Two sets of experiments were performed with mixtures of blackwater solids (BWS) with different bulk materials, namely bark (B), sawdust (S), peat (P), oat husks (O), shrimp waste (SW) and finally cereal bran (C) as an additive. Blackwater was collected from a student dormitory with 48 students served by vacuum toilets. The vacuum toilet and transport system (Jets™) contain a macerator and a dewatering unit rendering macerated blackwater with a dry matter content of approx. 14% (measured by drying overnight at 105°C) prior to storage at 4°C. The bulk materials were commercially available materials. A major part of the shrimp waste consisted of shells. The experiments were conducted under different temperature conditions and experimental design. The first experiment was a preliminary test to observe organic matter degradation with various mixtures of blackwater and the before mentioned bulking materials, as well as blackwater exclusively as a control. The mixtures were incubated at 37°C for 2 weeks and 55°C for 44 days. Air-tight and thermal resistant bottles with a lid equipped with a membrane for gas sampling were used for these experiments. Air was supplied to the samples by opening the bottles once a day and introducing air by pressure. The second experiment was designed to stimulate the process of composting in a self heating mode, without stirring, using a new combination of the tested bulk materials and additives, based on results of the first experiments (results not shown). The different mixtures and amount of each substrate of the second experiment are shown in table 2.2. In order to decrease the water content of the blackwater it was necessary to establish a proper ratio between the BWS and the bulking materials. The ratio was calculated based on the content of total solids (TS) of each substrate and the wished final TS (approx. 20%) for the mixtures (see table 3.1 for TS-content of each substrate).

Mixture no.	Substrates	Amount (kg/reactor)	No of reactors
1	BWS+O+C+S	1 (BWS)+0.05 (O)+0.05 (C)+0.025 (S)	3
2	BWS+O+C+B	1 (BWS)+0.05 (O)+0.05 (C)+0.025 (B)	3
3	BWS+O+C+P	1 (BWS)+0.05 (O)+0.05 (C)+0.025 (P)	3
4	BWS+O+C	1 (BWS)+0.05 (O)+0.05 (C)	2
5	BWS+P+SW	0.60 (BWS)+0.030 (P)+0.030 (SW)	2
6	BWS	1 (BWS)	2 (control)

Table 2.2. The composting mixtures and amount of each substrate.

15 vertical cylindrical polyethylene reactors with a volume of 2 L were designed for the second part of the experiments (height=32 cm, diameter=10 cm). To minimize the heat loses, the reactors were introduced

in thick Styrofoam boxes (height=55 cm, width=60 cm), made of 11 layers of Styrofoam (thickness of each layer=5 cm). The reactors were air tight to prevent gas losses. A perforated PVC sieve (2 mm) was installed at the bottom of each reactor. Aeration of the reactors was supplied with up-flow aeration from the bottom of the reactors with pressure pumps (Trixie Ap120, 120L/h, Australia), controlled by a time switch (24 Hour - In Time Switch, UK). Each reactor had its own pump in order to avoid differences of air diffusion due to the different texture of bulking material. During the experiment the air pump operated every 3rd hour for 30 min. The temperature in the composting material was measured continuously with temperature sensors buried in the core of the composite materials at 12 cm depth, and the ambient temperature was monitored as well. A data logger (Delta T Logger DL2e, software Ls2Win PC) was used to register temperature data continuously with a sampling rate of 4 hours. A schematic diagram of the reactor setup can be seen in figure 2.1.

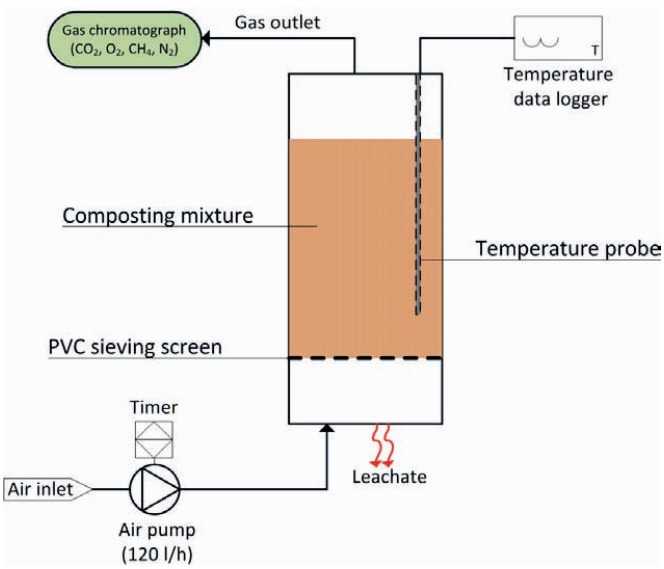


Figure 2.1. Experimental design of the composting reactors (Figure: Andreea Oarga).

Mixtures 1-3 were tested in triplicates, and mixtures 4-5 as well as the control (mixture 6) in duplicates. The experiment lasted for 62 days. At the beginning of the experiment 1 kg of each mixture was divided into three parts: One part for immediate analysis, another part for drying and the third part was frozen at -20°C for chemical analysis. The same procedure was followed at the end of experiment but with the whole composted material. During the process, the composting mass in the reactors was not mixed, but after 29 days of composting water was added. Total and volatile solids (TS and VS, respectively) were measured at the beginning and end of experiment as indicators for decomposition, substrate mineralization and compost maturity. Further analysis of the composting material included measurements

of conductivity, pH, total nitrogen, total carbon, ammonia-and nitrate-nitrogen, total phosphorus and total potassium. Selected results of these analyses will be shown in chapter 3.

The composting process in the preliminary test was monitored analyzing carbon dioxide, oxygen, nitrogen and methane by gas chromatography (Perkin Elmer, AutoSystem Gas Cromatograph, USA) with a thermal conductivity detector (TCD) and flame ionization detector (FID) (results not shown). In the second experiment the temperature and gas measurements were the parameters used to monitor the composting process. The temperature in the compost mass was measured as well as gas concentrations in the free space between the compost mass and the reactor's lid. The gas measurements were taken daily 20 minutes before each time the pump started its air inflow into the reactor. Carbon dioxide, oxygen and methane were analyzed using an infrared gas analyzer (GA2000 Plus, Geotechnical Instruments, UK). Gas was pumped out by the gas analyzer through silicone tubes installed in the lid of each composting reactor (results for gas measurements are not shown).

2.4 Pilot scale testing facilities in Greenland

2.4.1 Toilet facilities in tourist huts and sights in Qeqqata Kommunia, W-Greenland

As described in the introduction, the participation in a project funded by NORA was a part of the PhD project. The NORA project had a practical aim and was focused on selected tourist huts and sights in Qeqqata municipality. There are approximately 30 tourist huts in Qeqqata municipality, with toilet facilities in approximately 1/3 of them, in all cases bucket toilets (Gunnarsdóttir et al., 2011). The users of the tourist huts are hunters as well as local people and tourists. The visitors of the huts have to change the toilet bags themselves while a local cleaning company takes care of collecting the bags. The municipality has had problems with collection of the bags and maintenance of the huts for a long time, for which reason the aim of the project was to suggest a better toilet solution for the huts. Field work was carried out in the summer of 2010 where the conditions of one selected hut in the municipality was investigated as well as the soil conditions around the hut to evaluate its infiltration properties. Subsequently, solution suggestions based on the evaluations from the field work, were prepared (Gunnarsdóttir et al., 2011). The first solution consisted of a composting toilet with an insulated composting container underneath the toilet. A grid was inserted at the bottom of the container, allowing solids to settle on top of it while the liquid fraction is to be drained off with a drainage pipe to an infiltration system in the underground. Study of the soil conditions has revealed that the soil mainly consists of medium-coarse to coarse sand, which is well suited for infiltration (Jenssen and Siegrist, 1990). The analyses further indicated that a required infiltration capacity for the evaluated yearly number of users and thus volume of urine is fulfilled in the area (Gunnarsdóttir et al., 2011). Ventilation of the composting container is an important technical detail. A ventilation pipe, equipped with a mosquito net and wind fan, was led from the container and to a minimum height of 1 m above the roof. Two toilets of this type have been built and will be installed in the same hut during the spring 2012, testing different technical details, such as different diameter of the drainage pipes as well as usage of air solar collector for heating of the composting material in one of the

containers. A demonstrating model of the toilet solution is shown in figure 2.2. The side of the container facing the camera is made of Plexiglas. The container will be fit into the outside wall of the toilet room with the Plexiglas side of the container facing the outside of the hut, enabling sunlight to warm up the composting material.

Figure 2.2. A demonstrating model of the composting toilet that will be tested in a selected tourist hut in Qeqqata municipality (Picture: Ragnhildur Gunnarsdóttir).

Another toilet solution, also based on composting, has recently been installed at two additional tourist sites in Qeqqata municipality. The toilet solution is based on a solution which has been used with good results in the highlands of Iceland (Gunnarsdóttir et al., 2011). One of the composting toilets has been installed by a tourist hut owned by Qeqqata municipality, which is used regularly, e.g. by locals. The hut is located by Amerdloq Fjord. The other toilet has been installed by a popular tourist sight, Qerrortussup Majoria, frequently visited by both tourists and locals. The human excrements are collected in a barrel, perforated at the bottom, underneath the toilet seat. The liquid part of the excrements is drained of to the ground underneath the toilet. The barrels are replaced with empty ones as needed, and the full ones are stored in an outside cabinet for composting. A picture of this solution is shown in figure 2.3. Experiences with the solutions will be presented in section 3.3.1.

Figure 2.3. Composting toilet at the tourist sight Qerrortussup Majoria in Qeqqata Kommunia, W-Greenland (Picture: Erik Lomholt-Bek).

2.4.2 Toilet facilities in a single-family residence in Sisimiut, W-Greenland

Two toilet solutions have been tested in a single-family residence in the town Sisimiut. The sanitary solution in the residence prior to installation of the toilet facilities was a bucket toilet. The first solution tested in this residence was a commercially available urine-separating composting toilet (Villa 9010, figure 2.4) with a composting container for the faecal matter outside the residence. The urine was discharged from the toilet to a 25 L plastic container through a pipeline. The urine container was inserted into a wooden box outside the residence with room for two containers. The box was insulated on the inside with Styrofoam. The faecal matter was collected in a bucket underneath the toilet seat, and was emptied into the composting container approx. twice a week. The bucket was equipped with an electrically driven fan to minimize odour problems. The toilet was connected to a ventilation pipe which was led above the roof, and was additionally equipped with a mosquito net. The urine containers were collected by municipal workers and emptied into the sea at the same locality as emptying of toilet bags in Sisimiut takes place. The second toilet solution, tested in the same residence, was a low flush toilet (Miniflush from Gustavsberg) with collection of the blackwater in a tank, buried in the ground outside the residence. Experiences with both solutions are presented in section 3.3.2.

Figure 2.4. A urine separating composting toilet (Villa 9010) which was tested in a single-home residence in Sisimiut, West Greenland (Picture: Ragnhildur Gunnarsdóttir)

Chapter 3 Results

In the first section of the present chapter, results from physical analyses and temperature measurements from the second composting experiment (section 2.3.1.) will be presented (results from preliminary experiments not shown). The results from the long-term freezing and freezing and thawing experiments (Paper II) as well as the anaerobic digestion/aerobic storage experiments (Paper III) have been thoroughly dealt with in the results chapters of the respective papers. The second section of the present chapter contains a comparison of selected microbiological results from the long-term freezing experiment, the anaerobic/aerobic experiments as well as the composting experiments. The third section contains results gained from pilot scale testing facilities in Qeqqata municipality in West Greenland.

3.1 Physical analyses and temperature in composting reactors

Table 3.1 shows results from analyses of total and volatile solids (TS and VS, respectively) for each substrate type. The substrate types were the following: Blackwater solids (BWS), bark (B), cereal bran (C), oat husks (O), peat (P), sawdust (S) and shrimp waste (SW). Table 3.2 shows results from physical and microbial analyses, conducted before and after composting, of the five different substrate mixtures (1-5) and the reference mixture (6). The mixtures were the following: 1=BWS+C+O+S; 2=BWS+C+O+B; 3=BWS+C+O+P; 4=BWS+C+O; 5: BWS+P+SW; 6=BWS, reference sample.

Substrate	TS (%)	VS (% of dry weight)
BWS	14.3	92.3
B	83.2	94.0
C	87.6	93.7
O	68.4	95.7
P	64.3	97.0
S	92.9	99.8
SW	51.8	87.0

Table 3.1. Characteristics of the separate substrate types used in the composting experiments.

Mixture	1		2		3		4		5		6	
Parameter	B	A	B	A	B	A	B	A	B	A	B	A
TS (%)	24.3	22.3	24.2	32.9	22.5	19.7	22.3	23.4	14.7	17.9	14.3	19.6
VS (% of dry weight)	93.5	84.6	92.6	86.5	92.7	84.1	92.8	84.5	91.0	83.7	92.3	83.4
Tot-C (%)	45.3	42.3	45.6	43.7	44.7	42.3	44.0	41.1	45.7	41.5	na	39.2
Tot-N (%)	1.67	2.72	1.63	2.73	1.82	3.04	2.12	3.06	2.59	2.68	na	3.0
C/N ratio	27.2	15.5	28.0	16.0	24.5	13.9	20.8	13.4	17.6	15.5	na	13.1
Tot-K (g/kg)	6.4	10.5	7.1	9.2	8.5	10.6	8.0	12.1	5.2	5.0	na	6.2
Tot-P (g/kg)	16.3	25.8	13.1	22.9	17.6	25.3	16.5	29.1	20.0	26.9	na	35.8
Mass reduction (kg)	1.06	0.43	1.06	0.54	1.06	0.47	1.00	0.53	0.69	0.083	1.00	0.594
Mass reduction (%)	59.4		49.1		55.7		47.0		88.0		40.6	
pH	6.84	7.20	6.91	6.87	7.14	6.87	6.82	6.83	7.10	6.65	na	6.78
Faecal streptococci ($CFU \cdot 10^6/g$)	14.3	0.19	54.8	0.9	65.0	0.063	64.2	0.04	17.3	0.25	2.1	7.6
E. coli ($MPN \cdot 10^3/g$)	35.0	1.40	160	0.23	160	0.33	54.0	0.79	28.0	5.40	18.0	2.40

Table 3.2. Characteristics of the waste mixtures before (B) and after (A) composting (1: BWS+C+O+S; 2: BWS+C+O+B; 3: BWS+C+O+P; 4: BWS+C+O; 5: BWS+P+SW; 6: BWS, reference sample). Abbreviations: TS: Total solids, VS: Volatile solids, Tot-C: Total carbon, Tot-N: Total nitrogen, na: Not available.

The aim was to approach a TS content of approx. 20% in the mixtures in the beginning with the calculated ratio of BWS and bulking materials. Table 3.2 shows that the TS content was similar in mixtures 1-4 (approx. 22-24%), but was considerably lower in mixture 5 which contained BWS, P and SW. The TS content in this mixture was almost the same as in the reference sample, containing only BWS. Total phosphours (tot-P) and potassium (tot-K) increased during the experimental period in all mixtures, apart from tot-K in mixture 5 which was reduced. pH was relatively stable in all mixtures during

the experiment. The mass reduction was considerable, ranging from approx. 40-88%. However, the VS content was still high in all mixtures at the end of the experiment. The C/N-ratio of all mixtures was reduced during the composting experiment in all mixtures.

Figure 3.1 shows the temperature profile of each mixture (1-6) as well as the ambient temperature (T ref). The graph shows the profile that reached the highest temperatures within the duplicate/triplicate of each mixture.

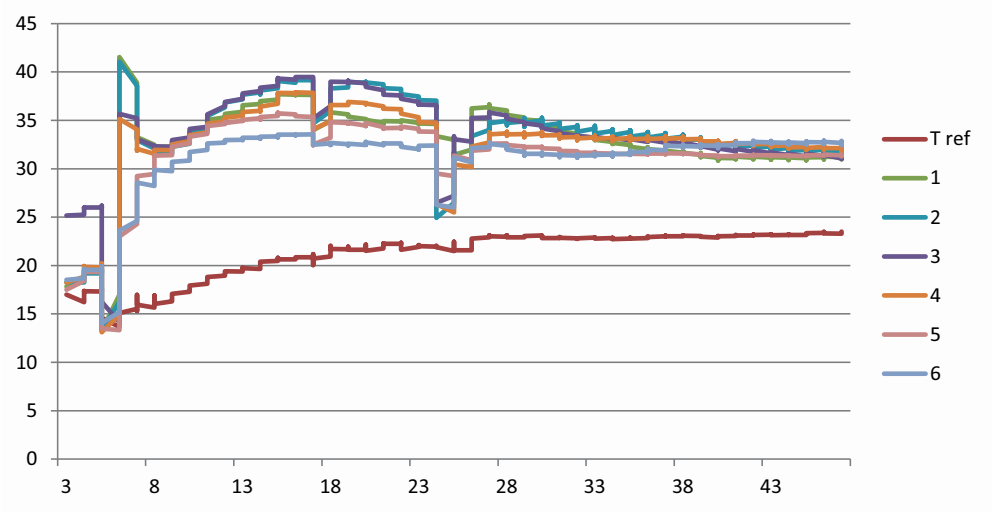


Figure 3.1. Temperature profiles for the different composting mixtures (1-6) as well as the ambient temperature (T ref). The unit on the x- and y-axis is days and temperature (°C), respectively.

The compost did not reach thermophilic phase. The maximum temperature registered was 41.5°C in mixture 1. The temperature was in the mesophilic range in mixture (1) between days 7 and 30, in (2) between 7 and 31, in (3) between 7 and 35, in (4) between 12 and 24, and in (5) between 12 and 17. The control sample (6) did not reach mesophilic range, reaching a maximum temperature of 33.5°C. The temperature drops were result of changing the air flow on day 7 and drying of material noticed after day 23. At day 29 the reactors were opened for adding water. The shortest period within the mesophilic range occurred in the mixture of BWS, P and SW (5).

3.2 Comparison of microbial results from laboratory experiments

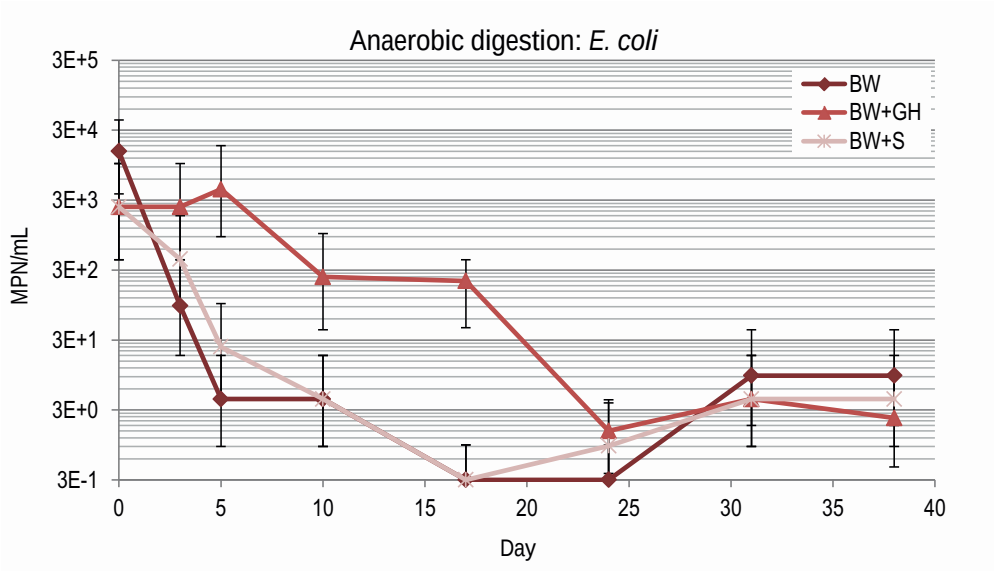
In this section the results for the microorganisms and microbial groups analyzed in more than one of the experiments will be treated in order to compare their resistance against the different treatment methods. The microorganisms and microbial groups that were analyzed in only one of the experiments, e.g. *Salmonella* (long-term freezing experiment, Paper II) will therefore not be dealt with in this section. The microorganisms and microbial groups that will be treated in this chapter are shown in table 3.3.

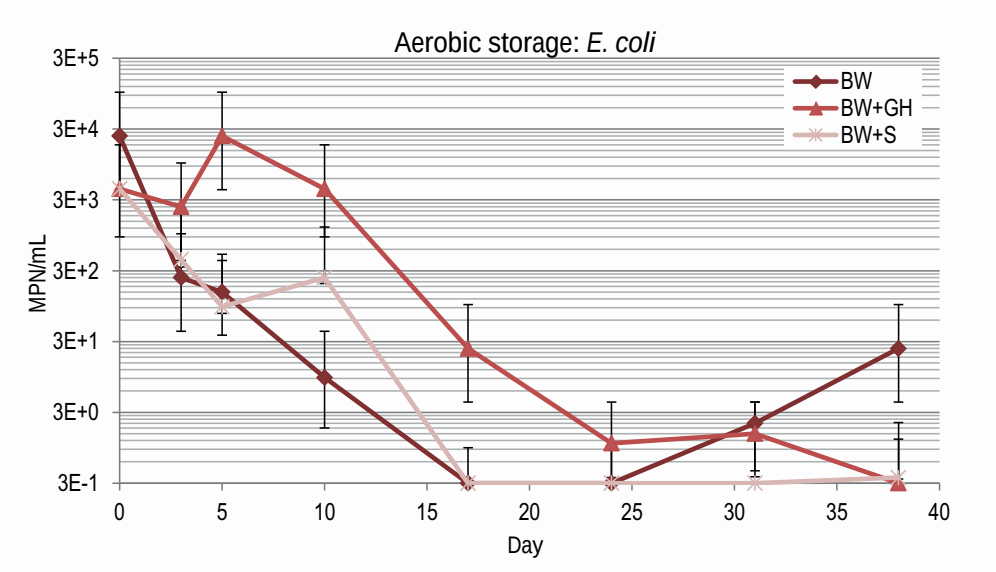
Microorganism/ microbial group	Paper	Treatment method
E. coli	II	LT-freezing
	III	AD and AS
	Section 2.3.1	Composting
S. faecalis	II	LT-freezing
	III	AD and AS
	Section 2.3.1	Composting
Amoxicillin and tetracycline resistant Enterobacteriacaeae	II	LT-freezing
	II	Freeze/thaw
	III	AD and AS
Somatic coliphages	II	LT-freezing
	II	Freeze/thaw
	III	AD and AS

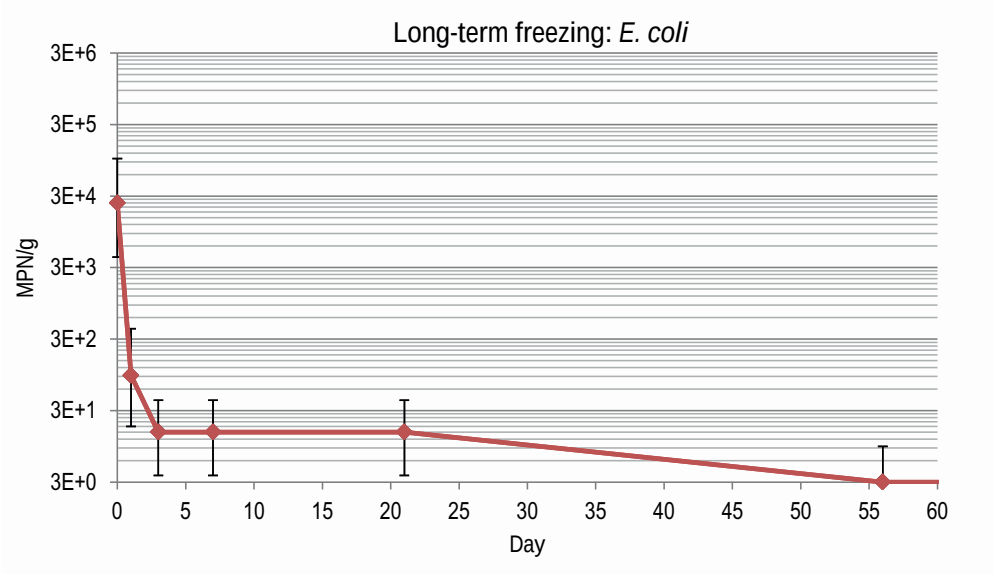
Table 3.3. The microorganisms and microbial groups of which results will be compared in this chapter. Abbreviations: LT=Long-term, AD=Anaerobic digestion, AS=Aerobic storage.

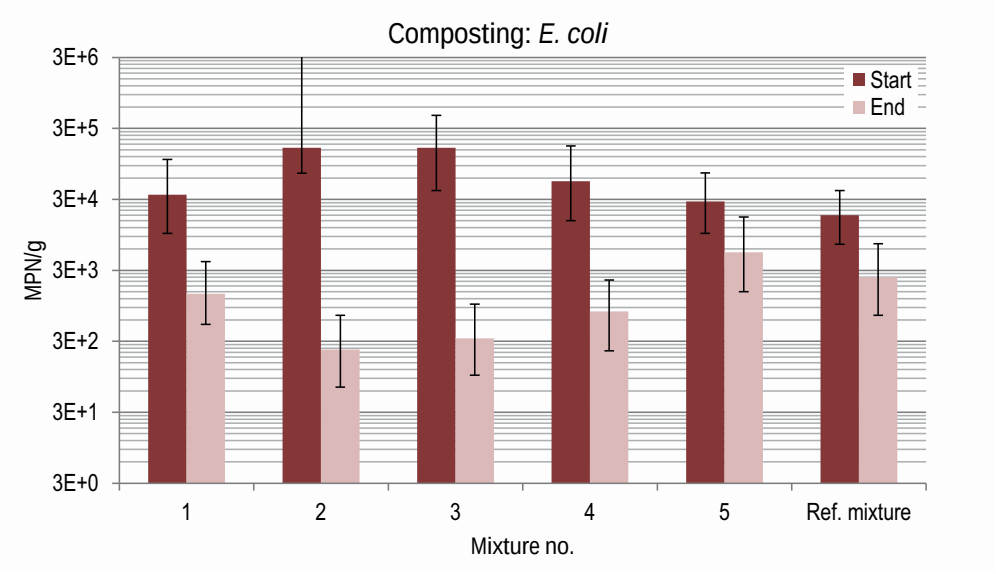
3.2.1 Escherichia coli

Analyses of *E. coli* were conducted in the anaerobic/aerobic batch experiments (Paper III), the long-term freezing experiment (Paper II), as well as in the composting experiment (section 2.3.1). The notations used in the graphs for anaerobic/aerobic batch experiments are the following: BW: Blackwater, BW+GH: Blackwater+Greenlandic halibut waste, BW+S: Blackwater+shrimp waste.









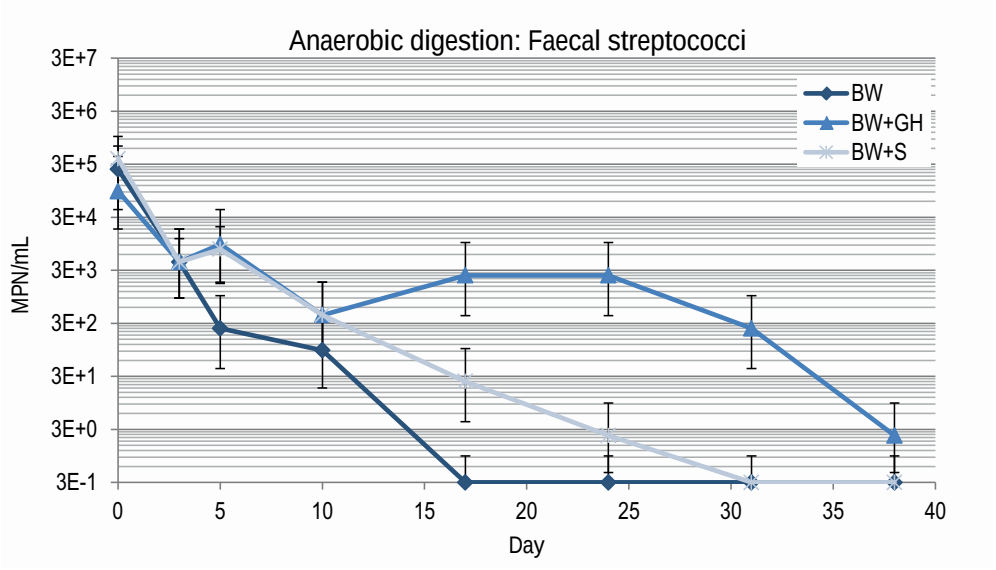
The initial level of *E. coli* was similar in all experiments. The reduction rate (reduction pr. time unit) was rapid during anaerobic digestion and similar under aerobic storage (Paper III). During the long-term freezing the reduction was also fast (Paper II), and after 56 days in frozen condition *E. coli* was under the detection limit and was not detected again during the 280 days long experiment (results for only the first 60 days showed in the graph). The reduction rate during the composting experiment (section 2.3.1) is not known since samples were taken exclusively before the experiment started and by the end of the

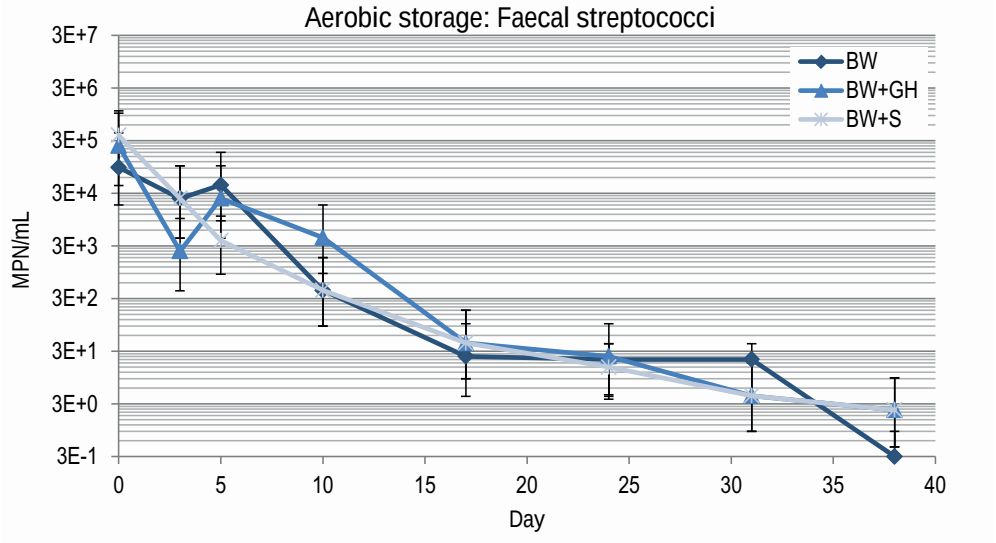
experiment. As the bar plot for composting mixture 2 shows, the upper limit of the confidence interval for the initial level of *E. coli* is unknown. This is due to the fact that high enough dilutions were not prepared, resulting in an MPN value of $>1.6 \cdot 10^5$ with an unknown upper confidence limit. Even though the composting process did not reach thermophilic temperatures in any of the mixtures the results indicate that the composting had a considerable effect on *E. coli*, particularly in mixtures 2, 3 and 4 (>2.84; 2.69 and 1.83 log, respectively). In mixture 5 the reduction was 0.72 log which was actually lower than in the reference mixture (number 6). This might be explained by the temperature profile of mixture 5 which was overall lower than for mixtures 1-4. The water content was higher in this mixture which could have had a negative effect on the composting process.

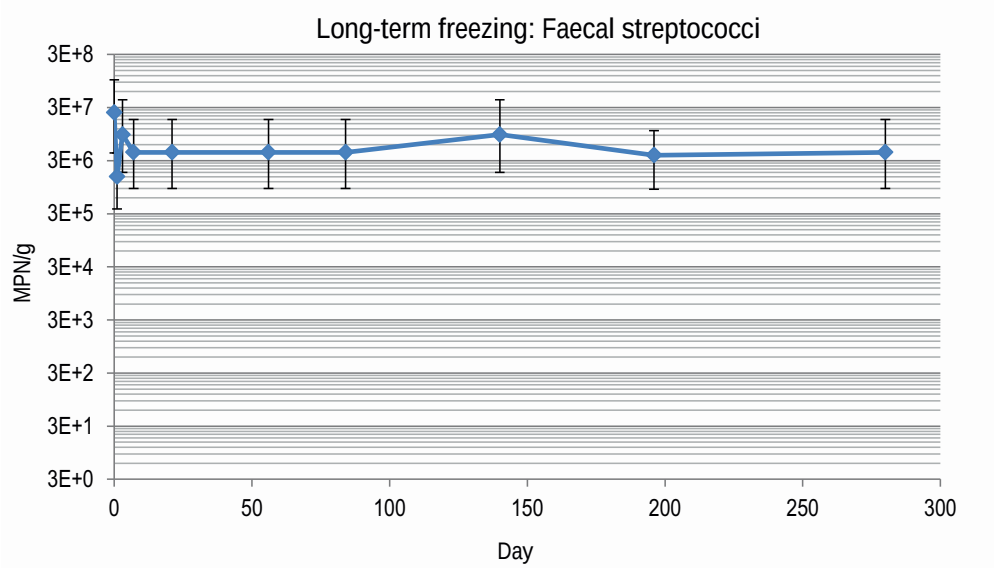
Recovery of possibly injured *E. coli* was done after the long-term freezing and the anaerobic digestion/aerobic storage experiments. No recovery was observed after the long-term freezing (Paper II) whereas approx. 2 log recovery was observed in the aerobic sample containing blackwater but none for the anaerobic samples (Paper III). This indicates a lethal effect of the freezing on *E. coli* and a sublethal effect of the mesophilic aerobic storage for 38 days.

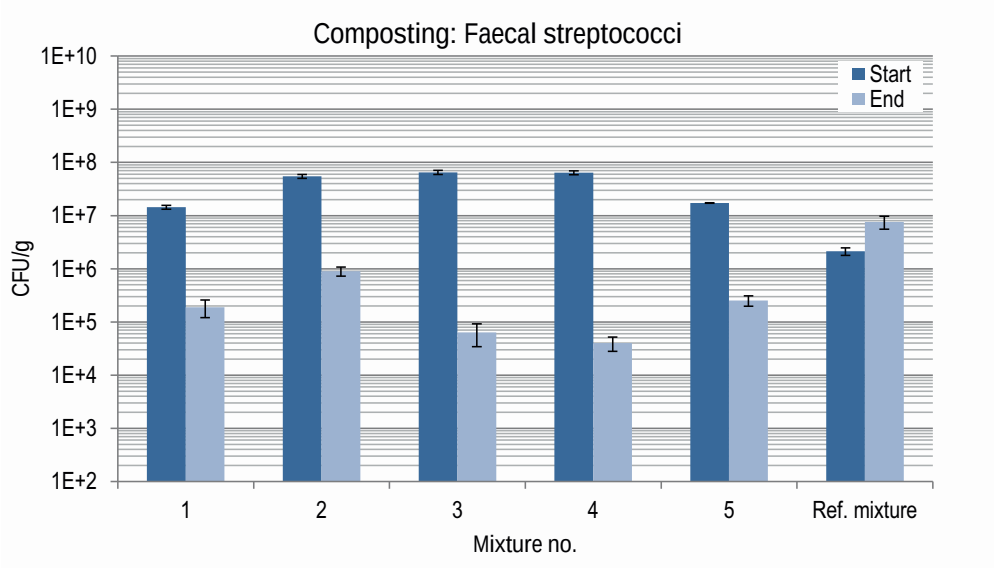
3.2.2 Faecal streptococci

Faecal streptococci were analyzed in the anaerobic/aerobic batch experiment (Paper III), the long-term freezing experiment (Paper II), as well as the composting experiment (section 2.3.1).







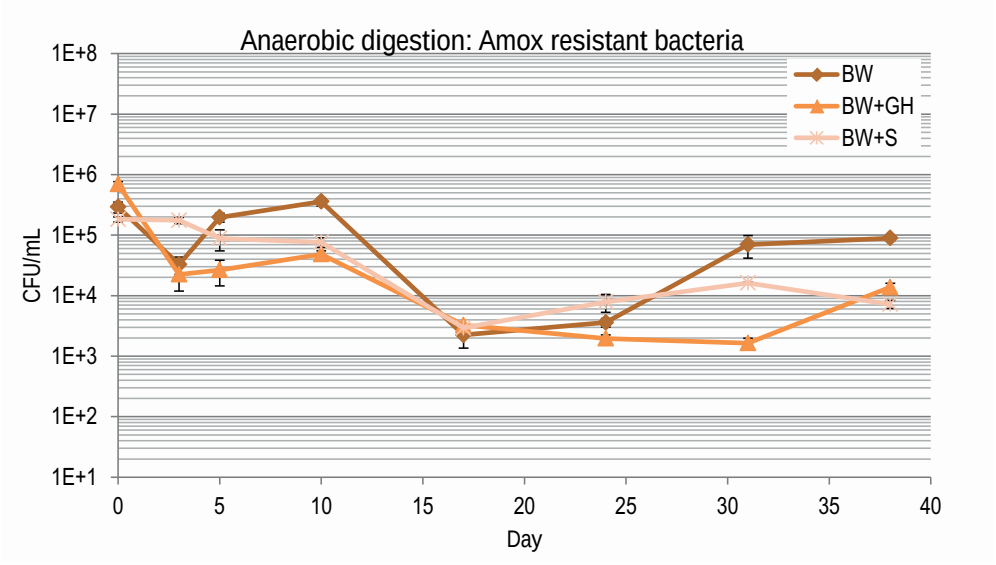


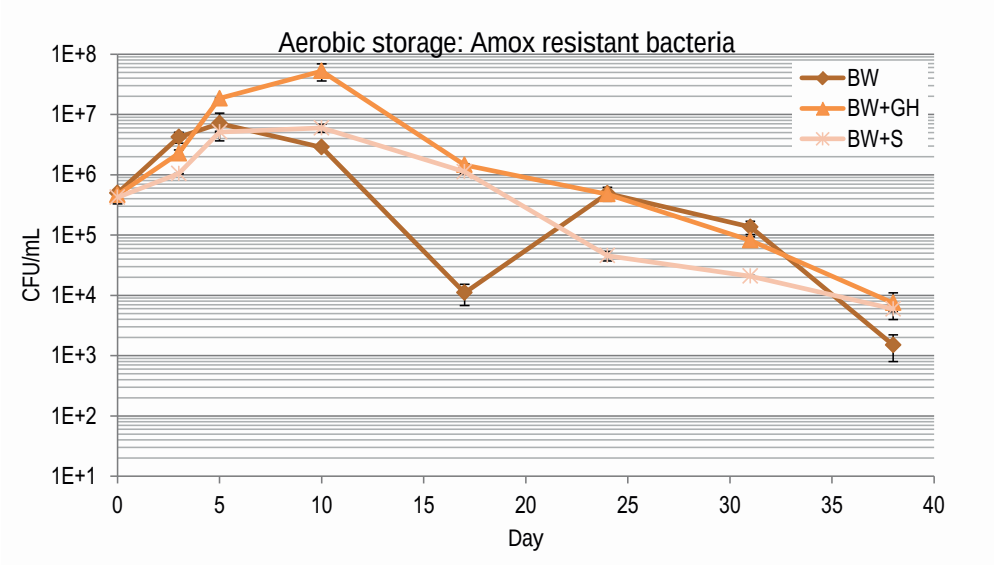
The reduction during anaerobic digestion and aerobic storage was large in both experiments but faster under aerobic conditions (Paper III). In the anaerobic mixtures containing BW, BW+GH and BW+S the reduction was 5.9, 4.6 and 6.1 log, respectively. In the aerobic mixtures containing BW, BW+GH and BW+S the reduction was 5.5, 5.0 and 5.2 log, respectively. On the other hand the freezing had a minimal effect on the faecal streptococci, less than 1 log reduction during the 280 days of freezing (Paper II).

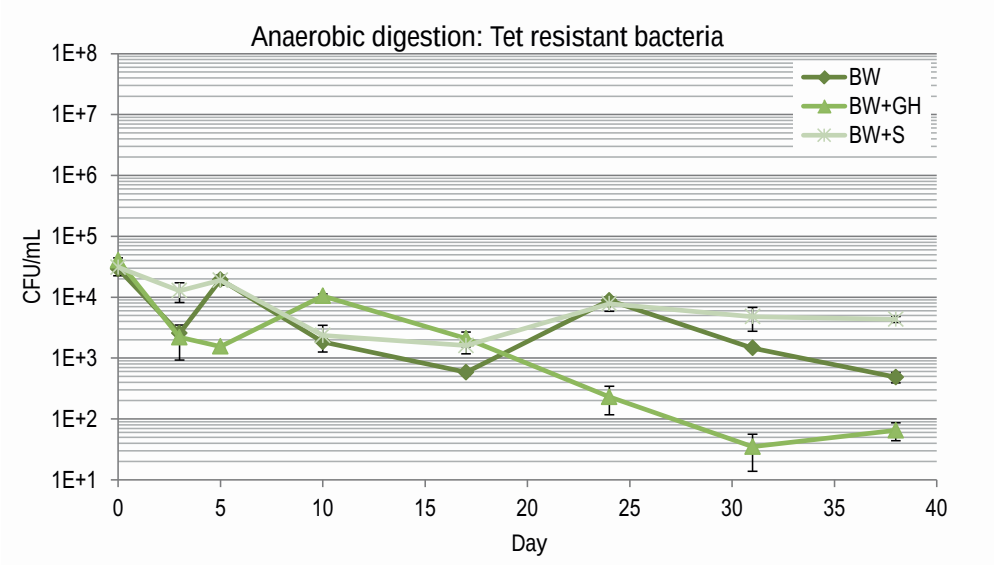
Again the composting had a substantial effect with the largest and second largest reduction occurring in samples 3 and 4 (3.0 and 3.2 log, respectively). The reduction was similar in mixtures 1, 2 and 5 (1.88; 1.78 and 1.83 log, respectively). Recovery treatment after the anaerobic digestion/aerobic storage experiments showed approx. 3 log recovery in the anaerobic sample containing BW+GH (Paper III).

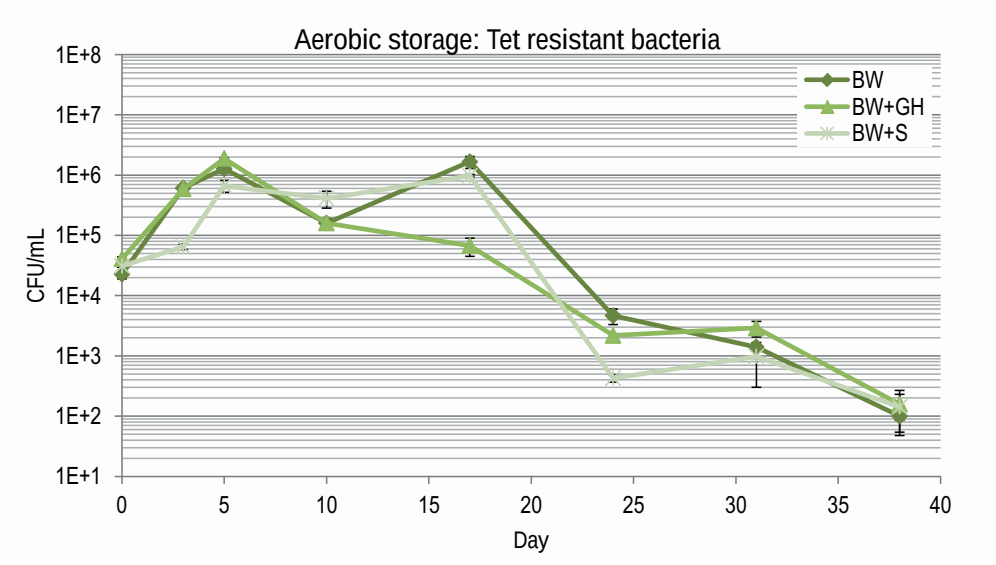
3.2.3 Antibiotic resistant Enterobacteriaceae

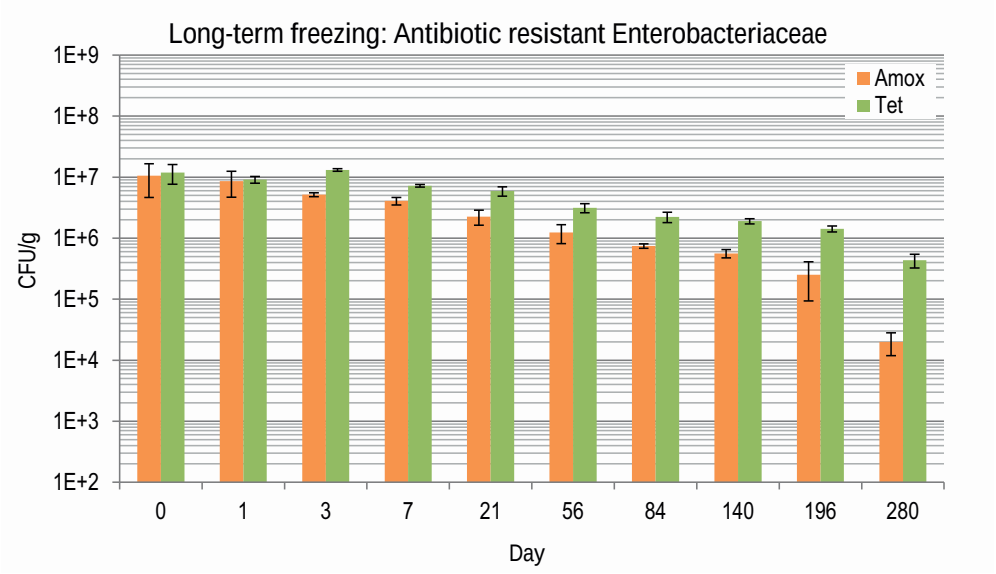
Antibiotic resistant bacteria belonging to the bacterial family Enterobacteriaceae were analyzed during the anaerobic/aerobic batch experiments (Paper III) as well as the long-term freezing and freeze/thaw experiments (Paper II). In the long-term freezing experiment bacteria resistant against four different antibiotics were tested (amoxicillin, ciprofloxacin, tetracycline and vancomycin) and in the freeze/thaw experiment amoxicillin resistant bacteria were tested. Amoxicillin and tetracycline resistant bacteria were analyzed during the anaerobic/aerobic batch experiments. Thus results for bacteria resistant against the two antibiotics that were analyzed in more than one experiment, namely amoxicillin and tetracycline, are presented in this section.

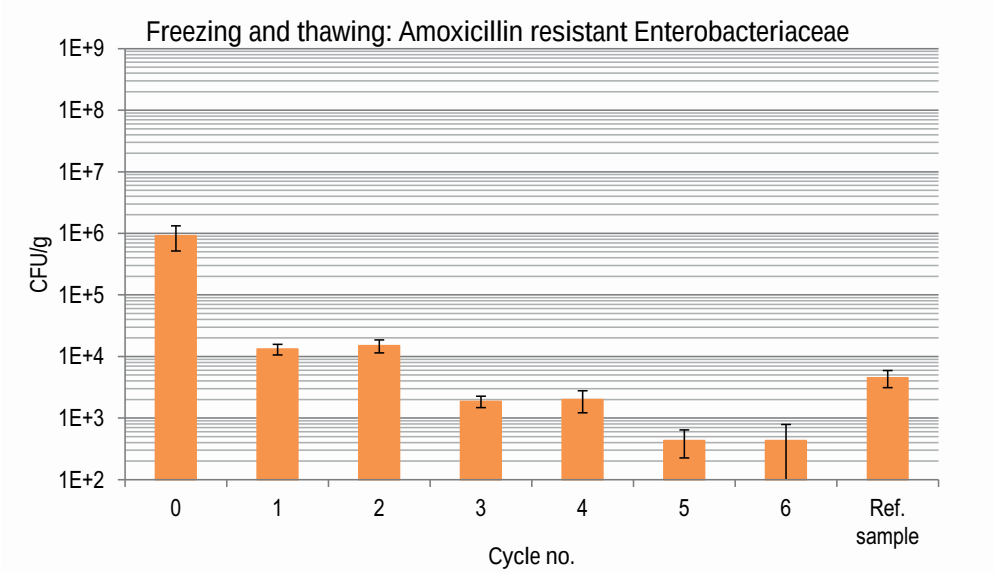

Anaerobic digestion: Amox resistant bacteria
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
0
5
10
15
20
25
30
35
40
Day
BW
BW+GH
BW+S


Aerobic storage: Amox resistant bacteria
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
0
5
10
15
20
25
30
35
40
Day
BW
BW+GH
BW+S


Anaerobic digestion: Tet resistant bacteria
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
0
5
10
15
20
25
30
35
40
Day
BW
BW+GH
BW+S


Aerobic storage: Tet resistant bacteria
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
0
5
10
15
20
25
30
35
40
Day
BW
BW+GH
BW+S





The reduction of amoxicillin resistant Enterobacteriaceae was similar during anaerobic digestion and aerobic storage in the mixtures containing BW+GH as well as BW+S (Paper III). Comparing the reduction in the sample containing BW, it was 2.52 log under aerobic conditions but only 0.51 log under anaerobic conditions (Paper III). During long-term freezing the reduction of amoxicillin resistant bacteria was 2.72 log (Paper II). Two different batches of dewatered wastewater were used for the long-term freezing experiment and the freezing and thawing experiment. The reduction during the two experiments can

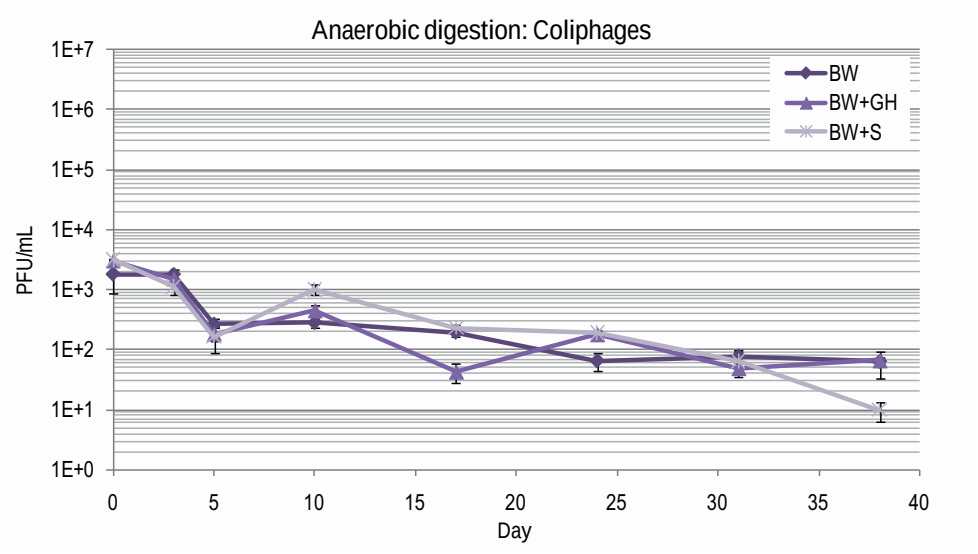
therefore not be compared directly. On the other hand, the reduction during the repeated cycles of freezing and thawing can be compared to the reference sample which was stored in the freezer while the freezing and thawing experiment took place (approx. 5.5 day). The six cycles of freezing and thawing resulted in a 3.33 log reduction while the reduction in the reference sample 1 log less, namely 2.31 log (Paper II), indicating an additional effect of the repeated freezing and thawing compared to storage in the freezer (the reference sample).

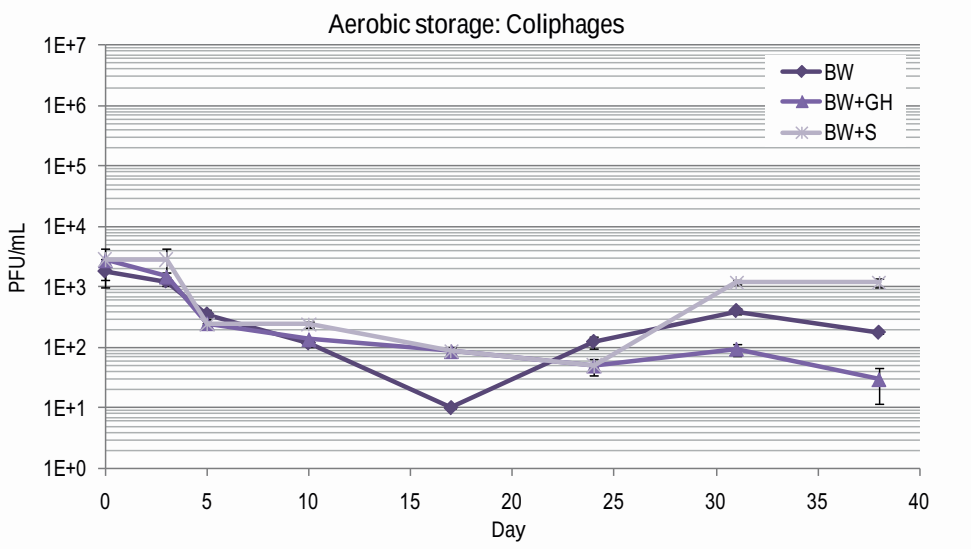
During anaerobic digestion and aerobic storage the reduction of tetracycline bacteria was similar in the samples containing BW and BW+GH. In the BW+S sample the reduction was significantly higher during aerobic storage, the overall reduction being 2.34 log while it was 0.85 log under anaerobic conditions (Paper III). The tetracycline resistant bacteria were more resistant against the long-term freezing than the amoxicillin resistant ones, and were reduced by 1.43 log after 280 days in frozen condition (Paper II).

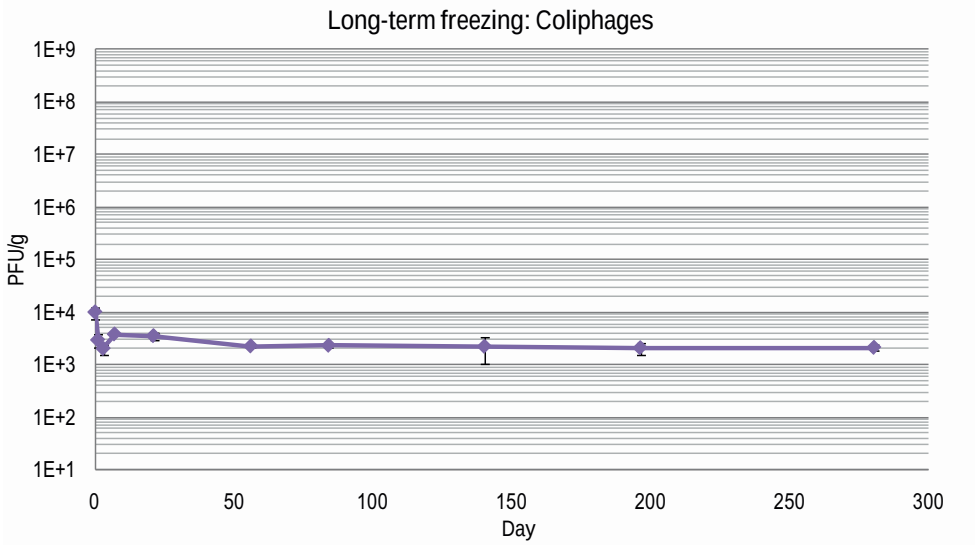
It is difficult to compare the effect of the different treatment methods/stress factors on the antibiotic resistant Enterobacteriaceae since it is a large family of many different gram-negative bacteria, e.g. *E. coli, Salmonella, Klebsiella* and *Shigella* and different batches of blackwater were used for each experiment. However, after the long-term freezing experiment (Paper II) and anaerobic digestion/aerobic storage (Paper III) a recovery treatment was conducted, and no or limited recovery was observed, indicating a lethal effect of both methods.

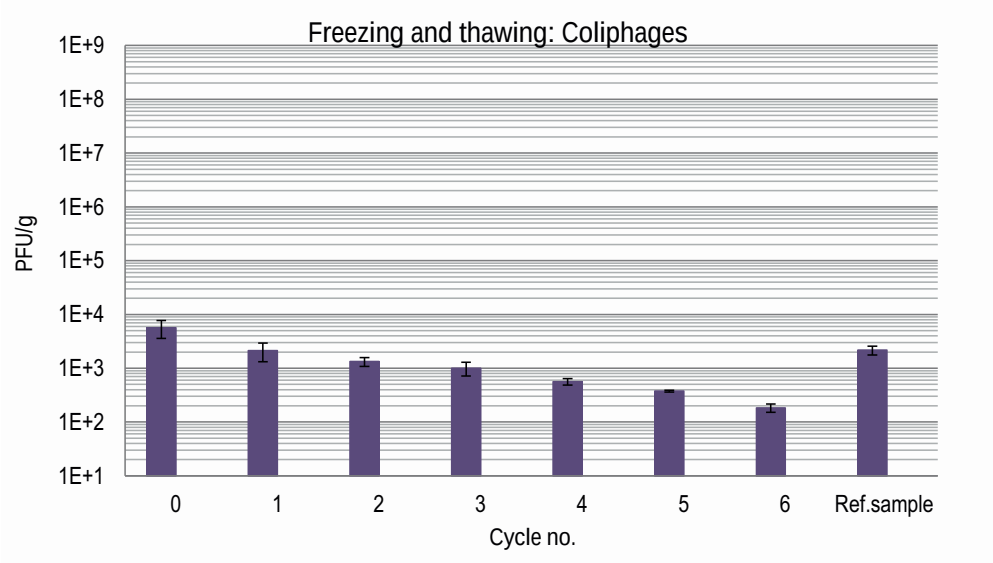
3.2.4 Coliphages

Coliphages were analyzed in the anaerobic digestion/aerobic storage experiments (Paper III), as well as in the long-term freezing and freeze/thaw experiments (Paper II). In the long-term freezing and freeze/thaw experiments the dewatered blackwater was inoculated with *E. coli* bacteriophage ΦX174 whereas indigenous coliphages were analyzed in the anaerobic digestion/aerobic storage experiments.









In the anaerobic/aerobic experiments the coliphages showed a better survival and even an increase in numbers under aerobic conditions, indicating that anaerobic digestion could be a more effective treatment method for viruses and phages than aerobic treatment methods (Paper III). The freezing had a limited effect on the phages, whereas the repeated freezing and thawing cycles had an additional effect (Paper II). The level of coliphages after 6 freezing and thawing cycles was significantly lower than the level in the reference sample ($p=0.0011$).

3.3 Pilot scale test facilities in Greenland

In the following two sections the results from testing of pilot scale test facilities in tourist huts and sights in Qeqqata Kommunia as well as in a single-home residence in Sisimiut will be presented.

3.3.1 Composting toilets in tourist huts and sights in Qeqqata Kommunia

The first composting toilet solution, designed for the tourist hut by the 1. Fjord in Qeqqata municipality will be installed in the spring 2012. The second toilet solution, which is based on a composting toilet solution from Iceland, was recently installed. User experience of the new toilets can therefore not be evaluated in this thesis. The second toilet solution has been used at tourist sites in the highlands of Iceland with good results, e.g. a large volume reduction of the composted black wastewater (Gunnarsdóttir et al., 2011). The new composting toilets in Qeqqata municipality will be observed by the municipality and ARTEK in the upcoming seasons, as well as the composting process which will monitored by e.g. temperature sensors in the composting containers.

3.3.2 Toilet facilities in a single-family residence in Sisimiut

The family experienced an improved in-door hygienic status with the installation of the urine separating composting toilet, e.g. with less odour problems than earlier experienced when using a bucket toilet. Maintenance and cleaning of the toilet was found to be easy. However, different challenges occurred, both technical, such as freezing of the urine pipe and odour from the composting container outside the house. The family also found it problematic to transfer the faecal matter themselves to the composting container. It was therefore decided to install a new type of toilet in the residence; a low flush toilet, connected to an underground holding tank. This toilet used approx. 1 L pr. flush, and the volume of the collection tank was designed for an emptying frequency of approx. every 3 months. The overall experience of the low-flush toilet was good, but again, some technical challenges occurred which had a negative effect on the user experience. There were for instance problems with the water trap which caused odour from the collection tank inside the toilet room. This was not due to the toilet construction, but the installation by inexperienced local plumbers who did not know how to remediate the situation. A lesson learned from the installation and use of the two alternative toilets was therefore the importance of training of technical personnel in maintenance of new toilet solutions. Regarding the usage of composting toilets in private homes, it is likely that solutions of that kind would be better accepted if hauling of the toilet waste was done by professionals instead of the home owner. Collective hauling done by trained personnel also minimizes the risk of contact to blackwater and spreading of diseases (Paper I) and secures uniform handling of the blackwater and safe final disposal. The experience gained from testing of the different toilet solutions is an important factor in optimizing possible future installation of similar toilet solutions under Greenlandic circumstances.

Chapter 4 Discussion

In this chapter the experimental results will be discussed, and subsequently the future outlook for possible wastewater treatment methods in Greenland. By the end of the chapter further research needs will be addressed.

4.1 Discussion of experimental results and recommendations

It has been addressed that discharging of untreated wastewater can have severe consequences for the Arctic marine environment, due to the vulnerability of the Arctic nature (AMAP, 2009). Discharging of Pharmaceuticals and Personal Care Products may be of special concern in the Arctic (Kallenborn et al., 2008). Spreading of antibiotic resistance is of global concern, and sewage is considered being a hotspot for antibiotic resistance genes (Zhang et al., 2009). It is therefore important to study their survival under different wastewater treatment processes, which has been done in the present PhD project, in addition to the survival of other microorganisms.

During the long-term freezing *E. coli* was non-detectable after 56 days and no recovery was observed, indicating a lethal effect of the freezing on *E. coli* (Paper II). It has been shown that coliforms may not be a good indicator for reduction of microorganisms during freezing, since they are more sensitive to freezing than e.g. streptococci (Sanin et al., 1994; Gao et al., 2009). In the anaerobic digestion and aerobic storage batch experiments the reduction of *E. coli* was rapid under both anaerobic and aerobic circumstances, apart from the mixtures containing blackwater and Greenlandic halibut, where the survival of *E. coli* was better for the first 24 days, presumably due to more available carbon than in the BW and BW+S samples (Paper III). The results of the subsequent recovery study indicated that a certain fraction of *E. coli* in the aerobic BW sample had been injured but capable of resuscitation (Paper III). Survival of *E. coli* in the composting experiment indicated that even though thermophilic temperatures were not reached in any of the mixtures the reduction of *E. coli* was considerable, apart from the mixture of blackwater, peat and shrimp waste (BW+P+SW) where the reduction was actually lower than in the reference mixture (section 3.2.1). This might be explained by the temperature profile of this mixture which was overall lower than for the other mixtures. The water content was also higher in this mixture which could have had a negative effect on the composting process. The temperature profiles of the composting mixtures were in general low which may have been due to different factors, having a negative effect on the composting process, such as the dry matter content which was approx. 22-24% in mixtures 1-4 in the beginning of the experiment. In mixture 5 it was lower, approx. 14%. Del Porto and Steinfeld (2000) found optimum moisture content for the composting process to be 45-70%, and Kalkoffen et al. (1995) as well as Boisen (1995) got similar results; 55% and 50-70%, respectively. The moisture content in the mixtures in the present experiment was approx. 76-86%, which is higher than the reported optimum moisture

conditions. This may have caused a limited oxygen access, resulting in a reduced microbial activity in the composting mixtures (Holtze and Backlund, 2003), and thus lower temperatures. The C/N-ratio is another factor that may have affected the composting process. The measured C/N-ratio before start-up of the composting experiment ranged from approx. 21-28:1 in mixtures 1-4, but was substantially lower in mixture 5; approx. 18:1. It has been shown that 30:1 is an optimum ratio (Del Porto and Steinfeld, 2000) and others have reported 27:1 (Boisen 1995) and 25:1 (Esrey, 1998). The C/N-ratio in mixtures 1-4 was near these numbers in the beginning but was considerably lower at the end of the experiment, approx. 13-16:1. Since sampling was done exclusively prior to startup of the experiment and at the end, it is not possible to evaluate how quickly the C/N-ratio was reduced, and thus how long it has been too low compared to the reported optimum values. The size of a composting reactor and thus the amount of composting material also influences the temperature development (Holtze and Backlund, 2003). Del Porto and Steinfeld, (2000) reported that high temperatures cannot be expected in ventilated composting systems consisting of small mass of composting material, which was the case in the present study. Moe et al. (2001) measured temperatures in source separating composting toilet systems in El Salvador, and found temperatures in the composting material ranging from 20-37.5°C with an average of 27.2°C. It has also been reported that when composting e.g. household waste, a rapid reduction of volume as well as pathogen levels can occur under high temperature conditions (Holtze and Backlund, 2003). On the opposite, volume and pathogen reduction in composting toilet systems has been shown to occur slowly and without development of high temperatures (Holtze and Backlund, 2003). The volume reduction in this kind of systems is related to activity of slower microorganisms while reduction of pathogens can occur due to competition and antagonism (Holtze and Backlund, 2003). However, higher temperatures may be expected under secondary composting of larger volume of composting material (Holtze and Backlund, 2003). In an experiment where material from composting toilets was transferred to an un-insulated secondary composting container the temperature in the composting material increased to 61-72°C within 3-11 days, even in frosty weather (Holtze and Backlund, 2003). The mass reduction during the present experiment was considerable, but the VS content was still high after the composting. The high liquid content may have been related to the high concentration of organic matter, which has a high liquid storing capacity (Kalkoffen et al., 1995). pH in the composting mixtures ranged from approximately 6.8-7.2, which is favourable for the substrate degrading microorganisms, but still not in a range (approximately 9 or higher) where it could have had a pathogen reducing effect in itself (Holtze and Backlund, 2003). Standard numbers for concentration of phosphorus in faecal matter have been reported to range from 13.4 to 17.0 g/kg TS (Del Porto and Steinfeld, 2000; Vinnerås, 2001; Wrisberg et al., 2001). The concentration of phosphorus in the composting mixtures before the experiment was started were within or close to this range, but increased during the composting process. Concentrations of potassium in the composting mixtures before the composting process took place were 5.2-8.5 mg/kg TS. This is lower than earlier reported numbers for potassium in faecal matter which range from 13.4-37.0 g/kg TS (Del Porto and Steinfeld, 2000; Vinnerås, 2001; Wrisberg et al., 2001). This may have been due to leaching of water soluble potassium (Holtze and Backlund, 2003) because of the flushing water in the vacuum toilets.

Regarding the effect of the different processes on faecal streptococci the long-term freezing did not affect them significantly and they showed less than 1 log reduction during the 280 days of freezing (Paper II). On the other hand the reduction of faecal streptococci was strong under both anaerobic and aerobic circumstances, but it was faster in the aerobic samples (Paper III). In the anaerobic mixtures the reductions ranged from 4.6-6.1 and in the aerobic mixtures from 5.0-5.5 log (Paper III). The composting also had a substantial effect with reduction ranging from 1.78 to 3.2 log (section 3.2.2). How rapidly the reduction of both *E. coli* and faecal streptococci during the composting experiment can however not be evaluated and compared to the other experimental results since samples were taken exclusively prior to experimental start-up and at the end of the experiment. Results of the recovery study after the anaerobic digestion/aerobic batch experiments indicated that a certain fraction of faecal streptococci in the anaerobic and aerobic samples containing BW+GH had been injured during the experimental period but still able to resuscitate under recovery treatment (Paper III).

Antibiotic resistant bacteria were analyzed in the long-term freezing and freezing and thawing experiments (Paper II) as well as the anaerobic digestion/aerobic batch experiments (Paper III). During long-term freezing antibiotic resistant bacteria were reduced slowly but constantly during the 280 days of freezing, but to a less degree than *E. coli.* However, repeated freezing and thawing cycles had an additional effect compared to long-term freezing on amoxicillin resistant enteric bacteria (Paper II). In the anaerobic digestion experiment the tetracycline resistant bacteria seemed to have survived better in the mixture of BW+S, which was the mixture having the lowest CH_4 yield whereas the decrease was greatest in the BW+GH sample which had the highest CH_4 yield (Paper III). This may have to do with increased bacterial competition in the more active reactors (Paper III). On the other hand, adding Greenlandic halibut and shrimp offal did not seem to have an effect on the amoxicillin resistant bacteria. Regarding the antibiotic resistant enteric bacteria in general the anaerobic environment seemed to have a limiting effect on growth of both amoxicillin and tetracycline resistant bacteria in the first part of the experiment, compared to the aerobic environment where growth of bacteria was observed for the first days.

The coliphages were analyzed in the long-term freezing and freezing and thawing experiments (Paper II) as well as the anaerobic digestion/aerobic batch experiments (Paper III). The results of the latter experiments indicated that the indigenous coliphages survived better under aerobic conditions, and even showed an increase in numbers at the end of the experiment. However the freezing did not have a significant effect on the inoculated coliphages analyzed in that experiment. On the other hand, alternating freezing and thawing cycles had an additional effect compared to long-term freezing, showing that each cycle, involving stress due to the freezing and thawing processes, had a further reducing effect.

Finally, *Salmonella* was analyzed, but only in the long-term freezing experiment where the inoculated *Salmonella* Enteritidis was found to be non-detectable after freezing for 4 and 7 days using XLD and BHI agar, respectively, as growth medium (Paper II). However, *Salmonella* showed some recovery, indicating that there still were viable bacteria left in the frozen sample and that a fraction of them has been injured

(Paper II). Since *Salmonella* is a pathogen, frequently found in wastewater, it is alarming that it can survive in an injured state for such long periods of freezing. Another subject of concern is that sublethal exposure to different stresses may enhance the survival of bacteria under subsequent stress conditions, resulting in cross-protection against other stresses (Abee and Wouters, 1999).

None of the tested processes had the ability to completely hygienize the blackwater, and based on the conducted laboratory experiments it is difficult to recommend one of the processes over another. Freezing has the potential of reducing microorganisms in wastewater and seemed to have a lethal effect on some microbial groups, as the coliforms, a sublethal effect on other microorganisms, such as *Salmonella* but only a minimal effect on others, e.g. the coliphages, which were used as an indicator of human viruses. Freezing alone, however is neither appropriate for total elimination of the microbiota in wastewater nor for reducing them to below accepted risk levels (Paper II). The results of the anaerobic digestion showed a substantial reduction of the analyzed microorganisms and microbial groups (Paper III). However, in big scale anaerobic digesters there is a continuous flow into the digesters of fresh substrate and thus microorganisms, meaning that their survival in big scale plants would be different and possibly better than what can be expected in batch experiments. It has e.g. earlier been stated that the faecal enterococci reduction is rarely more that 1-2 log units in mesophilic biogas plants, continuously fed with fresh biomass (Bendixen, 1994), which is less than what the results of the present batch experiments showed (Paper III). On the other hand, *E. coli* and faecal streptococci showed a considerable reduction during the composting experiment, even though it is questionable how well-functioning the composting process was in the present study. In composting systems on a bigger scale, including secondary composting and professional management, higher temperatures might be expected, resulting in a higher reduction of *E. coli* and faecal streptococci. However, if recommendation of selection of certain processes is to be done, other factors will have to be taken into consideration, such as cost and operational complexity. Table 4.1 compares the three tested processes; freezing/freezing and thawing, anaerobic digestion and composting; in terms of initial and operational cost, maintenance complexity, need of energy input and microbial reduction.

	Freezing	Freezing and thawing	Composting	Anaerobic digestion (simple)	Anaerobic digestion (advanced)
Initial cost	+	++	+	++	+++
Operational cost	+	++	+ to ++	++	+++
Maintenance complexity	+	+	++	++	+++
Energy input	Neutral	Positive	Positive*	Negative/Neutral **	Positive***
Microbial reduction					
E. coli	+++	NA	+ to +++	+++	NA
Faecal streptococci	+	NA	++ to +++	+++	NA
AR enteric bacteria	++	+++	NA	+ to +++	NA
Coliphages	+	++	NA	++	NA
Salmonella	+++	NA	NA	NA	NA

*Table 4.1. Comparison of the tested processes in terms of cost, complexity and microbial reduction, the latter one based on experimental results. +: Low, ++: Medium, +++: High. *Energy input probably required for at least part of the year. **The produced energy could be used for heating of the biogas digester, but energy input may still be required dependant on the local climate. ***The produced energy could be used for heating of the biogas digester, but additional energy input would be required if thermophilic digestion was to be used. Abbreviations: AR: Antibiotic resistant, NA: Not analyzed.*

Incorporating freezing in the design of wastewater treatment methods for Greenland where winter periods are long may be a cost-effective way to enhance the quality of treated wastewater. The initial and operational cost is low and maintenance of the process does not require technically skilled personnel. Additional energy is not needed since the natural conditions in Arctic climate ensure long periods of freezing, followed by a thawing period. The laboratory experiments indicated that microbial reduction

would vary, dependant on the microorganisms or microbial groups in question. If repeated freezing and thawing was to be used, thawing of the wastewater would require energy input, thus the higher initial and operational cost in table 4.1. The maintenance complexity would probably not be higher, and an advantage of alternating freezing and thawing over freezing is the higher microbial reduction. The initial cost of composting is low as well as the operational cost, given that local residue materials are available for use as bulking materials. If commercial bulking materials have to be used the operational cost would be higher. The complexity of the process is somewhat higher than the freezing and would thus require some training of the maintenance personnel. In the cold climate the composting material would freeze during winter without an energy input, but freezing might be beneficial for the structure of the material (Hedström and Hanæus, 1999) as well as microbial reduction (Paper II). During the short summer some energy input might be needed to accelerate the composting process. The microbial reduction would vary, dependant on diverse process parameters, such as temperature range (mesophilic/thermophilic). The anaerobic digestion is the process that would require the highest initial and maintenance cost if an advanced biogas plant was selected. The reducing effect on microorganisms would vary dependant on e.g. the operational temperature (mesophilic/thermophilic). The microbial reducing ability of advanced anaerobic digestion has not been mentioned in table 4.1, since the experimental results are based on mesophilic anaerobic digestion. However, an advanced biogas plant could include an additional hygienic step, e.g. additional heat treatment in a batch reactor, which would give a higher microbial reduction than mesophilic digestion (Christensen, 1998). The complexity of the process is higher than the freezing and the composting and might therefore require more comprehensive training of the maintenance personnel. However, the size and complexity of biogas plants vary, and small anaerobic digesters have been proven to be successful in small villages, but this has mainly been tested in warmer climate than in the Arctic (Bensah, 2009). An advantage of the anaerobic digestion is the energy production of the process, which can be used for heating of the digester or even further treatment of the degassed biomass.

One recommendation for wastewater treatment in Greenland is to combine the different treatment processes. The optimum combination of processes might be different for each community, depending on e.g. available substrates (including blackwater and other organic waste fractions), climate, access to technical assistance, settlement pattern, etc. Based on the experimental results and the effect of the tested processes in means of microbial reduction, a solution for a settlement could be to install modern composting toilets in the residences with subsequent onsite treatment of the blackwater. Professional collection and central secondary composting may also be beneficial in different ways, e.g. to secure a uniform handling of the composting material. It is also an advantage for the temperature development in the composting material to mix larger volumes of organic matter (Holtze and Backlund, 2003). Other organic waste fractions, such as household waste, can be composted along with the blackwater. Solar energy may be used during summer to heat the composting material, which is also the time of the year where the composting would take place. During winter it would be an advantage to utilize the natural freezing to enhance the microbial reduction and improve the structure of the material (Hedström and Hanæus, 1999).

Another possibility for the non-seweraged settlements in Greenland would be to replace the bucket toilets with low flush toilets and transport the blackwater with vacuum or pressure sewerage pipelines, to a centralized treatment plant, e.g. an anaerobic digester. The blackwater can also be stored in storage tanks outside the residences, or possibly in the basement of residences. However, this solution would require vehicles equipped with vacuum pumps to empty the tanks. It was discussed in Papers I and III that mesophilic digesters might be more robust than thermophilic since the latter ones are more sensitive against variations in operational conditions such as the organic loading rate, temperature and the characteristics of the influent sludge (van Lier, 1996; Kim et al., 2002). Besides, more energy would be needed to heat a thermophilic biogas plant in the cold climate. Other factors, such as lack of technical personnel in the settlements as well as remote locations, favour the use of small and simple biogas plants in the settlements (Paper I, III). However, mesophilic anerobic digestion would most likely not hygienize the blackwater (Larsen et al., 1994; Cabirol et al., 2001; Sahlström et al., 2004). If there is a surplus from the gas production it could possibly be used to hygienize it in a subsequent batch reactor under higher temperature conditions. Adding a hygienizing step to the anaerobic digestion process would, however, make the technical configuration of the treatment plant more complicated and thus more challenging to maintain. A biogas plant might also be heated by means of solar energy, but this would though be limited to the summer period, which in the Arctic is short. A possible surplus of gas could also be used to dewater the degassed biomass. Another possibility for dewatering of the digestate is to dewater it by incorporating natural freezing into the treatment processes, since freezing has been recognised as being a cost-effective and simple way to dewater sludge (Vesilind and Martel, 1990; Sanin et al., 1994; Martel et al., 1998; Hedström and Hanæus, 1999). By dewatering the degassed biomass, two fractions would be obtained: A liquid fraction, rich in nutrients, and a fibre part, less nutrient rich but more suitable for composting than the degassed biomass due to the higher dry matter content. The liquid fraction could be treated further in order to decrease the content of microorganisms, e.g. in Filtralite P™ filters which have been used with good results regarding reduction of both bacteria and viruses (Heistad et al., 2006), or in peat and/or sand filters if local soil conditions are suitable. Peat filters have been found to be highly effective for both viral and bacterial reduction in wastewater, as well as breakdown of organic matter and removal of e.g. trace metals (Simmering and Martin, 1997). Sand filters have been shown to be successful in reduction of e.g. *Salmonella* and coliforms as well as enterococci (Vanotti et al., 2005). The fibre part from a dewatering process can be post-treated by composting, possibly with the addition of organic household waste. In this way the three processes can be combined; a small, simple anaerobic digester, followed by dewatering by freezing or alternating freezing and thawing, and at the end composting (and filtration of the liquid part). This combination has the possibility of a good microbial reduction, done by combining relatively simple processes.

In the towns where bucket toilets are still in use, the same solutions as discussed for the settlements may be used. The solutions selected in the towns might even be more technically challenging since possibilities of technical supply are better than in the settlements. Parts of the towns have conventional flush toilets but are not connected to sewerage. Those residences have a storage tank for the blackwater

outside the residence. In some cases the greywater is discharged directly to the ground. A solution for treatment of wastewater from those residences that today store the blackwater in storage tanks might be to withhold the present solutions, but instead of discharging the blackwater to the sea it might be transported to an anaerobic digester. The greywater might be stored in a separate tank or treated onsite, e.g. by the use of biofilters. In most of the towns, e.g. in Sisimiut in West Greenland, there are waste incinerators, producing considerable yearly amount of waste heat. A biogas plant might possibly be located near a waste incinerator and the waste heat from the incinerating could be used for heating of the biogas plant. This might even allow for thermophilic anaerobic digestion, resulting in a higher biomass reduction and a better hygienized blackwater (Christensen, 1998). In the parts of the towns that are served by conventional sewerage pipelines, care has to be taken if considering installation of low flush toilets or vacuum toilets in the residences. Connecting toilets with reduced volume of flushing water to the existing sewerage system would reduce the velocity of the wastewater in the pipelines, possibly causing deposition of organic and inorganic matter which can lead to clogging or freezing in the pipelines. It might be most beneficial to maintain the flush toilet solutions, while introducing a treatment step prior to discharging to the recipient, such as simple mechanical treatment, primarily to remove particulate matter. That kind of treatment is widely used in the Nordic countries, such as in Iceland (UST, 2003) and parts of Norway (Vogelsang et al., 2006). Mechanical treatment might even be followed by further treatment, e.g. chemical precipitation or for smaller systems, sand filtration. Both these additional systems will reduce microbial discharge. However, in new built areas in the towns or where the sewerage pipelines need to be renewed, it might be beneficial to install low flush or vacuum toilets in the residences with connection to pressure or vacuum sewer pipelines, or have collection at the household level.

4.1.1 Alternative toilet solutions

As described in the introduction of the synopsis and Paper I, bucket toilets are still used in parts of the Greenlandic towns and almost all of the settlements. Besides, most towns and settlements in Greenland are located on islands or peninsulas where the available catchment areas are relatively small (Hansen and Niclassen, 1996). Vacuum toilets or low flush toilets are considered a good choice where water supply is scarce since the water usage pr. flush is considerably lower than what is used for conventional flush toilets. Vacuum and low flush toilets typically use 0.5 - 1 L pr. flush (Zifu et al., 2002; Jenssen et al., 2004), compared to conventional flush toilets which require approx. 4 - 9 L pr. flush (Zifu et al., 2002). When using composting toilets, collection and treatment can be on both household as well as central level. They might be a good alternative to the bucket toilets in non-seweraged residences, both in the towns and settlements. Modern composting toilets can be urine diverting or not. The urine diversion has several benefits, e.g. that urine is separated from the small amount of faecal matter where most of the pathogens are contained (WHO, 2006), reducing the volume to be hygenized substantially. Another benefit of urine diversion is that more pharmaceuticals and their metabolites are excreted via urine than via faeces (Winker et al., 2008), thus urine separation and separate handling of the urine is a promising approach to lower the pharmaceutical load of raw domestic wastewater, protecting the aquatic environment from pharmaceuticals. The faecal matter can be treated by composting and the urine can be

stored until it is safe to dispose or use as fertilizer, e.g. in South Greenland where agriculture is practised. Testing of a urine diversion composting toilet in a private home in Sisimiut showed that solutions of that kind are likely to be better accepted by the users if hauling of the toilet waste is done by professionals instead of the home owner. At remotes locations where urine cannot be properly infiltrated or used, e.g. in tourist huts, composting toilets without urine diversion might be a better solution (Paper I).

Both composting and low flush toilet solutions facilitate composting or anaerobic digestion of blackwater since the human excreta is not mixed with large amounts of water. Low flush toilets give the opportunity to have both collection and treatment at the household level, or collection at the household level and centralized processing. For centralized processing either pump and haul systems or pressure sewer and vacuum sewer systems are alternatives to gravity sewers. Pressure sewer and vacuum sewer systems need the same insulation as traditional sewers, but are less vulnerable to gradient changes that may occur in permafrost areas. Vacuum and pressure sewer systems also allow for usage of smaller diameter pipelines (U.S. Congress, 1994). By decentralized collection of blackwater and onsite/in-house treatment of the greywater, for instance by using biofilters, the need for expensive secondary sewer collection systems can be reduced or even eliminated (Heistad, 2008; Karabelnik et al., 2010).

4.1.2 Future research tasks

The task of selecting suitable treatment methods for Greenland and the different conditions in each settlement and town is a challenge. However, the small settlements where bucket toilets are used and no sewage pipeline systems exist might give a good opportunity to test the above mentioned toilet solutions and treatment methods. The conducted laboratory studies and results hereof can however not give a complete picture of a well suited wastewater treatment solution for Greenlandic conditions. First of all only three methods were tested, and other methods might be suitable for the Greenlandic communities as well, e.g. septic tanks, usage of UV-radiation, and others. Secondly, more laboratory studies are needed in order to refine the methods and adjust them to the Arctic climate and circumstances. However, the results from this work can be seen as an indication of the methods being well suited in Greenland, and as a first step towards a more sustainable way to handle wastewater in the Arctic, namely by treating it as a resource rather than waste.

Wastewater treatment systems are mainly developed for temperate regions and little is done to specifically address the needs of the Arctic. In Paper I the advantages of using decentralized treatment methods were discussed, and it was shown that the blackwater can be safely treated or collected onsite. However, solutions for greywater treatment in Greenland are another subject that should be focused on, especially since it is discharged directly to the ground in almost all settlements and parts of the towns. Compact onsite methods for greywater treatment exist (Heistad, 2008; Karabelnick et al., 2010), but need further development to be better suited for Arctic conditions. The use of pressure or vacuum sewers under Arctic conditions should also be explored. Such alternative transport systems are interesting from

an economical point of view and can transport wastewater to areas suited for local treatment systems without the need for a large collection network.

The long-term freezing and repeated freezing and thawing experiments showed the advantages of freezing as a possible treatment method against certain microorganisms. Studies of possibilities to take advantage of the cold climate when designing treatment methods for Arctic regions should therefore also be conducted. Development and testing of small and simple treatment units, for instance composting toilets and anaerobic digestion plants, under cold conditions would also be valuable for small and often remotely located Arctic communities, like the Greenlandic settlements. It would also be relevant to conduct studies that combine the different treatment methods which have been tested separately in this PhD project.

It has earlier been addressed, that substances in the wastewater, such as heavy metals and pharmaceutical residues might pollute the recipients in Greenland. However, no regular measurements of the concentrations of those substances in the Greenlandic wastewater streams are done and thus it cannot be evaluated whether or not the discharging of them is harmful for the environment (Danish EPA, 2005). Earlier studies from other parts of the Arctic marine environment have shown a slower breakdown of various pharmaceuticals and personal care products (PPCPs) under Arctic conditions due to the cold climate, lack of sunlight, and other factors (Kallenborn et al., 2008), but this has not been studied in the Greenlandic marine environment. Such analyses are needed to better assess the risk of pharmaceutical residues and microbial agents, in the aquatic environment of Greenland. It is relevant to study the effect of different wastewater treatment methods, as the processes tried in this PhD project, for degradation of PPCPs.

Chapter 5 Conclusion

The tested processes did not have the ability to fully hygienize the blackwater, but some of the microorganisms and microbial groups were reduced substantially during the laboratory experiments, e.g. antibiotic resistant bacteria, which is important in order to reduce the spreading of antibiotic resistance. Other factors also play a role when selecting a suitable treatment method, e.g. operational and maintenance cost. Combining the methods might enhance the microbial reduction. This can be done in various ways, dependant on the community in question and the available organic waste fractions as well as technical facilities. One recommendation for the settlements is to combine composting and freezing or alternating freezing and thawing. Another alternative could be to use small and simple biogas plants, followed by dewatering of the degassed biomass, either by utilizing possible surplus of energy from the biogas plant or natural freezing, which could be a more cost-effective way. Subsequently, the liquid part can be treated by filtration and the fibre part can be composted. These combinations of relatively simple processes have the possibility of a good microbial reduction. In the non-seweraged parts of the towns, the same combination could be utilized, but more advanced biogas plants could also be used, for instance with additional heat treatment, even by utilizing waste heat from the waste incinerators. For the seweraged parts of the towns it might be most beneficial to maintain the flush toilet solutions, while introducing a treatment step prior to discharging to the recipient, such as simple mechanical treatment, primarily to remove particulate matter. Mechanical treatment might even be followed by further treatment, e.g. chemical precipitation or for smaller systems, sand filtration.

During the long-term freezing *E. coli* was non-detectable after 56 days and no recovery was observed subsequently, indicating a lethal effect of the freezing on *E. coli* (Paper II). In the anaerobic digestion and aerobic storage batch experiments the reduction of *E. coli* was rapid under both anaerobic and aerobic circumstances (Paper III). The results of the subsequent recovery study indicated that a certain fraction of *E. coli* in the aerobic sample of blackwater has been injured but capable of resuscitation (Paper III). Survival of *E. coli* in the composting experiment indicated that even though thermophilic temperatures were not reached in any of the substrate mixtures the effect on *E. coli* was considerable, apart from in the mixture of blackwater, peat and shrimp waste where the reduction was lower than in the reference mixture (section 3.2.1). The temperature profiles of the composting mixtures were in general low which may have been due to different factors, such as the C/N-ratio, moisture content or size of the reactors used in the experiment.

Faecal streptococci was not reduced significantly during long-term freezing (Paper II) but showed a strong reduction under both anaerobic and aerobic circumstances, though being faster in the aerobic samples (Paper III). The composting had a substantial effect on the faecal streptococci as well (section 3.2.2). Results of the recovery study after the anaerobic digestion/aerobic batch experiments indicated that a certain fraction of faecal streptococci in the anaerobic and aerobic samples containing blackwater

and Greenlandic halibut has been injured during the experimental period but were able to resuscitate under recovery treatment (Paper III).

During long-term freezing antibiotic resistant bacteria were reduced slowly but constantly, but to a less degree than *E. coli* (Paper II). Repeated freezing and thawing cycles had an additional effect compared to long-term freezing on amoxicillin resistant enteric bacteria (Paper II). In the anaerobic digestion experiment the tetracycline resistant bacteria survived better in the mixture having the lowest CH_4 yield whereas the decrease was greatest in the mixture with the highest CH_4 yield (Paper III). In the first part of the experiments the anaerobic environment seemed to have a limiting effect on growth of both amoxicillin and tetracycline resistant bacteria, compared to the aerobic environment where growth of bacteria was observed for the first days.

Results of the anaerobic digestion/aerobic batch experiments indicated that the indigenous coliphages survived better under aerobic conditions, and even showed an increase in numbers towards the end of the experiment. However the freezing did not have a significant effect on the inoculated coliphages analyzed in that experiment. Repeated freezing and thawing cycles were shown to have an additional effect in reducing the coliphages compared to long-term freezing.

Salmonella was only analyzed in the long-term freezing experiment where the inoculated *Salmonella* Enteritidis was found to be non-detectable within one week of freezing (Paper II). However, *Salmonella* showed some recovery, indicating that there were still viable bacteria left in the frozen sample and that a fraction of them has been injured (Paper II).

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Appendix: Papers I – III

Paper I

Gunnarsdóttir, R., Jenssen, P.D., Jensen, P.E.,Villumsen, A., Kallenborn, R., 2012. A review of wastewater handling in the Arctic with special reference to Pharmaceuticals and Personal Care Products (PPCPs) and microbial pollution. Accepted for publication in Ecological Engineering.

http://dx.doi.org/10.1016/j.ecoleng.2012.04.025

A review of wastewater handling in the Arctic with special reference to Pharmaceuticals and Personal Care Products (PPCPs) and microbial pollution

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Key Words

Arctic; wastewater; public health; pharmaceutical residues; antibiotic resistant bacteria; composting; anaerobic digestion

Abstract

Treatment of wastewater is often inadequate or completely lacking in Arctic regions. Wastewater contains different kinds of substances that can be harmful for the environment and human health, including residues of pharmaceuticals and personal care products. Bioaccumulation and biomagnifications of chemicals in the food web are of concern. This can affect fishery that is a significant industry in many Arctic coastal regions. Wastewater from human settlements may also contain antibiotic resistant bacteria and pathogens that can cause negative impacts on human health and the environment. In the Arctic, especially, the direct release of untreated sewage may have severe consequences for the receiving

environment due to low biological diversity, low ambient temperatures and consequently high vulnerability of the Arctic ecosystem to environmental contaminants.

Bucket toilets are common in remote settlements but are also used in towns. In settlements having inadequate sanitary facilities the risk of contracting diseases, such as hepatitis A, is unacceptably high. Conventional centralized wastewater collection systems and treatment plants are a challenge to build in the Arctic and expensive to operate. Thus alternative methods are needed. Possible solutions are improved dry or low flush toilets with collection of toilet waste at the household level and subsequent centralized treatment by dry composting or anaerobic digestion. Both treatment methods facilitate co-treatment of wastewater along with other organic waste fractions and provide a byproduct that is environmentally safe and easy to handle. Combining the above with decentralized greywater treatment will reduce the costs for expensive infrastructure.

1. Introduction

It is challenging to design, construct and operate wastewater collection systems in the Arctic because of permafrost conditions, hard rock surfaces, freezing, flooding in the spring, limited quantity of water, high costs of electricity, fuel and transportation, as well as a settlement pattern with limited accessibility, especially in the rural parts of the Arctic. The cold climate influences the efficiency of biological treatment processes in particular, but also chemical processes (Smith and Low, 1996). The most important present types of wastewater collection systems in the Arctic are listed in figure 1 (Smith and Low, 1996):

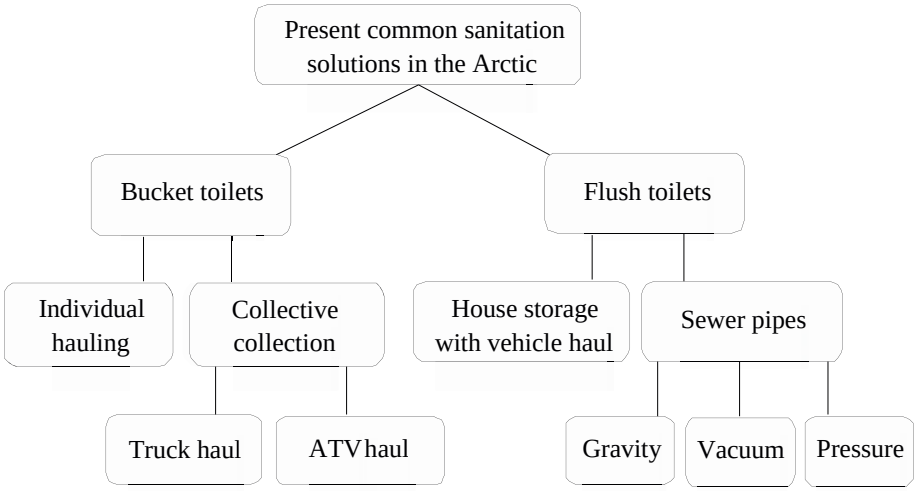

Figure 1. The main types of wastewater collection systems in the Arctic. Abbreviation: ATV: All Terrain Vehicle.

Bucket toilets are still widely used in many of the northern communities, such as in Canada's northern areas and some parts of Alaska and Greenland. This particular toilet solution has been considered a problem for many years with respect to uncontrolled spreading of nutrients, diseases and potential pollution issues. As indicated in figure 1 the hauling of waste from bucket toilets is either done individually or collectively. The health and convenience level is considered being low when hauling is done individually due to limited water usage and varying individual disposal practices (Smith and Low, 1996). Those factors are improved when the hauling is done collectively by municipal or private organized operators. The initial construction costs of sanitation systems consisting of bucket toilets are low whatever hauling system is selected, and so is the operational cost of individual hauling, while the cost of collective hauling can be high, dependant on the usage rates. Flush toilets provide high inhouse health and convenience level. The selection of sewer pipe system depends on i.a. the topography; gravity systems can be used in gently sloping terrain, while vacuum systems can be used in level to gently sloping terrain. The advantage of pressure sewer is that it can be used under every topographical

situation. The gravity systems require the least maintenance but flushing of low-use lines may be required. The pressure and vacuum systems use smaller diameter pipes and the water requirement is low for the latter one. (Smith and Low, 1996)

The main options for drinking water systems in cold regions are (Smith and Low, 1996):

- Self-haul systems
- Vehicle-haul systems
- Piped systems

The volume of wastewater from each dwelling depends on the water supply system; i.e. households with self-haul drinking water systems produce much less wastewater than those on piped water.

In many Arctic regions wastewater treatment is inadequate or even completely lacking. Greenland is an example of an Arctic region where no treatment of industrial or domestic wastewater exists. In the Greenlandic towns the residents have pressurized in-home drinking water. The dwellings have either traditional water flush toilets or bucket toilets. Those who have water flush toilets in the larger towns are either connected to a sewer or the blackwater (wastewater from toilets) is stored in a holding tank outside the residence while the greywater is led out directly to the terrain. In the small settlements of Greenland some dwellings have pressurized in-home drinking water while other residents have self-hauled water, typically obtained from a community water point. Bucket toilets are used in almost all settlements in Greenland where approx. 8500 out of a total population of approx. 57 000 inhabitants lived in 2012 (Stat Greenland, 2012). Routine collection of the bags from the bucket toilets and pumping of the holding tanks is organized by the municipalities or local companies, but individual haul is also done in some settlements. Handling of wastewater from tourist huts in Greenland is another challenge since they do not have running water supply and are often remotely located.

In indigenous people's communities in Alaska five levels of service have been established to categorize the different methods to dispose of human sewage (U.S. Congress, 1994). These are summarized in table 1.

Level	Sanitary solution	Collection and discharge
A	Pit toilets, privies and bucket toilets	The buckets are carried by the residents to a disposal site, but in some cases they are emptied on the ground in the immediate vicinity of the residence or carried to nearby pit bunkers by residents.
B	Bucket toilets	Bucket toilets are hauled by a community employee. Individual residents haul the waste to bins at central collection points, and when filled the bins are hauled to the community sewage lagoon by snowmobile or truck.
C	Flush toilets	Holding tanks and hauling of wastes to a disposal area is done by a truck service. The tanks, which are emptied periodically by a pump or vacuum collection vehicle operated by the community, are either large insulated tanks located outside the residence or smaller containers located inside the home.
D	Flush toilets	Septic tanks and leach fields
E	Flush toilets	Piped sewerage

Table 1. Five levels of service in indigenous people's communities in Alaska (U.S. Congress, 1994).

Level A is the most rudimentary service, and the health and convenience level of this service is considered low due to limited water use and different individual disposal practices (Smith and Low, 1996). Regarding level D septic tanks only work in regions with well-drained soil above the seasonal water table. Level E is considered to provide the highest technical and safety level and includes flush toilets and piped sewerage. However, construction of these systems is often difficult and expensive due to the remoteness of the villages and the harsh environment. For level D and E a year round water supply for flushing must be supplied (U.S. Congress, 1994).

In Canada municipal wastewater effluents are considered being one of the largest threats to the quality of the water (Environmental Signals, 2003). There is a great difference in the level of wastewater treatment between Canadian municipalities that discharge to coastal versus fresh inland waters. In 1999 most of the coastal municipalities served by sewers had primary or no wastewater treatment and only a minority had secondary treatment (Environmental Signals, 2003). On the contrary about 84% of the inland municipalities served by sewers received secondary or tertiary wastewater treatment while 15% received only primary treatment (Environmental Signals, 2003). In large urban areas in Canada, such as Victoria in British Columbia, wastewater is not treated before discharged into the Pacific Ocean (Colt et al., 2003).

In 1990 wastewater from approx. 5% of the inhabitants in Iceland was treated, while in 2002 that number had increased to over 60% (UST, 2003). However, this was mainly in the capital Reykjavík, where 62% of the inhabitants lived in 2002. In other parts of the country more than half of the wastewater was discharged untreated to the recipients. The treatment method mostly used outside the capital city was septic tanks (UST, 2003). Figure 2 shows the division of treatment methods used in Iceland in 2002.

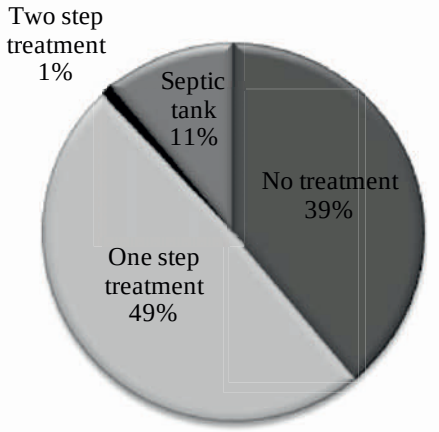

Figure 2. Wastewater treatment methods for all inhabitants in Iceland in 2002 (UST, 2003).

In Iceland one step treatment includes mechanical and/or chemical treatment to lower the content of suspended particles, e.g. by precipitation or filtration. Two step treatment includes an initial precipitation or filtration step and a second step that includes biological treatment of the wastewater, often succeeded by precipitation (UST, 2003). In Iceland it is allowed to use one-step treatment when discharging wastewater from 10,000-150,000 person equivalents (PE) in coastal areas categorized as being less sensitive, and from 2,000-10,000 PE when discharging to estuaries (UST, 2003). However, it is allowed to use one-step treatment for more than 150,000 PE when discharging to less sensitive areas if it can be shown that more developed treatment methods do not improve the environmental status of the recipient. Sludge from septic tanks and treatment plants is landfilled in most cases as there is no tradition for using sludge in agriculture in Iceland. However, treatment plants for sludge have been built in certain parts of the country where the sludge is mixed with lime with the purpose of being used as fertilizer (UST, 2003).

In other parts of Scandinavia simple mechanical treatment is also common, such as along the western and northern coast of Norway where that kind of treatment is found to dominate at small and medium sized wastewater treatment plants, primarily being used for the removal of particulate matter (Vogelsang et al., 2006).

The here presented comparison of national Arctic sewage and wastewater treatment strategies is only meant as an illustration and not to give an exhaustive comprehensive overview on the circum-Arctic situation.

Disposal of organic matter and nutrients (nitrogen and phosphorus) are not of great concern in Arctic regions when discharged directly to the sea, due to low population density and large receiving water bodies (Danish Environmental Protection Agency, 2005). If the water change in the recipient is poor those substances can however deteriorate the quality of the marine environment and even cause eutrophication. However, wastewater contains a variety of substances that can be harmful for the

environment as well as human health. Those substances include anthropogenic pollutants like industrial chemicals, oil, grease (Eriksson et al., 2002), metals (Palmquist and Hanæus, 2005), residues of pharmaceuticals and personal care products (PPCPs) (Kallenborn et al., 2008; Vasskog et al., 2009), pathogenic microorganisms and parasites (Bitton, 2005) as well as antibiotic resistant bacteria (Batt and Aga, 2005). The Arctic nature is vulnerable to environmental contaminants because of low biological diversity, lack of nutrients and extreme seasonal variations in light (AMAP, 2009). Therefore the direct release of untreated sewage may have severe consequences for the receiving (aqueous) environment (Kallenborn et al., 2008, Bergheim et al., 2010; Bach et al., 2009 and 2010). With increasing populations in the Arctic communities it becomes even more vital to improve the status of wastewater treatment in these regions. In addition, future projections show that the continuous loss of permafrost expected in the next two decades will add additional challenges to technological solutions chosen for sewage treatment (AMAP, 2011).

This article will discuss challenges and possible solutions of wastewater treatment in the Arctic with focus on pharmaceutical residues as well as pathogens and antibiotic resistant bacteria.

2. Methods

Standard literature search was performed in a selection of available bibliographic databases including ISI Web of Science, the DTU Library (Technical Information Center of Denmark), PubMed databases and the open accessible Scholar Google database. Relevant research papers were identified using scientific key word search procedures by combining scientific terms like "Arctic", "cold climate", "wastewater", "public health", "composting", "anaerobic digestion", "antibiotic resistant bacteria", "pathogens", "pharmaceutical residues", covering the subjects and chapters identified as relevant for the here performed review. Relevant secondary references within each paper were also reviewed and included. In addition, important

information (including reports of limited availability) was identified by approaching and discussing with colleagues as well as direct requests to networks of Arctic experts.

3. Results

Discharging of untreated wastewater to fresh inland waters or the sea can have various impacts on the environment, such as biomagnifications and bioaccumulation of chemicals in the food chain, chronic and acute toxicity to aquatic life from chemical pollutants, as well as increased nutrient levels. In the Arctic there are periods of ice cover on lakes, rivers and the sea. During those periods the concentration of dissolved oxygen gradually decreases (Smith and Low, 1996). In the spring Arctic waters may therefore be especially vulnerable to discharge of nutrient rich effluents.

3.1 Pharmaceuticals in the marine environment

Pharmaceuticals in the environment are becoming a subject of global concern, potentially having environmental consequences (Zuccato et al., 2006). One concern is the risk of exposure to and uptake in the food web with the potential for hazardous effects on human health and the environment. As fishery is a significant industry in many Arctic coastal regions, pollutant contamination of marine species exploited for human consumption is a major concern. Some pharmaceuticals such as anti-inflammatory drugs, antidepressants and antibiotics are not completely eliminated in the human body and can therefore enter the sewage system as the parent compound and their biologically active metabolites (Vasskog et al, 2009). In the Arctic where wastewater treatment is lacking these compounds enter the receiving water often as the parent compound and their biologically active metabolites. Some of the chemicals identified as pharmaceuticals and personal care products (PPCP) can increase the risk of development of antibiotic resistant microorganisms (Büyüksönmez and Şekeroğlu, 2005; Kallenborn et al., 2008) and it has been shown that extended exposure to low doses of antibiotics leads to the selective propagation of resistant bacteria which can transfer the resistance genes to other bacteria, even other species (Batt and Aga,

2005; Kallenborn et al., 2008; Bergheim et al., 2010). Kallenborn et al. (2008) concluded that pharmaceutical residues are degraded significantly slower in the cold Nordic aquatic environment compared to release scenarios on lower latitude. Removal of medicine residues by photodegradation is limited during the winter in the Arctic when sunlight is limited, and the cold climate in the Arctic slows down the rate of degradation of medicine residues in the environment (Kallenborn et al., 2008). The biodegradation and non-biotic elimination of the antibiotic Benzyl-penicillin was studied at different temperatures (5, 12.5 and 20°C). The degradation process followed similar pathways at the three different temperatures. What differed significantly was the degradation velocity, which was shown to be temperature dependent (Bergheim et al., 2010). In this way a threefold reduction of the transformation velocity was found when lowering the ambient experimental temperature from 20 to 5°C (Bergheim et al., 2010).

Human exposure to endocrine disrupters in the environment is also of critical concern and the long-term impacts are still unknown (Liu et al., 2009). Compounds with potential endocrine disrupting effects enter the environment via long range transport (AMAP, 2009) and direct emissions from landfills, agricultural runoff and various effluent discharge pathways (Salste et al., 2007). Aquatic ecosystems have been studied for the effect of endocrine disrupters from wastewater discharged continuously to the recipients, directly linking steroidal estrogen concentrations in wastewater treatment plant effluents with the production of vitellogenin (female specific egg-yolk protein) in fish downstream of the plants (Routledge et al., 1998; Larsson et al., 1999; Rodgers-Gray et al., 2000). Purdom et al. (1994) showed that concentrations of the synthetic birth-control pharmaceutical ethinylestradiol as low as 0.1 ng/L caused elevated levels of vitellogenin in male rainbow trout. Lorenzen et al. (2005) examined the persistence and pathways of dissipation of testosterone in agricultural soils at different temperatures, showing that the

rates of testosterone dissipation and metabolite appearance and subsequent dissipation were temperataure dependent with rates decreasing with decreasing temperatures.

In those parts of the Arctic where treatment of domestic and hospital wastewater is absent e.g. in Sisimut, Greenland (Bach et al 2009 and 2010), discarded and excreted pharmaceuticals are discharged without any elimination. Concentrations of antibiotics in hospital effluents are typically higher by a factor of 100 compared to municipal sewage and what is found in sewage treatment plants (Kümmerer, 2009). Weigel et al. (2004) studied the prevalence of selected pharmaceuticals in different sewage samples from Tromsø (~68 000 inhabitants) in Norway as well as in seawater from Tromsø-Sound, the recipient of the wastewater. The selected pharmaceuticals were, among others, the analgesic, anti-pyretic and non-steroidal anti-inflammatory drug ibuprofen and its metabolites, and the insect repellent *N,N*-diethyl-3-toluamide (DEET) as well as caffeine, which was included as a tracer for domestic sewage. It was concluded that regardless of the strong tidal current in the Tromsø-sound, and dilution with the presumably non-polluted North Atlantic water, caffeine and DEET were detected in all seawater samples. Furthermore ibuprofen and/or its metabolites were detected in most seawater samples. It has previously been shown that ibuprofen and its metabolites are easily eliminated in sewage treatment plants and under limnic conditions, so apparently low temperatures and low biological activity in the Tromsø-Sound hindered their rapid transformation (Weigel et al., 2004).

3.2 Pathogens

Wastewater contains numerous pathogenic microorganisms and parasites. There are three categories of pathogens found in the environment (Leclerc et al., 2002):

- Bacterial pathogens: Some of these pathogens are enteric bacteria, such as *Salmonella* and *Shigella*, while others, e.g. *Legionella, Mycobacterium avium, Aeromona*, are indigenous aquatic bacteria.
- Viral pathogens: These pathogens are released into the aquatic environments but are unable to multiply outside the host cells. The infective dose is generally lower than for bacterial pathogens.
- Protozoan parasites: The parasites are released into the aquatic environments as oocysts and cysts and they are quite resistant to environmental stress and disinfection. They do not multiply outside their hosts.

Other pathogens transmitted via the feco-oral route are the human polio-virus (Ghendon and Robertson, 1994), the bacteria *Clostridium tetani* that causes tetanus (Edlich et al., 2003), as well as the virus causing hepatitis A that can be transmitted through water and food and is associated with poor sanitation (WHO, 2008). E.g. it has been reported that the potential for Natives in Alaska to contract hepatitis A and other diseases is unacceptably high in villages where bucket toilets are still used and water supply (for excrement processing as well as for human consumption) is limited (U.S. Congress, 1994). The relationship between inadequate sanitation and higher rates of respiratory tract infections (Gessner, 2008; Hennessy et al., 2008; U.S. Department of Health and Human Services, 2006), skin, and gastrointestinal tract infections has also been documented among rural Alaska natives (Hennessy et al., 2008). Outbreak of epidemics of other diseases, such as impetigo, bronchitis, serious ear infection, meningitis as well as hepatitis A and B in remote Alaskan communities is often ascribed to poor sanitary facilities (U.S. Congress, 1994). More than 70% of all hepatitis A cases reported throughout the State of Alaska in 1988 occurred in Native villages with honey bucket systems (U.S. Congress, 1994). Throughout parts of the Arctic regions and rural Alaska, the outbreak of disease, such as chronic influenza-like symptoms to hepatitis and enteric diseases, is often a result of exposure to human waste and deficient personal hygiene (U.S. Congress, 1994). When transporting the human waste to disposal sites or

lagoons, spillage occurs on community roads and boardwalks. The exposure of residents in rural Alaska, particularly children, is therefore frequent (U.S. Congress, 1994). In some of the agricultural parts of Iceland severe incidences of *Salmonella* infections in animals have occurred and it is believed that they were caused by installation of septic tanks without usage of secondary treatment, i.e. a drain field (UST, 2003). Zoonotic pathogens (i.e. pathogens transmissible from vertebrate animals to humans, and vice-versa) have been found in mammals and marine birds from the Northwestern Atlantic (Bogomolni et al., 2008). The pathogens may have been acquired via contamination of coastal water by sewage, run-off and medical waste. The findings indicate that vertebrates in the Northwest Atlantic are reservoirs for potentially zoonotic pathogens that may be transmitted to humans.

3.3. Antibiotic resistant bacteria

Bacteria that have previously been exposed to antibiotics enter sewage from private households and hospitals, and sewage is therefore considered being a hotspot for antibiotic resistance genes (Zhang et al., 2009). A broad range of bacteria resistant to multiple antibiotics have been found in mammals and marine birds from the Northwestern Atlantic (Bogomolni et al., 2008). Marine vertebrates can act as vectors for pathogens and resistant bacteria globally (Bogomolni et al., 2008). Inadequate wastewater treatment in the Arctic is therefore not only a local problem but potentially a global one as well. Antibiotic resistance has been found in marine bacteria (Neela et al., 2007) as well as in bacteria living in coastal waters or estuaries polluted with sewage water (Kümmerer, 2004; Kimiran-Erdem et al., 2007). Antibiotic resistant *Escherichia coli* isolates, originating from Arctic birds, have even been found in remote places such as the Arctic Sea (Sjölund et al., 2008). Resistance genes can therefore be found in regions where no selection pressure exists. Engemann et al. (2006) showed that light exposure in the receiving waters of wastewater effluents is important and should be maximized in order to maximize the loss rate of the resistance genes tet(W), tet(M), tet(Q) and tet(O) after release. Other antibiotic resistance genes are

similar in chemical nature so it can be expected that they are sensitive to light as well. It is therefore likely that the Arctic winter with limited sunlight is unfavorable regarding removal of resistance genes.

4. Discussion

The out-phasing of bucket toilets would help to reduce waste water related diseases and improve the living conditions of northern communities (Heinke and Prasad, 1979; U.S. Congress, 1994), and treatment of wastewater would help reducing damage to the ecosystem. Considering suitable treatment methods, the low temperatures influence the efficiency of biological treatment processes in particular (Ekama and Wentzel, 2008). This makes it challenging and sometimes expensive to implement conventional treatment methods used in temperate zones. Septic tanks are a common conventional on-site treatment method where floatable and deposited material from the wastewater is removed while the liquid and solid components are treated anaerobically. The tanks can be installed in the ground outside the residences if the soil conditions permit. However, in cold regions permafrost and surface bedrock complicates installation and due to the cold climate, it is necessary to insulate the tanks. In areas with permafrost or deep frost penetrations the septic tanks may even have to be installed in a heated area under the house or a heated shed.

Another on-site treatment opportunity is aerobic systems or package treatment plants that are widely used in Scandinavia. The package plants include variations of the activated sludge process, aeration, trickling filters with or without chemical precipitation (USEPA, 2000; Johannessen, 2012). Even though secondary or tertiary treatment is achieved in theory, practical experience from Norway and Sweden show that this is not the case (Yri et al., 2007; Hübinette, 2009; Johannssen, 2012). The systems cannot perform without regular maintenance (Johannessen, 2012), and quality of operation, varying loads and loss of solids are the major factors causing poor performance (Smith and Low, 1996; Johannessen, 2012). Trials in Greenland observed byt the authors show that package treatment plants are vulnerable to

freezing and may require both good insulation and heating. This makes the systems expensive in extremely cold climate. Due to temperature vulnerability, cost, varying performance and maintenance need, traditional package treatment systems are not recommended in the Arctic. In parts of Scandinavia mechanical/chemical methods are preferred over biological (UST, 2003; Vogelsang et al., 2006). One reason for this is that mechanical and chemical processes are less dependent of temperature of the incoming wastewater. Mechanical and chemical methods also require less space than biological methods and this affects the cost of treatment plants when they have to be covered. If conventional treatment is selected in the Arctic, mechanical and chemical unit processes are probably more suited than biological.

Simple mechanical treatment methods, such as screening, remove large objects from the sewerage stream. In smaller and less modern treatment plants manually cleaned screens may be used, but mechanical cleaning should be preferred to avoid the risk of disease spreading (Winther et al., 1998). Simple mechanical treatment may also include a primary sedimentation stage where the sewage flows through large tanks in which sludge is allowed to settle. The collected solids are later landfilled or burned at the wastewater treatment plant itself or in a solid waste incinerator. The collected sludge and screenings can also be treated further by e.g. anaerobic digestion (Hyaric et al., 2010) or composting.

Another interesting option is, however, to collect and handle the blackwater separate from the greywater. The approximately 1.5 L/day that a human excretes contribute with a considerable fraction of the PPCP (Winker et al., 2008), pathogens, organic matter and nutrients (Gallagher and Sharvelle, 2010) in wastewater. Composting and anaerobic digestion of black water are treatment methods that could be well suited for this material. Both processes may, however, be more challenging to run in cold climate than in more temperate zones, thus sufficient insulation and heating of reactors are needed. Both anaerobic digestion and dry composting methods open up for co-treatment of organic household waste and organic waste e.g. from food processing industries.

4.1 Composting

Composting is an aerobic, controlled self-heating, solid phase biodegradation process of organic material, comprising mesophilic and thermophilic phases which involves numerous microorganisms (Ryckeboer et al., 2003). Alternative sanitation technologies, such as composting toilets, appear to be an improvement over bucket toilets because they reduce the possibility of users in contact with fresh human waste and they may thus reduce overall, long-term health costs (U.S. Congress, 1994). Composting toilets eliminates the need for e.g. a sewage lagoon that is often used in north America (Smith and Low, 1996) to treat the wastewater containing human excreta and they may also provide a byproduct that is more environmentally safe and easier to handle (U.S. Congress, 1994; Stenström, 2001).

Composting in cold climate is challenging, but it has been tested successfully at a Norwegian research station in the AntArctic (Hanssen et al., 2005). Solar heating of composting material could be a possibility in the Arctic, at least in the summer, while during winter material would accumulate and possibly freeze unless heated by other means. However, freezing has shown to reduce some pathogens and indicator bacteria in sludge and wastewater (Sanin et al., 1994; Torrella et al., 2003), and freezing also helps to dewater and improve the structure of the compost material (Hedström and Hanæus, 1999). It might therefore be beneficial when developing wastewater treatment methods for Arctic communities to utilize the cold climate and include freezing and thawing in the treatment processes. When human excrements are composted it is necessary to dewater the excrements since they contain 80-85% urine (Jennera et al., 2005). The effect of freezing and thawing on pathogen reduction in this type of material has not been investigated. Where possible the excess liquid can be disposed of by soil infiltration. This may be a challenge in some parts of the Arctic where thawed soil layers are scarce. This can be solved by using commercial biofilters of different materials. Systems for composting at household level as well as

collection systems for professional centralized processing can be designed (Hanssen et al., 2005). The latter reduce the household level hygienic risk (Hanssen et al., 2005).

Removal of pharmaceutical compounds, which may be one of the key issues in wastewater treatment in the Arctic, show highly varying elimination rates for different compounds in traditional wastewater treatment plants (Vasskog et al., 2009). The potential for removal or breakdown of medicine residues or other organic chemicals is potentially larger in an intense thermophilic composting process than in traditional wastewater treatment. Vasskog et al. (2009) investigated the depletion of selective serotonin reuptake inhibitors (SSRIs) during sewage sludge composting and found that concentrations of all the SSRIs in question, as well as some of their metabolites, had a significant decrease during the composting process. Büyüksönmez and Şekeroğlu (2005) also tested the efficacy of the composting process to degrade 10 different PPCPs found in biosolids generated during municipal wastewater treatment. The results suggested that composting could be an effective treatment alternative for biosolids. Dolliver et al. (2008) studied degradation of the antibiotics chlortetracycline, monensin, sulfamethazine and tylosin in spiked turkey litter during managed composting compared to unmanaged composting. The results showed that some management of the composting, such as mixing to optimum water content and stockpiling, can reduce concentrations of antibiotics. After 35 days of thermophilic temperatures chlortetracycline showed >99% reduction, while monensin and tylosin reduction ranged from 54 to 76% (Dolliver et al., 2008). There was no degradation of sulfamethazine during the study. Other studies on antibiotic degradation during composting have shown >99% removal of oxytetracycline during 35 days of beef manure composting, compared to less than 15% reduction during incubation of the manure at room temperature (Arikan et al., 2007).

Heat inactivation is considered to be one of the most reliable methods for sanitation (Sahlström, 2003). Studies have been conducted to assess the effectiveness of composting to destroy pathogens possibly present in raw sewage sludge. Wiley and Westerberg (1969) studied the survival of *Salmonella newport,* poliovirus type 1, *Ascaris lumbricoides* ova and *Candida albicans*, and found that after 43 hours of composting, no viable indicator organisms could be detected. Their results indicated that aerobic composting of sewage sludge would destroy the indicator pathogens when a temperature of 60-70°C is maintained for a period of 3 days (Wiley and Westerberg, 1969). To achieve thermophilic conditions in compost the containers have to be well insulated, especially under Arctic conditions. Tønner-Klank et al. (2007) found that it was only possible to ensure a homogeneous temperature in compost of faecal material when amendments were made and when composting containers were insulated. Vinnerås et al. (2003) studied a mixture of faecal matter, food waste and old compost matter, used as an amendment, mixed in a 90-L bin. The average surrounding temperature during the experiment was 10°C. Thermal composting resulted in a temperature of over 65°C. By using insulation and turning the compost it was possible to ensure a 5 $\log_{10}$ reduction of pathogens (Vinnerås et al., 2003). The relatively low ambient temperature in this particular experiment shows that it is possible during Arctic summers to reach thermophilic conditions.

One of the advantages of composting is the degradation of organic matter. A 50-60% reduction of volatile solids (VS) during anaerobic digestion due to liquefaction and gasification and a volume reduction of two-thirds has been reported (Finstein et al., 1980). Vinnerås et al. (2003) found that almost 75% of the organic matter in a compost of faeces and food waste during a pilot scale experiment had decomposed after 35 days, and Lopez Zavala et al. (2005) found a 56%, 70% and 75% reduction of total solids (TS), VS and chemical oxygen demand (COD), respectively, in experimental laboratory-scale bioreactors when testing the efficiency of bio-toilets.

4.2 Anaerobic digestion

Anaerobic digestion involves a series of biological processes where a number of microorganisms break down biodegradable material under anaerobic circumstances. During the process methane and carbon dioxide rich biogas is produced, suitable for energy production. After the digestion the nutrient-rich digestate can be used as fertilizer. Anaerobic digestion has been used for industrial and domestic purposes to treat waste and produce energy (Wang, 2010). The fishing industry is one of the most important industries in many Arctic coastal regions, such as in Greenland where it is the biggest one, generating about 14,000 tons of waste products each year whereof only 20% is utilized (Nielsen et al., 2006). This waste fraction could be used for biogas production along with other organic waste. In many settlements in the Arctic, municipal collection of blackwater is practiced. Instead of direct discharge to the sea it could be transported to a central anaerobic digester. In small remote settlements in the Arctic there is often a lack of technically skilled personnel and logistical problems can also occur during operational resupply (Smith and Low, 1996). It is therefore important to use technically simple digestion systems. Small anaerobic digesters have been proven to be successful in small villages but this has yet mainly been tested in warmer climate than in the Arctic (Bensah, 2009). Small anaerobic digesters are easier to run at mesophilic than thermophilic conditions because thermophilic digestion can be more sensitive to operational conditions such as the organic loading rate, temperature and the characteristics of the influent sludge (Kim et al., 2002; van Lier, 1996). Taking this, as well as the cold climate in the Arctic, into account it might be practical to run anaerobic digesters at mesophilic conditions, especially in the smaller settlements.

Information about breakdown and behavior of PPCPs during anaerobic sludge digestion is limited and even contradictory. Khan and Ongerth (2002) stated that most PPCPs persisted in the aqueous part of

digested sludge. Another study showed that oestrogens were not degraded substantially under methanogenic conditions (Andersen et al., 2003). In contrast Kreuzinger et al. (2004) indicated that the breakdown of natural oestrogens was accelerated during anaerobic digestion. Holbrook et al. (2002) found that between 51% and 67% of the oestrogenic activity contained in the influent wastewater was either biodegraded during the treatment of wastewater or biosolids, or was made unavailable to the extraction/detection procedure used during treatment.

The reducing effect of anaerobic digestion on various microorganisms has been studied by many and it has been shown that the survival of pathogenic bacteria during anaerobic digestion is mainly dependant on the temperature (Dumontet et al., 1999). Inactivation of bacteria due to temperature is also related to time (Olsen and Larsen, 1987). T_{90}, which is the time required for a decrease by one logarithmic unit (log_{10}) or 90% reduction of viable counts of a population of microorganisms, can be counted in hours in thermophilic digestion and in days in mesophilic digestion, compared to weeks and months in conventional treatment (storage) (Gibbs et al., 1995; Larsen et al., 1989). For instance, Kumar et al. (1999) studied the removal of different microorganisms during anaerobic digestion of cattle dung in batch digesters at room temperature and mesophilic conditions (35°C). They found a complete removal of *Escherichia coli* (*E. coli*) and *Salmonella typhi* after 25 days at room temperature and 15 days at 35°C. Complete removal of *Streptococcus faecalis* took longer time; 20 and 40 days at 35°C and room temperature, respectively. In a mesophilic biogas plants, continuously fed with fresh biomass, the faecal enterococci reduction is rarely more that 1-2 log units (Bendixen, 1994). Others have observed similar reduction, as for instance Bonjoch and Blanch (2009) who found a 1 log unit reduction for enterococci populations in sludges and biosolids used in mesophilic anaerobic digestion for a period of 20 days. Berg and Berman (1980) analyzed the destruction of viruses compared to faecal coliforms, total coliforms and faecal streptococci under mesophilic conditions (about 35°C) where the average residence time of the

digested sludge was 20 days. They found that the numbers of viruses recovered from the raw sludges were reduced by about 90% and faecal streptococci more the 90%. Faecal coliforms were reduced by about 98% and numbers of total coliforms by a little more than 99%. Sanders et al. (1979) found that poliovirus type 1 is inactivated after 4.17 days at 35°C. The ability of anaerobic digestion to reduce the quantity of antibiotic resistant bacteria in wastewater solids has been studied. Statistically significant reductions in the quantities of antibiotic resistant genes were found to occur at 37, 46 and 55°C with the removal rates increasing as a function of temperature (Diehl and Lapara, 2010). In this study it was found that the quantities of tetracycline resistance determinants (*tet*(A), *tet*(O), *tet*(W) and *tet*(X)) and integrase genes from class 1 integrons were substantially affected by temperature. Those results were generally consistent with reductions observed in full-scale treatment facilities during previous work by the same authors. Abdul and Lloyd (1985) found that during anaerobic digestion at 37°C counts of defined strains of *E. coli* were rapidly reduced. They tested both antibiotic resistant and sensitive strains and found that antibiotic resistant strains were more persistent. In experiments in which feeding was daily for periods of 10 days, the percentage reduction of the counts of resistant *E. coli* W3110T (R300B), J53 (R136), J53 (R144) during 10 days retention time from influent to effluent were 95.5, 94.5 and 93.3% respectively while the reductions for sensitive *E. coli* (strains J53, MP1, C600) were 99.9, 100 and 99.4% respectively. In both resistant and sensitive strains there were immediate decreases in numbers over a 10 h period. They also found that some of the strains isolated from the digesters had the ability to grow anaerobically, so that anaerobiosis was found not to be the only cause of rapid die-off.

In addition to the treatment effect on the wastewater the mass reduction of the organic waste fractions during anaerobic digestion is significant (Borowski and Szopa, 2006; Nges and Liu, 2010; Novak et al., 2011). Salsabil et al. (2010) reported that anaerobic digestion was globally more effective in sludge reduction compared to aerobic digestion. However, one of the biggest advantages is the bioenergy that is

produced during the process. Anaerobic digestion is an endogenous process and where possible, it might be a good solution to locate a biogas digester next to a solid waste incineration plant for heating of the digester in the winter. It might also be possible to use the generated gas for heating in the winter and possibly solar heating during summer. Many Arctic communities are dependent on fossil fuels that in most cases are imported. Utilizing the organic matter in the blackwater together with other organic waste fractions to produce biogas could therefore turn out to be economically as well as environmentally beneficial for Arctic communities.

4.3 Toilet solutions

There are several alternative toilet types available today that could be a good solution for many Arctic communities. For Arctic communities and households, simple technologies with high technological efficacy are the preferred strategy for new sanitary structure. Low flush or composting toilets might be good alternatives to bucket toilets and conventional flush toilets in Arctic areas. The two types and sub-types are shown in figure 3. These toilet solutions do not mix the human excreta with large amounts of water are suitable if composting or anaerobic digestion of black water is to be applied. Low flush toilets give the opportunity to have both collection and treatment at the household level, or collection at the household level and centralized processing. For centralized processing, pressure sewer and vacuum sewer systems are alternatives to gravity sewers. They need the same insulation as traditional sewers, but are less vulnerable to gradient changes that may occur in permafrost areas. Vacuum sewer systems also allow for usage of smaller pipelines and thus low flush toilets (U.S. Congress, 1994). By decentralized collection of human excrements and onsite/in-house treatment of the greywater, for instance by using biofilters, the need for expensive secondary sewer collection systems can be reduced or even eliminated (Heistad, 2008; Karabelnik et al., 2010).

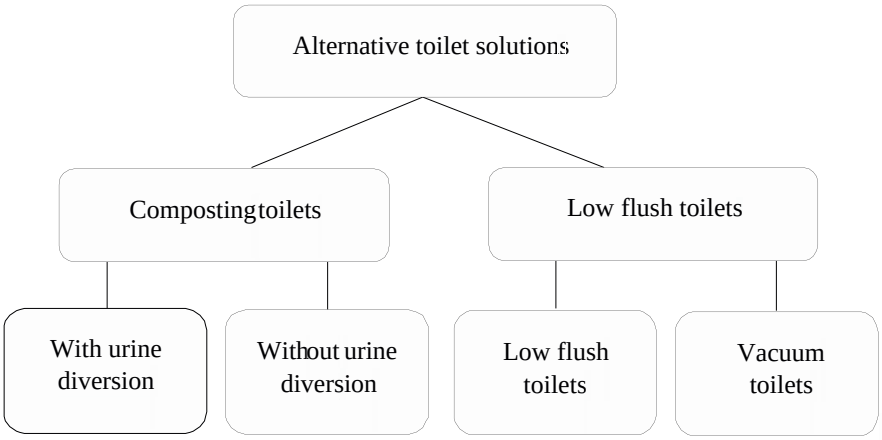

Figure 3. Alternative toilet solutions for Arctic areas.

Vacuum toilets or low flush toilets are considered a good choice where water supply is scarce (U.S. Congress, 1994). Vacuum toilets typically use about 1 L pr. flush (Zifu et al., 2002) while low flush toilets use approx. 0.5-1.5 L pr. flush (Jenssen et al., 2004) compared to conventional flush toilets which require approx. 9 L pr. flush (Zifu et al., 2002). However, if these types of toilets are to be installed in communities which are already served by conventional sewerage pipelines, care has to be taken when connecting this kind of toilets to the sewer since the reduced volume of flushing water will also reduce the velocity of the wastewater in the pipelines, possibly causing depositing of organic and inorganic matter in the pipelines which can lead to clogging or freezing.

For composting toilets, collection and treatment is always on household level. Composting toilets are a good alternative to the bucket toilets in non-sewered residences. Modern composting toilets can be urine diverting or not. The urine diversion has the benefit that urine is separated from the small amount of faecal matter where most of the pathogens are contained (WHO, 2006), reducing the volume to be hygenized substantially. The faecal matter can be treated by composting and the urine can be stored until

it is safe to dispose or use as fertilizer. The general recommended storage time for urine is 6 months under most conditions (WHO, 2006). Another benefit of urine diversion is that more pharmaceuticals and their metabolites are excreted via urine than via faeces (Winker et al., 2008). Urine separation and separate handling of the urine is therefore a promising approach to lower the pharmaceutical load of raw domestic wastewater and to protect the aquatic environment safely from pharmaceuticals. Composting toilets without urine diversion may still be a better solution at remote locations, for instance Arctic tourist huts or other locations where the urine cannot be properly infiltrated or used.

5. Development needs

Wastewater treatment systems are mainly developed for temperate regions and little is done to specifically address the needs of the Arctic. This paper advocates decentralized treatment and shows that the excreta can be safely treated or collected onsite, but the greywater also needs treatment. Compact onsite methods for greywater treatment exist (Heistad, 2008; Karabelnick et al., 2010), but need further development to be better suited for Arctic conditions.

The use of pressure or vacuum sewers in Arctic conditions should be explored. Such alternative transport systems are interesting from an economical point of view and can transport wastewater to areas suited for local treatment systems without the need for a large collection network.

This paper points to possible environmental and health risks caused by inadequate wastewater treatment in the Arctic. However, additional analyses are needed of to better assess the risk of pharmaceutical residues and microbial agents, in the aquatic environment of the Arctic.

Studies of possibilities to take advantage of the cold climate when designing treatment methods for Arctic regions should also be done. This could for instance be to include freezing in treatment of wastewater, which has not been addressed in this article. Development and testing of small and simple treatment

units, for instance composting toilets and anaerobic digestion plants, under cold conditions would also be valuable for small and often remotely located Arctic communities.

6. Conclusions

Treatment of wastewater is often inadequate or completely lacking in Arctic regions. Bucket toilets are still widely used in Arctic regions, even in villages and towns, and the risk of exposure to human waste and spreading of diseases is unacceptably high. Out-phasing the use of this toilet type will improve public health, but also indoor comfort. Conventional collection and treatment systems are expensive to build and operate under Arctic circumstances. Onsite or decentralized treatment will reduce the need for expensive collection systems. Possible alternative wastewater treatment methods for Arctic communities are dry composting or anaerobic digestion of excreta collected at household level using dry or water saving toilets. This opens up for co-treatment of wastewater and other organic waste fractions. Non-ordinary toilet solutions such as vacuum toilets and urine separation toilets may be particularly well suited under Arctic circumstances. The blackwater contains pharmaceuticals and their metabolites and separate treatment of the excreta fraction facilitates their removal. Since a higher portion of many pharmaceuticals and their metabolites are excreted via urine than via faeces, urine separation techniques are a promising approach to lower the pharmaceutical load of raw domestic wastewater and to protect the Arctic aquatic environment from pharmaceuticals. Composting and anaerobic digestion have been shown to be effective sanitation methods, where the temperature appears to be the most important factor. Composting also seems to be a promising method to degrade PPCPs but there is a lack of information about breakdown of these substances during anaerobic digestion.

Further development of simple and robust treatment units for the small and often remotely located Arctic communities is needed. Such units should include composting processes and take advantage of the freezing as to enhance treatment.

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Paper II

Gunnarsdóttir, R., Müller, K., Jensen, P.E., Jenssen, P.D., Villumsen, A., 2012. Effect of long-term freezing and freeze/thaw-cycles on indigenous and inoculated microorganisms in dewatered blackwater. Submitted to Environmental Science and Technology.

Effect of long-term freezing and freeze/thaw-cycles on indigenous and inoculated microorganisms in dewatered blackwater

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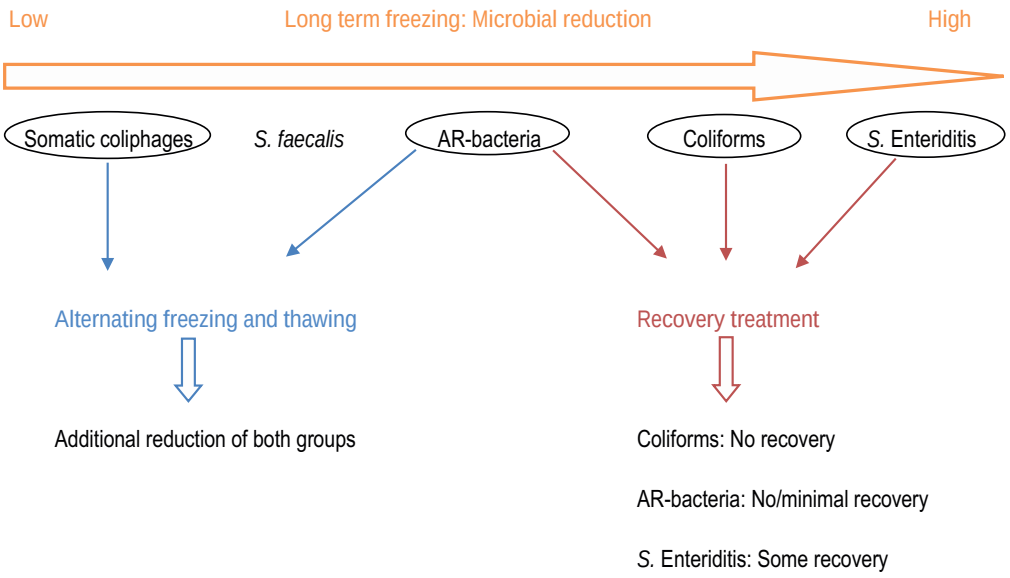
Keywords: Freezing, freeze/thaw, recovery, indigenous microorganisms, *Salmonella*, coliphage ΦX174, antimicrobial resistance, dewatered wastewater, wastewater treatment, cold climate, Arctic



Abstract

Wastewater treatment in many arctic regions is inadequate, even non-existing. Natural freezing of wastewater in those areas may be beneficial in various ways, e.g. for reduction of microorganisms. The aim of this study was to investigate the effect of long term freezing, and repeated freezing and thawing, on indigenous and inoculated microorganisms in dewatered blackwater. At the end of the long term freezing experiment (10 month), a recovery study was carried out, including the microbial groups that had shown that largest reduction. Indigenous faecal streptococci (*S. faecalis*) were more resistant to long-term freezing than the coliform group. Total number of indigenous Enterobacteriaceae resistant to amoxicillin, ciprofloxacin, tetracycline and vancomycin (AR-bacteria) decreased slowly but constantly over the 10 month freezing period. Inoculated *Salmonella* Enteriditis (*S.* Enteritidis) rapidly decreased during freezing and were non-detectable within a week. However they exhibited some recovery after 10 months of freezing. Inoculated *E. coli* bacteriophage ΦX174 showed limited reduction during the long term freezing. Repeated freezing and thawing increased the reduction of all tested microbial groups markedly. The experiments showed that freezing has the potential of reducing microorganisms in wastewater and might be a cost-effective way to enhance the quality of treated wastewater in arctic areas.

1. Introduction

Wastewater treatment in many arctic regions is inadequate or even non-existing, especially in small and remote communities. Wastewater effluents contain different anthropogenic pollutants[1] and pharmaceutical residues[2] as well as pathogenic parasites and microorganisms[3], including antibiotic resistant bacteria[4]. Discharging of untreated wastewater to the recipients can have a negative effect on people's health as well as the vulnerable arctic environment[5]. Due to special technical needs and infrastructure in the arctic climate it is, however, challenging and expensive to build centralized collection and treatment systems in those areas, thus alternative treatment methods are needed[6]. Natural freezing can be beneficial in different ways, as it is recognized as being a cost-effective wastewater treatment technique that can be a reliable dewatering method for wastewater sludge[7,8]. Freezing has also proven to be an effective way to remove organic and inorganic contaminants from different industrial wastewaters, the latter one being done by concentrating the aqueous solution by partial freezing and subsequent separation of the ice crystals[9]. Furthermore it has been shown that freezing can reduce microbial levels in wastewater. The freezing time seems to be an important factor in the reduction of microorganisms such as *E. coli*[10], faecal and total coliforms[11], *Salmonella* and phages[7,11]. However, the effect of long-term freezing lasting more than two months on indicator organisms in wastewater has not been tested, and studies on susceptibility of antibiotic resistant bacteria to freezing are also scarce[12].

Freezing has been shown to have a dissimilar effect on different microbial groups. Gram-negative bacteria, such as coliforms, have been shown to be more susceptible to freezing than gram positive bacteria, such as faecal streptococci[7,12]. The value of faecal coliforms as a measure of reduction efficiency for freezing has thus been questioned[13].

Repeated freezing and thawing can be more effective in reducing microbial levels in wastewater but knowledge on the effect of the freeze/thaw processes on living cells, including antibiotic resistant bacteria, has only been studied by a few[12,14]. Gao et al.[12] compared the freezing resistance of vancomycin susceptible and resistant strains of *Enterococcus faecalis* (*E. faecalis*), but no difference was observed. The effect of alternating freeze/thaw cycles on phages in wastewater has to the knowledge of the authors not yet been studied.

Many bacteria subjected to freezing, thawing or frozen storage are believed to be killed but are in fact only injured as a result of sublethal structural and/or physiological changes[13]. When bacteria are sublethally stressed by chemical or physical treatment, they show various damages, such as breakdown of essential cellular components like RNA and enzymatic damage and increased cell wall permeability, making the cells more sensitive to various antimicrobial agents used in selective media[15,16]. Thus, injured bacteria lose the ability to grow on certain selective media at elevated temperatures[15] and to form colonies[17]. If the environment becomes more suitable for injured cells, they have the possibility to recover which is of concern as it can pose a human health threat[10].

Many of the studies regarding the effect of freezing on microorganisms are performed using aqueous solutions inoculated with laboratory microbial strains[10,12,14] and only a few have tested the susceptibility of indigenous indicator organisms in sludge and wastewater[7,11]. Indigenous microorganisms in wastewater might be more resistant to freezing than laboratory strains. E.g. indigenous viruses in raw sludges have appeared to be inactivated by anaerobic digestion much more slowly than laboratory strains of viruses inoculated into such sludges[18]. Furthermore, wastewater may be a more protective environment for the microorganisms than pure water. Bacteriophages´ survival in the environment has been reported to depend not only on temperature but on water quality as well[19]. Dewatering of wastewater is necessary during some processes of wastewater treatment. The increased dry matter content might however provide an extra protection for microorganisms contained in the wastewater[20]. Dewatered wastewater has, to the knowledge of the authors, not been used previously in studies of freezing or repeated freezing and thawing.

Based on this our aim was to investigate the applicability of long-term frozen storage as a method of sanitation of dewatered blackwater (DBW) containing gram-postive as well as gram negative bacteria, including the pathogen *Salmonella,* antibiotic resistant enteric bacteria and coliphages. We accomplished this by three experimental studies: 1) The effect of long-term freezing of dewatered blackwater at -18°C for 10 months, to mimic long winter periods in arctic regions, on indicator organisms, antibiotic resistant enteric bacteria, and *S.* Enteritidis; 2) the effect of repeated freeze/thaw cycles on some of the microbial groups showing most resistance against long-term freezing; and 3) recovery of the microorganisms most affected by the long-term freezing.

2. Methods and Materials

2.1. Wastewater collection and sample preparation

Blackwater was collected from a student dormitory with 48 students served by vacuum toilets. The vacuum toilet and transport system (Jets™) contain a macerator and a dewatering unit rendering macerated blackwater with a dry matter content of approx. 15% (measured by drying overnight at 105°C) prior to storage at 4 °C. Two batches of DBW were used in the experiments; one for the long-term freezing and recovery study and another for the freezing and thawing study. Immediately prior to the experiments, each DBW batch was homogenized mechanically with an industrial mixer, divided into samples of 50 g and transferred into double layer plastic bags in which it was further mixed and finally transferred into sterile 100 mL plastic bottles with a screw cap.

2.2. Microbial analyses

2.2.1. Experimental microorganisms and preparation of inocula

Before beginning the experiments, the content of the coliform group, faecal streptococci/enterococcus group, enteric bacteria resistant to the antibiotics amoxicillin, ciprofloxacin, tetracycline and vancomycin, somatic coliphages and *Salmonella* was determined. *Salmonella* and somatic coliphages were non-detectable (detection limit 100 CFU/g and 10 PFU/g, respectively), thus they were inoculate. Prior to inoculation with *Salmonella enterica ssp. enterica serovar* Enteritidis (*S.* Enteritidis), samples were autoclaved in a table autoclave (CertoClav Typ CV-EL 10L, Kelomat Austria) for 45 min at 121ºC and 1.3 bar to prevent indigenous microbial interference. This decreased the level of indigenous bacteria to non detectable (detection limit 100 CFU/g), determined with non-selective brain heart infusion (BHI) agar.

Inoculation of *S.* Enteritidis and somatic coliphages was performed with *S.* Enteritidis ATCC 13076 and *Escherichia coli* bacteriophage ΦX174 ATCC 13706-B1 which was grown before and after inoculation in presence of *Escherichia coli* C ATCC 13706 (*E. coli* C) as host. *S.* Infantis jeo 4208 (78121/5) (fully susceptible)[21] was used as a negative control strain for MacConkey agar plates containing amoxicillin, ciprofloxacin and tetracycline (separate plates for each antibiotic). *E. faecalis* ATCC 2921 (vancomycin sensitive) was used as negative control strain for PSE agar plates containing vancomycin. Bacteria strains were provided by the Danish National Food Institute. The somatic coliphage and the bacterial host were purchased at LGC Standards in Borås, Sweden. For the preparation of the inocula, *S.* Enteritidis was grown from a frozen culture (stored at -80°C in LB medium containing 15% glycerol) on blood agar overnight at 37°C wherefrom a colony was inoculated into brain heart infusion (BHI) broth which was incubated overnight at 37°C under gentle shaking. After growth the cultures were harvested, washed twice and re-suspended in sterile physiological salt water (0.9% NaCl) to get a final cell concentration of approx. 10^8 CFU/ml, corresponding to an OD_{600nm} of 0.5. Immediately after, it was inoculated into the homogenized 50 g samples of DBW to obtain a concentration of 10^6 CFU/g. The inoculated samples were mixed thoroughly. The coliphage ΦX174 stock solution used for inoculation was prepared according to Eaton et al.[22]. A coliphage suspension used as a positive control for the coliphage analyses was prepared by diluting the phage stock filtrate to 30 to 80 PFU/mL in tryptone broth. Stock filtrate and control suspension were stored at 4°C until use. The procedure for inoculation of the stock filtrate into the DBW subsamples was the same as for the *S.* Enteritidis. The inocula were adjusted to a final concentration of 10^5 PFU/g.

2.2.2. Enumeration methods

Growth media and detection methods for each type of microorganism or microbial group are listed in Figure S1 in Special Information. For enumeration of the coliform group and faecal streptococci/enterococci the MTF technique was used according to Eaton et al.[22]. Triplicate experiments were performed and most probable number (MPN) values were calculated from the number of positive

tubes as described in Blodgett [23]. Triplicate analyses of *S.* Enteritidis, somatic coliphages and antibiotic resistant bacteria were performed by plating of two technical replicates.

At each enumeration step, the DBW samples were diluted 10-fold with 0.9% NaCl by manual shaking and with a mini shaker at medium speed before transferred to the different growth media. For the analyses of total coliforms, lauryl sulphate (LS) broth was used as growth medium for the presumptive phase and brilliant green lactose bile (BG) broth for the confirmed phase. Both media were incubated at 37°C for 48 ± 3 h. EC medium was used for selection of faecal coliforms while tryptone water and Kovac's reagent were used for confirmation of *E. coli*. Both growth media were incubated at 45°C for 24 ± 2 h. Growth media for the presumptive phase of analyses of faecal streptococci was azide dextrose (AD) broth and Pfizer selective Eneterococcus (PSE) agar was used for the confirmed phase. Tubes containing AD broth were incubated for 48 ± 3 h at 37°C while the PSE agar plates were incubated for 24 ± 2 h at 37°C. Brownish-black colonies with brown halos on the PSE agar plates confirmed the presence of faecal streptococci. To confirm if the faecal streptococci belonged to the enterococcus group the colonies were transferred into brain-heart infusion (BHI) broth containing 6.5% NaCl and incubated at 45°C overnight. *S.* Enteritidis were enumerated by direct plate counts on both XLD and BHI agar and incubated at 37°C overnight. Somatic coliphages were enumerated using a variant of the double-agar-layer method according to Eaton et al.[22], using tryptone bottom and top agar as growth medium. The host, *E. coli* C, was prepared according to Eaton et al.[22]. The plates were incubated at 37°C overnight. Antibiotic resistant bacteria were enumerated using MacConkey agar (for amoxicillin, tetracycline and ciprofloxacin) and PSE agar (for vancomycin). MacConkey agar is selective for Enterobacteriaceae while the PSE agar is used for enumeration of faecal streptococci. The concentrations of the selected antibiotics in the agar were based on available information of MIC values of Enterobacteriaceae and fecal streptococci (according to EUCAST breakpoints). Stock solutions of the antibiotics were prepared with appropriate solvents and stored at -20 ºC prior to use in the experiment. The antibiotic stock solutions were inoculated into the fluid sterilized agar at a temperature below 50°C before to obtain the following concentrations:

- Amoxicillin, 16 µg/mL
- Ciprofloxacin, 4 µg/mL
- Tetracycline, 16 µg/mL
- Vancomycin, 8 µg/mL

The inverted plates were incubated at 37°C for 2-4 days, until countable colonies had grown.

2.2.3. PCR analyses of the 16 S rDNA

PCR (Polymerase Chain Reaction) amplification of the gene encoding the 16 S rRNA was performed on the extracted DNA of different colonies growing on the MacConkey and PSE agar plates containing antibiotics. PCR was carried out in a 50 µl mixture including 0.5 µl forward (5'-GACTACCNGGGTATCTAATCC-3') and reverse primer (5'-TGACGGGCGGTGTGTACAA-3') (20 pmol/µl)[24], using an annealing temperature of 50°C. The PCR fragments were purified with the GFX purification kit (GE Healthcare) and sent for sequencing to Macrogen (Seoul, Korea). The sequence analyses were performed using the software Vector NTI (Invitrogen). The sequences were BLASTed against GenBank (National Center for Biotechnology Information) database to find the closest match to the colony types in question.

2.3. Experimental setup

2.3.1. Long term freezing and recovery experiment

The long-term freezing study lasted 10 months, where after a recovery study was carried out with the microorganisms showing the highest reduction. The experiment included 30 50 g samples of which ten were autoclaved and inoculated with *S.* Enteritidis, ten were inoculated with the somatic coliphage ΦX174, and ten were used for analyses of indigenous bacteria (coliform group, faecal streptococci/enterococci and antibiotic resistant enteric bacteria). The samples were frozen in a standard freezer at an average temperature of -18°C, which was used in all experiments. One of each sample type was taken out for analysis on day 0 (before freezing), 1, 3, 7, 21, 56, 84, 140, 196 and 280 to thaw overnight at 4°C after which the microbial analyses were performed. After 10 months of freezing the coliform group, resistant enteric bacteria and *S.* Enteritidis were included in a MPN recovery study. Recovery in tryptone soy broth has proven to be successful for *E. coli* and members of Enterobacteriacae at temperature ranges 17-45°C and incubation times ranging from 30 min to 8 h[17,25]. Resuscitation treatment for the coliform group and resistant enteric bacteria was performed in tryptone soy broth (TSB) at 20°C. An additional recovery treatment was done at 10°C to mimic a typical arctic summer climate. Bacteria that survive freezing often have an extended lag phase[25]; hence the incubation time was prolonged to overnight for recovery at both 10 and 20°C. The recovery study was performed using the last samples (of day 280) of the long term freezing experiment. Thus, the initial values in the recovery study were the same as the final values in the long term freezing experiment. The DBW sample for recovery of indigenous bacteria was diluted 10-fold with 0.9% NaCl by shaking manually and with a mini shaker at medium speed before inoculated into TSB. Two dilution series incubated at 10 and 20°C respectively were prepared with TSB (to observe any reproductive microbial activity) and with 0.9% NaCl (to observe whether the elevated temperatures alone were enough for recovery of the microorganisms). Each dilution series was done in triplicate. After incubation, all tubes showing turbidity were transferred to LS broth followed by the standard procedure for analyses of the confirmed phase of total coliforms, faecal coliform and *E. coli* (see section 2.2.2). For analyses of recovery of antibiotic resistant bacteria, a loopfull

(approx. 10 µL) from each tube showing growth was streaked out on agar plates containing one of the four mentioned antibiotics. Plates showing growth after incubation at 37°C for about 2 days were noted as being positive. The procedure for recovery of *S.* Enteritidis was modified after Malorny et al.[26]. A serial dilution series of DBW samples was inoculated in buffered peptone water in triplicate and incubated at 37°C for 18-20 h. After incubation, 0.1 mL from all tubes showing turbidity was spread on BHI and XLD agar plates. The inverted plates were incubated at 37°C overnight. Plates showing growth were noted as being positive. Colonies from BHI plates were transferred to XLD plates to confirm the presence of *Salmonella.*

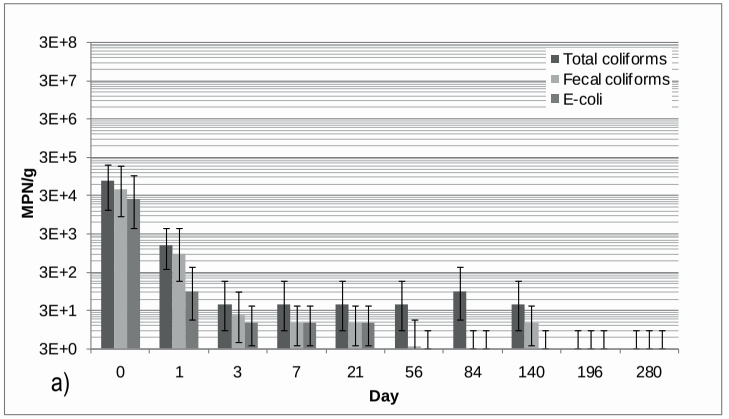
2.3.2. Freezing and thawing experiment

The effect of alternating freeze-thaw cycles was investigated for the microbial groups showing little reduction during the first five months of the long term freezing experiment: somatic coliphages and amoxicillin resistant enteric bacteria. Six cycles were accomplished, one cycle consisting of 10 h at -18°C followed by overnight thawing at 4°C. Four samples of DBW were used in the experiment. Two samples were subjected to repeated freezing and thawing (one for detection of amoxicillin resistant enteric bacteria, and one inoculated with the coliphage ΦX174), while two samples (identical to the prior) were stored at constant -18°C during the full period of the experiment (approx. 5.5 days) and used as reference. After each freeze-thaw cycle subsamples were taken from the two first samples for microbial analyses. Reference samples were analysed simultaneously with analyses after the last freezing and thawing cycle.

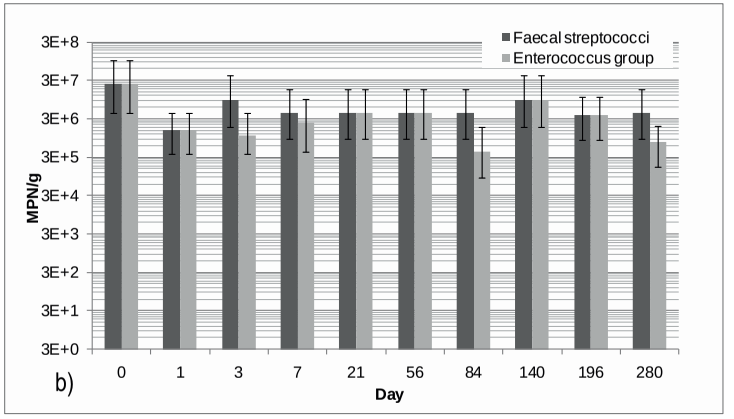
3. Results

3.1. The impact of long term freezing and recovery on the microorganisms in DBW

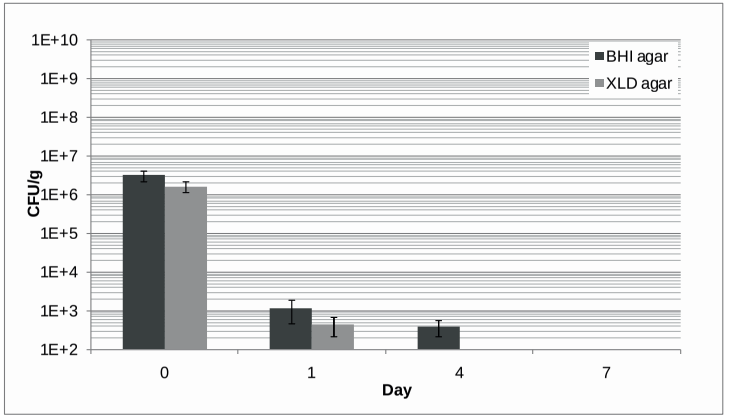
Figure 1a-e shows the results for the different bacterial groups.


3E+8
3E+7
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
MPN/g
Total coliforms
Fecal coliforms
E-coli
0
1
3
7
21
56
84
140
196
280
Day

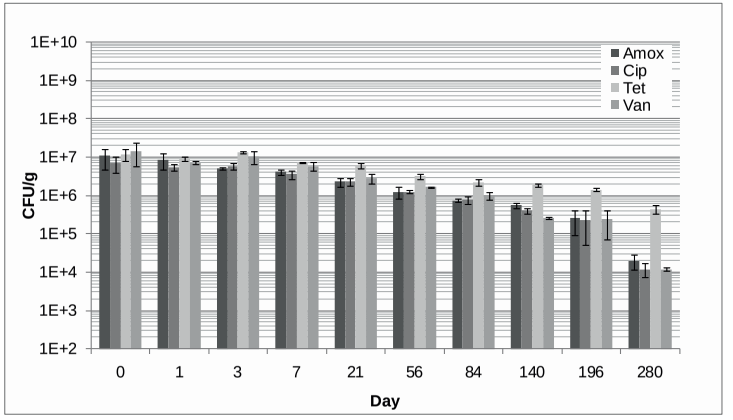
a)


3E+8
3E+7
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
MPN/g
Faecal streptococci
Enterococcus group
0
1
3
7
21
56
84
140
196
280
Day

b)


1E+10
1E+9
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
CFU/g
BHI agar
XLD agar
0
1
4
7
Day

c)


1E+10
1E+9
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
CFU/g
Amox
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0
1
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7
21
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84
140
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280
Day

d)

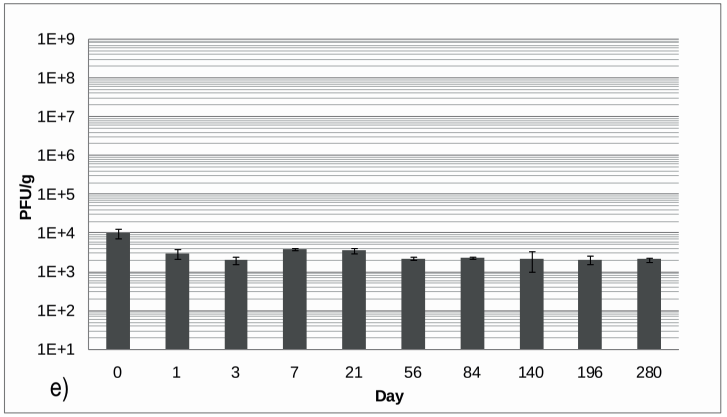

Figure 1. Effect of long-term freezing on a) the coliform group, b) faecal streptococci/enterococcus group, c) S. Enteritidis, d) total count of antibiotic resistant enteric bacteria, e) Escherichia coli bacteriophage ΦX174. The lower limit on the y-axis is the detection limit in all cases. Error bars represent the standard deviation of three independent replicates.

The background concentration (day 0) of total and faecal coliforms and *E. coli* was $7.4 \cdot 10^4$, $4.3 \cdot 10^4$ and $2.4 \cdot 10^4$ MPN/g respectively (Figure 1a). The largest reduction in levels of the total and faecal coliforms as well as *E. coli* occurred between day 0 and 1; 1.69, 1.66 and 2.41 log, respectively. The levels of faecal coliforms and *E. coli* were alike until day 56 (month 2) where the level of *E. coli* was below the detection limit (<3 MPN/g) which was the case for the faecal coliforms on day 84 (month 3). The faecal coliforms were detected again at day 140 (month 5), but only in a low number; approx. 10 MPN/g. This indicates that the level of faecal coliforms has been just under the detection limit the month before. The total coliforms were detectable until day 196 (month 7).

The background concentration of faecal streptococci equalled the concentration of faecal streptococci belonging to the Enterococcus group and was $2.4 \cdot 10^7$ MPN/g (Figure 1b). The faecal streptococci were more robust against the long-term freezing than the coliform group but as for the coliforms the largest reduction of the faecal streptococcus and the enterococcus group occured between day 0 and 1; 1.2 log for both. During the whole experiment, a reduction of 0.75 log was observed for the faecal streptococci. The part of the faecal streptococci belonging to the enterococcus group showed a higher reduction of 1.51 log.

The inocula of *S.* Enteritidis in the DBW samples were $3.26 \cdot 10^6$ CFU/g on BHI agar and $1.65 \cdot 10^6$ CFU/g on XLD agar (figure 1c). Already after 1 day of freezing, *S.* Enderitidis declined markedly to a concentration of $1.18 \cdot 10^3$ CFU/g and $4.5 \cdot 10^2$ CFU/g on BHI and XLD agar, respectively. *S.* Enderitidis was non-detectable at day 4 and 7 on XLD and BHI agar, respectively.

The initial concentration of total enteric bacteria resistant to amoxicillin, ciprofloxacin, tetracycline and vancomycin was $1.06 \cdot 10^7$, $7.04 \cdot 10^6$, $1.18 \cdot 10^7$ and $1.43 \cdot 10^7$ CFU/g respectively (figure 1d). Like for the other microbial groups the largest reduction occurred during the first days of the long-term freezing period. For the amoxicillin resistant bacteria it occurred between day 1 and 3, for ciprofloxacin and tetracycline resistant bacteria between day 3 and 5, and for vancomycin resistant bacteria between day 0 and 1. Overall, the antibiotic resistant bacteria showed less reduction than the coliform group and *S.* Enderitidis. The tetracycline resistant bacteria exhibited the least reduction, having a final concentration of $4.37 \cdot 10^5$ CFU/g while bacteria resistant to the other antibiotics had concentrations of approx. $1\text{-}2 \cdot 10^4$ CFU/g. By the end of the experiment the total viable counts of amoxicillin, ciprofloxacin, tetracycline and vancomycin resistant enteric bacteria had been reduced by 2.72, 2.77, 1.43 and 3.08 log, respectively.

Figure 1e shows the effect of freezing on the bacteriophages which was overall limited. The initial level was $1.0 \cdot 10^4$ PFU/g and on day 1 the level declined to $2.93 \cdot 10^3$ PFU/g, which corresponds to the largest reduction during the long-term freezing experiment; 0.53 log. After that the reduction was only weak. At the end of the experiment (280 days), the level was approx. $2 \cdot 10^3$, which corresponds to a reduction of 0.68 log during the whole experiment.

16 S rDNA sequences from selected colonies of antibiotic resistant bacteria were BLASTed against the GenBank database (Table S1 in Supporting Information). It was chosen only to present the results for the colony types that dominated the growth on the MacConkey agar plates containing the antibiotics amoxicillin, ciprofloxacin and tetracycline (Figure 2). Other colony types than those shown in figure 2 were identified but in lower levels and in some cases sporadically.

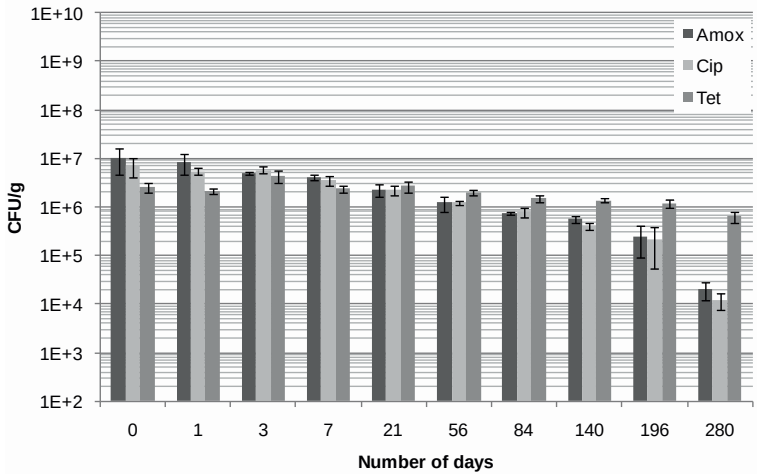

Figure 2. Dominant colony types on MacConkey agar containing the antibiotics ciprofloxacin, amoxicillin or tetracycline identified during the long-term freezing experiment. The lower limit on the y-axis is the detection limit. Error bars represent the standard deviation of three independent replicates.

The amoxicillin resistant colony type had a best match with two strains; a Rainbow trout intestinal bacterium or *Providencia heimbachae*; which is a gram-negative bacterium[27]. The best match for the ciprofloxacin resistant colony type was an uncultured bacterium and *Lactobacillus sakei*. Lactobacilli are, with the possible exception of the small intestine, not a dominating bacterial group in humans but are found in the gastro-intestinal microbiota of almost all adults and are commonly found in faeces[28]. The best match for the tetracycline resistant colony type was *Enterococcus faecium*.

The initial concentration of the microbial groups used in the recovery study (antibiotic resistant bacteria as well as *S.* Enderitidis) corresponded to the concentration of the last sampling day (month 10) of the long-term freezing experiment. The results of the recovery study, where an MTF method was applied, had the unit MPN/g. The results of the long-term freezing study, where direct plate counts were used, had the unit CFU/g. In order to compare the results, it was estimated in which dilutions the plate counts would have shown growth, if an MTF method would have been applied. This was compared to the dilutions where growth was detected in the recovery study. No recovery was detected for the coliforms or amoxicillin and ciprofloxacin resistant bacteria, Recovery of tetracycline and vancomycin resistant bacteria was minimal (approx. 1 log) and only when using TSB as recovery media, incubated at 20°C;

hence 10°C were insufficient (results shown in Supporting Information table S2). As for the *S.* Enderitidis the level was under detection limit (<100 CFU/g) on both BHI and XLD agar before the recovery study was started. Results from the recovery study are shown in table 1. The table shows in which dilutions and triplicates growth was detected, indicated with a "+" for growth and "–" for no growth.

S. Enteritidis (before recovery <100 CFU/g)	-1	-2	-3
BHI agar	+++	++ +	---
XLD agar	+++	---	---

Table 1. Effect of recovery in buffered peptone water and subsequent growth on BHI and XLD agar of S. Enderitidis. A "+" indicates growth in the respective dilution of one triplicate whereas a" –" indicates no growth.

From the results it can be interpreted that there were still some viable *Salmonella* in the frozen sample, and furthermore that a fraction of them have been injured since they could not grow on the XLD agar. Some part of the *S.* Enderitidis may have undergone metabolic injury, resulting in an inability to form colonies on the selective XLD agar, which uninjured cells can tolerate[29]. Since the DBW samples used for enumeration of *S.* Enteritidis were autoclaved before inoculation of *S.* Enteritidis, interference of indigenous microorganisms with the Salmonella counts on BHI agar were avoided. It was therefore possible to determine the fraction of injured cells by comparing the levels of growth on BHI and XLD agar after recovery. It was assumed that colonies developed on the BHI plates represented both injured and uninjured cells whereas only uninjured cells developed on the XLD agar. Since recovery on BHI agar was one log higher than on XLD agar (see Table 1) it can be estimated that the fraction of injured cells after growth in BPW was approx. 90%.

3.2. The impact of repeated freeze/thaw cycles on the most robust microbial groups in DBW

During the freezing and thawing experiment, where only MacConkey agar containing amoxicillin was used, four different colony types were identified by sequence analysis of the 16 S rDNA gene. The identified colonies and their closest match from GenBank can be seen in table S3 in Supporting Information. There were two colony types that dominated the growth on the MacConkey plates during the freezing and thawing experiment (types 1 and 2). Type 1 had a best match with *Proteus vulgaris*, which is a gram-negative bacterium within the family Enterobacteriaceae[30]. Type 2 had a best match with an uncultured bacterium or *Enterobacter* sp, a gram-negative bacterium. Colony types 3 and 4 were only found sporadically and in low concentrations. Types 3 and 4 had a best match with *Klebsiella* sp. or *Raoultella planticola,* and uncultured bacterium or *P. heimbachae*, respectively. Figure 3 presents the results for the total count as well as for the two dominating colony types of the amoxicillin resistant microbial group present in the second batch of DBW after each cycle.

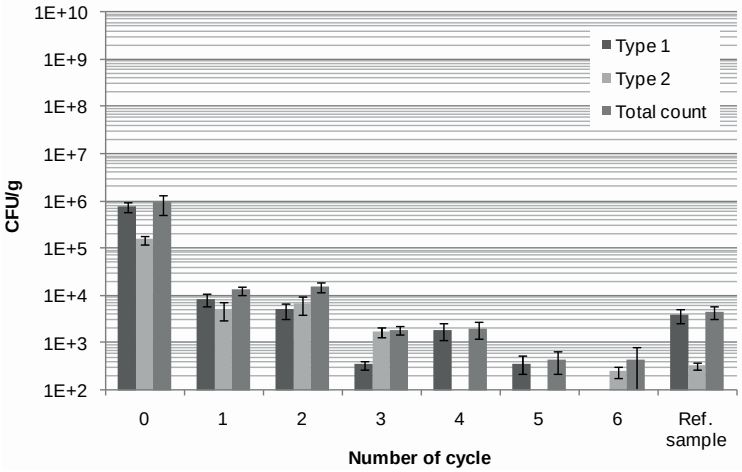

Figure 3. Effect of 6 freeze/thaw cycles on amoxicillin resistant enteric bacteria. The third column shows the total counts for each cycle. The lower limit on the y-axis is the detection limit. Error bars represent the standard deviation of three independent replicates.

Type 1 and 2 showed the largest reduction after the first cycle of freezing and thawing. Type 1 had a concentration of $3.93 \cdot 10^3$ CFU/g in the reference sample, which is similar to the level after the first freezing and thawing cycle ($8.52 \cdot 10^3$ CFU/g). After the first cycle type 1 gradually declined until after cycle 6 where it was non-detectable (detection limit <100 CFU/g). The same pattern can be seen for type 2, which was found to be non-detectable after cycle 4. After cycle 6, this type was, however, detected again in low concentration (250 CFU/g), indicating that after cycle 4 and 5, it may have been just below the detection limit. The levels of the respective colony types in the reference sample were also reduced compared to the initial levels. Type 1 had a concentration of $3.92 \cdot 10^3$ CFU/g in the reference sample, which is a bit lower than the level after the first freezing and thawing cycle ($8.52 \cdot 10^3$ CFU/g). Type 2 had an initial concentration of $1.53 \cdot 10^5$ CFU/g and was just above the detection limit in the reference sample; $3.33 \cdot 10^2$ CFU/g; indicating that the combination of the initial freezing and subsequent storage in the freezer during the experiment (5.5 days) had a similar effect as four freeze/thaw treatments, which brought the level down to non-detectable.

Figure 4 presents the results for the coliphages during the freezing and thawing experiment.

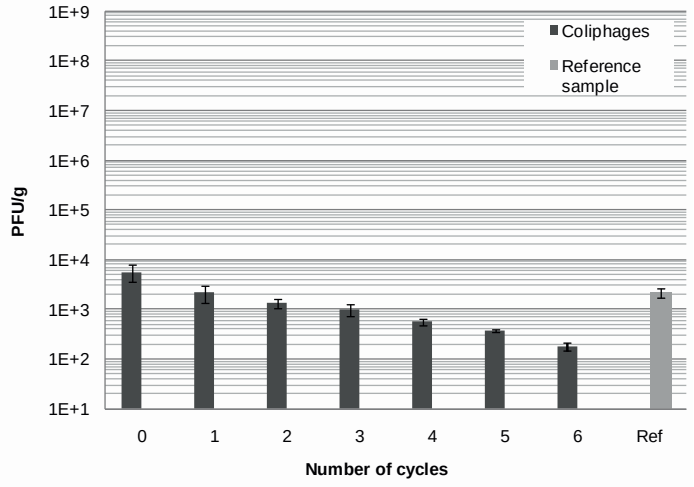

Figure 4. Effect of six freeze/thaw cycles on Escherichia coli bacteriophage ΦX174. The lower limit on the y-axis is the detection limit. Error bars represent the standard deviation of three independent replicates.

The level of coliphages after 6 freezing and thawing cycles was significantly lower than the level in the reference sample (p=0.0011). The coliphages declined during the freezing and thawing experiment from $5.67 \cdot 10^3$ PFU/g to $1.83 \cdot 10^2$ PFU/g, resulting in 1.49 log reduction (figure 4). In contrast, the number of coliphages in the reference sample ($2.13 \cdot 10^3$ PFU/g) corresponded largely to the amount of coliphages after the first freezing and thawing cycle ($2.17 \cdot 10^3$ PFU/g), indicating that each freezing and thawing cycle has had an additional effect on the reduction of coliphages.

4. Discussion

4.1. Long-term freezing and recovery

The results from the long-term freezing experiment showed that after 3 days of freezing total and faecal coliforms and *E. coli* had decreased with 3.24, 3.27 and 3.2 log, respectively. In contrast Torrella et al.[11] reported that total and faecal coliforms decreased with 0.17 log after 4 days of freezing. Sanin et al.[7] also observed only a limited reduction (0.1 log) of faecal coliforms in sludge samples when frozen at -18°C and stored for 1 day. Both studies analyzed indigenous coliforms but their susceptibility can be different between various wastewater samples because of uncontrolled parameters in raw wastewater, such as fat droplets, particles, different kind of dissolved wastes, etc., which can influence the survival capacity of the bacteria[11]. The increased dry matter in the dewatered wastewater in our study does at least not seem to have provided extra protection of the coliform group, rather opposite. Gao et al.[14] studied the effect of frozen storage for 30 days at different temperatures on inoculated *E coli* in water samples, and observed

about 1.5 log reduction after 20 days at -15°C with a more rapid inactivation initially which gradually slowed down and remained almost constant as storage time approached 30 days. Thus the inoculated *E. coli* neither seem to have been more sensitive against freezing than the indigenous bacteria, nor does the reduction seem to be faster in pure water than wastewater.

The total and faecal coliforms in our study achieved a steady state after 3 and 7 days of freezing, respectively, and were detectable until day 196 and 84 of the experiment, respectively. At day 140 the faceal coliforms were detected again, but were under the detection limit at day 196 and 280. *E. coli* got to a steady state after 3 days and was non-detectable at day 56. The study performed by Sanin et al.[7] lasted for 28 days. The authors found that frozen storage had little effect on the reduction of fecal coliforms after 7 days of freezing. Torrella et al.[11] observed that total and faecal coliforms in raw wastewater were inactivated particularly during initial freezing stress of the first days to first weeks, and by the end of their study (60 days), a population of survivors still remained in the frozen wastewater. Our results are in accordance with those results and further show that total and faceal coliforms remained viable for a long time after 28 and 60 days, the maximal experimental periods of the mentioned studies[7,11].

Comparing the two microbial indicator groups, the coliform group showed a larger overall reduction than the faecal streptococci/enterococcus group when frozen in DBW, which is in accordance with earlier studies[7,12,31]. The overall reduction of faecal streptococci during the long-term freezing experiment was limited; 0.75 log. Sanin et al.[7] studied the reduction of faecal coliforms and faecal streptococci in aerobically digested sludge when frozen at -25°C for 7 days, and found a reduction of 1.9 log for the faecal coliforms while the faecal streptococci were reduced by only 0.21 log. Incorporation of natural freezing in wastewater treatment for cold regions might therefore not be effective against gram-positive bacteria.

Common for all of the microbial groups during the long-term freezing was that the highest reduction occurred in the beginning of the experiment, indicating that the initial freezing caused the largest damage to the cells. Subsequent long-term freezing had an additional effect since all groups, except for the faecal streptococci/enterococcus group and the coliphages, showed further reduction after the first days in frozen condition.

The antibiotic resistant bacteria showed a slow but constant reduction throughout the experiment, the tetracycline resistant bacteria being most resistant to freezing. 16 S rDNA sequencing of dominating antibiotic resistant colony types indicated that the dominant tetracycline resistant bacterium was *Enterococcus faecium*. Tetracycline resistance in *Enterococci* is acquired[32,33]. The effect of long term freezing on this bacterium was limited (Figure 2). This could be due to that gram-positive strains are more resistant to freezing, but the freezing resistance might also have to do with the tetracycline resistance. Earlier studies have indicated that e.g. *E. coli* strains resistant to antibiotics, i.a. tetracycline, were more persistent during mesophilic anaerobic digestion of pig slurry than sensitive strains[34].

The best match for the dominant ciprofloxacin resistant colony type was an uncultured bacterium and *Lactobacillus sakei*. Lactobacilli are often found in faeces[28]. It has been reported that Lactobacillus has a high natural resistance against ciprofloxacin[35] and Razzak et al.[36] reported that 93.3% out of 15 Lactobacilli isolates tested were ciprofloxacin resistant. Based on this it is not unlikely that the ciprofloxacin strain belonged to Lactobacilli. As the Lactobacilli are highly resistant to ciprofloxacin, it is possible that they may have overgrown other bacteria having a lower level of resistance that could therefore not be detected. These bacteria might also pose a human health threat.

The dominant amoxicillin resistant colony type had a best match with two strains; a Rainbow trout intestinal bacterium or *Providencia heimbachae*. The first isolation of *P. heimbachae* from a human was reported by O'Hara et al.[37] and some strains of *Providencia spp.* are pathogenic to humans[38]. No pathogenic resistant bacteria were identified but the antibiotic resistant bacteria pose a reservoir of antibiotic resistance genes that may be transferred to pathogenic bacteria.

After 2 months of frozen storage, 22.3% of the coliphages were still viable. Torrella et al.[11] found that after 2 months of freezing only 10.2% of the initial population of indigenous somatic coliphages in wastewater samples were still viable but they also experienced that the viruses were slowly but constantly inactivated. This was not the case for the coliphages in this study where the concentration, after the initial reduction, achieved a quasi steady state throughout the 10 month-long experiment which is more in line with what Olson et al.[39] found. Although freezing of viruses at ultra-low temperature (-80°C) preserves their infectivity, losses of active viruses can occur due to the freezing process[39]. In frozen samples initial viral loss is primarily attributed to ice crystal formation which generates shearing forces which can disrupt viral coat proteins and destroy nucleic acids[40]. After freezing predation by protozoans and bacteria is stopped, resulting in a quasi steady state after the initial viral reduction[39]. Various factors affect the numbers and behavior of phages in water environments, including densities of host bacteria and phages, association of phages and bacteria with solids, presence of organic matter, and temperature. Sanin et al.[7] found that a longer freezing time improved the reduction of indigenous phages in sludge samples. In their study, the phages declined to negligible levels (approx. 5 log reduction) after 1 week of storage at -18°C, which contradicts to the results from the present experiment. Thus, on one hand, the inoculated bacteriophages used in our study did not seem to be more sensitive to freezing than indigenous ones. On the other hand, it has earlier been reported that bacteriophages are protected from environmental stress when water quality is poor, such as in wastewater[39], and it has further been suggested that viruses attach to particles in water which makes them more stable and keeps them in active state due to the shrinkage that occurs when a virus adsorbs to a surface[41]. Sanin et al.[7] and Torrella et al.[11] used sludge and raw wastewater, respectively, in their studies whereas dewatered blackwater was used in our study. The dry matter content of the raw wastewater used in the study of Torrella et al.[11] is not mentioned, but it is generally very low in raw sewage sludge[42]. The content of suspended solids of the aerobic and anaerobic sludge used in the study by Sanin et al.[7] was 1.84% and 1.52%, respectively. The increased/higher dry matter content of the dewatered blackwater used in the

present experiments (approx. 15%) may therefore have protected the bacteriophages from damage caused by the freezing to a higher degree than in sludge and wastewater.

During long-term freezing, inoculated *S.* Enderitidis was detected for longer time (7 days) on the non-selective BHI agar than on the selective XLD agar (4 days). These results are similar to the study of Torrella et al.[11]. Sanin et al.[7] showed that freezing for 1 day at -18°C resulted in a 0.9 log reduction of *Salmonella*, which is much lower than in the present experiment where 1 day of freezing resulted in a reduction of 3.4 and 3.6 log, using XLD and BHI agar for detection, respectively. In the present study an inoculated *Salmonella* strain was used whereas the *Salmonella* studied by Sanin et al.[7] and Torrella et al.[11] were indigenous. The microorganisms in the sludge samples of Sanin et al.[7] had a longer time to adapt to the environmental conditions before the freezing and were possibly more resistant to the stress exposure than the inoculated bacteria used in the present study. This is not supported by the results of Torrella et al.[11], who also analyzed indigenous *Salmonella* in wastewater, but variances between various components in the wastewater samples could also explain the different susceptibility of the *Salmonella*.

The recovery study was performed on samples that had been frozen for 10 months and no recovery for the coliform group was detected. The same accounts for antibiotic resistant bacteria, where little or no recovery was detected. This is opposite to what previous studies on resuscitation of *E. coli* frozen at -78°C[25] and species of Enterobacteriaceae, rapidly frozen to -22°C and subsequently stored for 1 week at that temperature, have shown where recovery at 17-25°C for 1-2 h in tryptone soy broth has resulted in good or almost full recovery[17]. This indicates that the long-term freezing has had a lethal effect on a fraction of the antibiotic resistant bacteria and the coliform group, even though a part of them remained viable for a long period; total and faecal coliforms until day 196.

On the other hand, *S.* Enteritidis that had shown a rapid reduction in the beginning of the long term freezing experiment, showed some recovery, indicating that there were still viable bacteria left in the frozen sample and that a fraction of them has been injured. Since *Salmonella* is a pathogen, frequently found in wastewater, it is alarming that it can survive (in an injured state) for such long periods of freezing. Another subject of concern is that sublethal exposure to different stresses may enhance the survival of bacteria under subsequent stress conditions, resulting in cross-protection against other stresses[43]. The results of the present study emphasise that the coliform group may not be a suitable indicator organism when measuring the efficiency of freezing as a treatment method.

4.2. Freezing and thawing experiment

Regarding the amoxicillin resistant bacteria colony type 1 had a closest match from GenBank with *Proteus vulgaris. Proteeae* constitute a part of the normal flora of the human gastrointestinal tract and are widespread in the environment[44]. Moreover *Proteus* ranks third as the cause of uncomplicated cystitis, pyelonephritis, and prostatitis, especially in hospital-acquired cases[44]. Colony type 2 had a best match with an uncultured bacterium or *Enterobacter* sp which is a gram-negative bacterium that has been

recognized as an increasingly important pathogen[45]. Most *Enterobacter sp.* are naturally resistant to older antibiotics and furthermore they have the ability to develop resistance rapidly to newer antimicrobial agents[45]. They are intrinsically resistant to amoxicillin[46]. In contrast to colony type 2, the repeated freezing and thawing had an additional reductive effect on colony type 1. The initial freezing and subsequent thawing had the strongest effect on colony type 1 whereas it seemed that the combination of the initial freezing and subsequent storage in the freezer during the experiment (approx. 5.5 days) had a similar effect on colony type 2 as 4 freeze/thaw treatments.

The results from the freezing and thawing experiment showed that each cycle had an additional effect on the reduction of the coliphages. Comparing the scarce reduction in the reference sample in the freezing and thawing experiment as well as during the long term freezing experiment to the additional effect caused by repeated freezing and thawing, it might be valuable for viral reduction to incorporate freezing and thawing in the design of wastewater treatment methods for cold regions.

4.3. Overall results

This study shows that freezing has the potential of reducing microorganisms in wastewater. It seemed to have a lethal effect on some microbial groups, as the coliforms, and a sublethal effect on other microorganisms, such as *Salmonella*. Incorporating long-term freezing and repeated freezing and thawing in the design of wastewater treatment methods for cold regions where winter periods are long can therefore be a cost-effective way to enhance the quality of treated wastewater. However, freezing is not appropriate for total elimination of the microbiota in wastewater but it might be a valuable additional process in wastewater treatment in cold regions.

As indigenous coliform bacteria in the DWB were most susceptible to freezing, they may not be suitable indicator organisms to measure the reduction efficiency of pathogenic bacteria during freezing. The faecal streptococci/enterococcus group were more robust against freezing. Enteric bacteria, resistant to amoxicillin, ciprofloxacin, tetracycline and vancomycin, showed a slow but constant reduction during long term freezing. The coliform group as well as antibiotic resistant enteric bacteria showed limited or no recovery at 20°C overnight, suggesting that the level of viable but injured bacteria was low. Inoculated *S.* Enteritidis rapidly decreased during freezing but did, however, show some recovery after 10 months of freezing. Inoculated *E. coli* bacteriophage ΦX174 showed limited reduction during the long term freezing but repeated freezing and thawing increased the reduction noticeably.

Supporting Information

A scheme showing the microorganisms and microbial groups analyzed in the present studies can be found in Supporting Information, where numeration methods and growth media are listed as well. The Supporting Information also contains tables showing results of recovery treatment of antibiotic resistant

bacteria, as well as results from PCR which was performed on the extracted DNA of different colonies growing on MacConkey and PSE agar plates containing antibiotics, during the long term freezing and freezing and thawing experiments. The sequences were BLASTed against GenBank database to find the closest match to the colony types in question.

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SUPPORTING INFORMATION

Title of submitted manuscript

Effect of long-term freezing and freeze/thaw-cycles on indigenous and inoculated microorganisms in dewatered blackwater

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Number of pages, figures and tables

Number of pages S1-S6

Number of figures: 1 (Figure S1, page S3)

Number of tables: 3 (Table S1, page S4; Table S2, page S5; Table S3, page S6)

Figure S1

Growth media and detection methods for each type of microorganism or microbial group are listed in Figure S1.

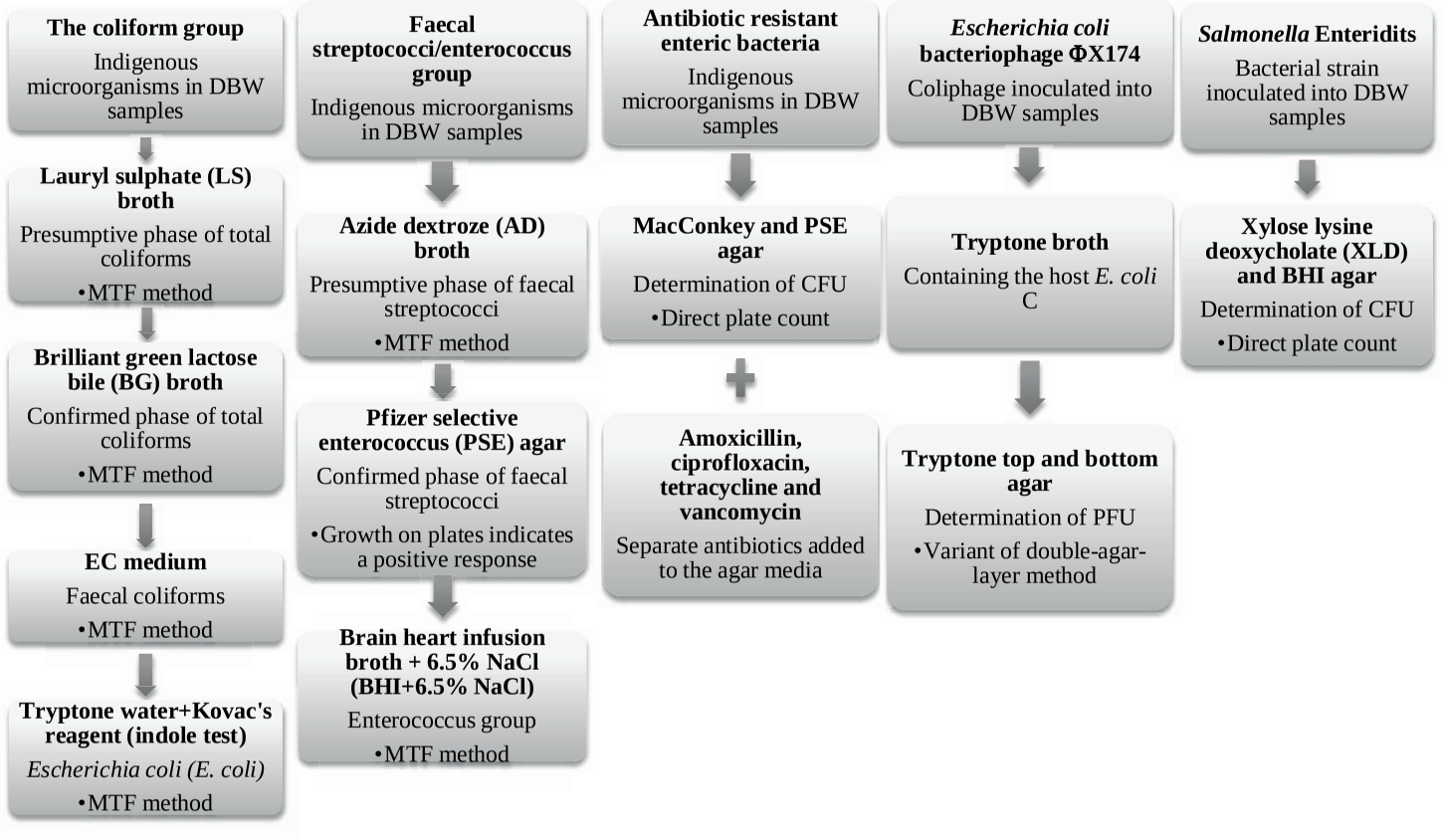

Figure S1. Microorganisms and microbial groups analyzed. Numeration methods and growth media are listed for each microorganism/microbial group. Abbreviations: MTF=Multiple tube fermentation method. CFU=Colony Forming Units. PFU=Plaque Forming Units.

Table S1

16 S rDNA sequences from selected colonies of antibiotic resistant bacteria, identified during the long-term freezing experiment, were BLASTed against the GenBank database (National Center for Biotechnology Information). It was chosen only to present the results for the colony types that dominated the growth on the MacConkey agar plates containing the antibiotics amoxicillin, ciprofloxacin and tetracycline. Other colony types than those shown in Table S1 were identified but in lower levels and in some cases sporadically.

Antibiotic	*Description*	*Accession number*	*E value*
Amoxicillin	*Rainbow trout intestinal bacterium D22, or*	AY374116.1	*0.0*
	Providencia heimbachae strain : DSM 3591	NR 042412.1	*0.0*
Ciprofloxacin	*Lactobacillus sakei strain kimshi007, or*	JF781305.1	0.0
	Uncultured bacterium clone PB1_aai28g03	EU777810.1	0.0
Tetracycline	*Enterococcus faecium gene, or*	AB512765.1	0.0
	Uncultured organism clone ELU0047-T268-S-NIPCRAMgANa_000466	HQ761284.1	0.0

Table S1. Dominating colony types identified during the long-term freezing experiment and their closest match from GenBank. The colony types were identified by amplification and sequence analysis of the 16S RNA gene.

Table S2

Table S2 shows the results for recovery treatment of tetracycline and vancomycin resistant bacteria (grown on MacConkey and Pfizer selective Enterococcus agar, respectively). No recovery was detected for amoxicillin and ciprofloxacin resistant bacteria, hence the table presents only results for tetracycline and vancomcyin resistant bacteria The tables show in which dilutions and triplicates growth was detected, which is indicated with a "+" for growth and "–" for no growth. The level of the microorganisms before recovery is given in parenthesis after the antibiotic´s name.

Tetracycline (before recovery $4.35 \cdot 10^5$ CFU/g)	-1	-2	-3	-4	-5	-6
0.9% NaCl 10°C	+++	+++	+++	---	---	---
0.9% NaCl 20°C	+++	+++	+++	+-+	---	---
TSB 20°C	+++	+++	+++	+--	---	---
TSB 20°C	+++	+++	+++	+++	+++	--+

Vancomycin (before recovery $1.22 \cdot 10^4$ CFU/g)	-1	-2	-3	-4	-5	-6
0.9% NaCl 10°C	+++	+++	---	---	---	---
0.9% NaCl 20°C	+++	+++	--+	---	---	---
TSB 10°C	+++	+++	-+-	---	---	---
TSB 20°C	+++	+++	+++	+-+	-+-	---

Table S2. Effect on antibiotic resistant enteric bacteria of recovery in tryptone soy broth (TSB) and 0.9% NaCl at 10 and 20°C and subsequent growth on MacConkey agar plates (tetracycline) and PSE agar plates (vancomycin). A "+" indicates growth in the respective dilution of one triplicate whereas a" –" indicates no growth.

Table S3

During the freezing and thawing experiment, where MacConkey agar containing amoxicillin was used as growth media for bacteria belonging to Enterobacteriaceae, four different colony types were identified by sequence analysis of the 16 S rDNA gene. The identified colonies and their closest match from GenBank can be seen in table S3.

Colony type	*Description*	*Accession number*	*E value*
1	*Proteus vulgaris*	AB679997.1	0.0
2	Uncultured bacterium clone Con1068, or	JN394064.1	0.0
	Enterobacter sp. CMG24314	EU162036.1	0.0
3	*Klebsiella* sp. B19, or	GU594299.1	0.0
	Raoultella planticola strain ATCC 33531	NR_024996.1	0.0
4	Uncultured bacterium clone nbt106b08, or	EU539770.1	0.0
	Providencia heimbachae strain : DSM 3591	NR_042412.1	0.0

Table S3. Colony types identified on MacConkey agar containing amoxicillin during the freezing and thawing experiment and their closest match from GenBank. The colony types were identified by amplification and sequence analysis of the 16S RNA gene.

Paper III

Gunnarsdóttir, R., Heiske, S.., Jensen, P.E., Schmidt, J.E., Villumsen, A Jenssen, P.D., 2012. Effect of anaerobiosis on indigenous microorganisms in blackwater with fish offal as co-substrate. Manuscript for Water Research.

Effect of anaerobiosis on indigenous microorganisms in blackwater with fish offal as co-substrate

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Keywords: Anaerobic digestion, aerobic storage, indigenous microorganisms, coliforms, faecal streptococci, coliphages, resistance, wastewater, fish waste, biogas yield, cold climate, Arctic

Abstract

Many Arctic communities are in lack of sound waste and wastewater handling and proper sanitation, raising environmental and hygienic concerns. The energy supply in those communities often depends on costly long-distance oil delivery. Introducing local biogas plants in Arctic communities could be a potential way to approach these problems. The aim of this study was to compare the effect of mesophilic anaerobic digestion with aerobic storage on the survival of selected indigenous microorganisms and microbial groups in blackwater, including the effect of addition of Greenlandic halibut and shrimp offal. The methane (CH_4) yield of the different substrate mixtures was also determined in batch experiments. By the end of the experiments recovery study was carried out to determine possible injury of the microorganisms analyzed in the experiments. The CH_4 yield was highest for mixture of blackwater and Greenlandic halibut offal; 619.7 ml/(g VS). Digestion of blackwater alone resulted in a CH_4 yield of 438.6 ml/(g VS), which was actually higher than co-digestion of blackwater and shrimp offal, which had a CH4 yield of 346.4 mL/(g VS). During the anaerobic digestion tetracycline resistant bacteria showed the least reduction in the mixture resulting in the lowest CH_4 yield while the highest reduction was observed in the mixture with the highest CH_4 yield. The reduction of faecal streptococci was large under both anaerobic and aerobic circumstances but faster in the aerobic samples. Coliphages showed a better survival under aerobic conditions. The addition of fish offal had a different effect on survival of *Escherichia coli* (*E.* coli) and faecal streptococci in the anaerobic samples, but had no effect on survival of coliphages. The results of the recovery study indicated that a certain fraction of *E. coli* in the aerobic blackwater sample and

faecal streptococci in the anaerobic and aerobic samples containing blackwater and Greenlandic halibut has been injured only and able to resuscitate during recovery.

1. Introduction

Many Arctic communities consist of small remote settlements that lack sound waste and wastewater handling and proper sanitation. Moreover, their energy supply often depends on costly long-distance oil delivery. Raising energy prices and vulnerability of supply have increased interest in renewable options. Additionally, wastewater contains a variety of substances, including anthropogenic pollutants and residues of pharmaceuticals and personal care products (PPCPs) as well as pathogenic microorganisms, parasites and antibiotic resistant bacteria, which can be harmful for the environment as well as human health. The introduction of local biogas plants in Arctic communities could be a potential way to approach the challenges regarding waste and wastewater handling (Gunnarsdóttir et al., 2012). The treatment of organic waste in a biogas reactor comes with several benefits: Reduction of waste/wastewater, stabilization, improved dewatering properties, production of renewable energy (Mata-Alvarez et al., 2000) as well as hygienization (Luste and Luostarinen, 2010). Biogas plants could therefore improve waste and wastewater handling as well as secure energy supply, ensuring the sustainability and endurance of peripheral communities. However, implementing biogas plants in the Arctic involves challenges, such as low temperature, logistical problems and absence of technical personnel (Smith and Low, 1996). Small anaerobic digesters are easier to run at mesophilic than thermophilic conditions because thermophilic digestion can be more sensitive to operational conditions such as the organic loading rate, temperature and the characteristics of the influent sludge (Kim et al., 2002; van Lier, 1996). Taking this as well as the cold climate in the Arctic into account it might be practical to run anaerobic digesters at mesophilic conditions, especially in the smaller settlements.

The fishing industry is one of the most important industries in many Arctic regions and is for instance the biggest one in Greenland today, generating about 14,000 tons of waste products each year whereof only about 20% is utilized (Nielsen et al., 2006). Fish offals and fish oil have been shown to have a promising biogas yield (Callaghan et al., 1999; Ahring, 2003; Mshandete et al., 2004), thus an option for this waste fraction could be to use it for biogas production along with other organic waste. In many settlements in the Arctic, municipal collection of blackwater (wastewater from toilets) is practiced. Instead of direct discharge to the sea it could be transported to a central anaerobic digester. The anaerobic digestion of food waste and animal manure has been extensively studied (Neves et al., 2008; Ahn et al., 2010; El-Mashad and Zhang, 2010) while studies on blackwater combined with other waste fractions are not as common (Lim, 2011). One of the concerns about usage of blackwater for anaerobic digestion is the hygienic aspect of the process. Many have studied the effect of anaerobic digestion on various microorganisms, e.g. members of the coliform group (Berg and Berman, 1980; Kumar et al., 1999; Bonjoch and Blanch, 2009; Watcharasukarn et al., 2009) who in many studies have shown a higher

reduction than for instance enterococci (Berg and Berman, 1980; Bonjoch and Blanch, 2009). The value of coliforms as a measure of reduction efficiency of anaerobic digestion has thus been questioned (Bendixen, 1994). The reduction of antibiotic resistant bacteria has also been studied (Abdul and Lloyd, 1985; Diehl and Lapara, 2010), for instance by Abdul and Lloyd (1985) who found that antibiotic resistant *E. coli* strains had a better survival than sensitive ones during mesophilic anaerobic digestion. It has also been shown that the survival of pathogenic bacteria during anaerobic digestion is highly dependent on the temperature (Dumontet et al., 1999). However, the effect of anaerobiosis compared to aerobic treatment and the effect of addition of different kinds of substrate for anaerobic digestion on survival of microorganisms has not been extensively studied.

The digestate from anaerobic digesters is sometimes treated further by e.g. composting. If the digestate contains a fraction of injured microorganisms appearing dead from prior treatment there is a possibility of recovery if the environment becomes more suitable for the injured cells, and this can present a health threat (Gao et al., 2007).

The aim of this study was threefold: 1) To compare the effect of mesophilic anaerobic digestion with aerobic storage under the same temperature, moisture and substrate conditions on survival of selected different microbial groups; 2) to compare the effect of additional substrate, namely Greenlandic halibut and shrimp offal, on the different microbial groups; 3) to determine the methane (CH_4) yield of different substrate mixtures, containing blackwater and Greenlandic halibut and shrimp offal.

2. Methods and Materials

2.1. Sample collection and preparation

The blackwater used in the experiments was derived from a student dormitory with 48 students served by vaacuum toilets. The vacuum toilet and transport system (Jets™) contains a macerator and a dewatering unit. The blackwater was stored at 4±0.5°C prior to use in the laboratory experiments. The shrimp offal, mainly consisting of crushed shells and heads, was collected from a shrimp factory in Sisimiut in W-Greenland while the Greenlandic Halibut offal was collected in Uummannaq in NW-Greenland. The samples were stored frozen during shipment and were homogenized at arrival at the laboratory using an industrial blender. After homogenization the samples were stored frozen prior to use in the experiments. Total and volatile solids (TS and VS) in the blackwater and the fish offal samples were determined by drying overnight at 105°C followed by heating to 550°C for 2 h. Results can be found in table 2.

2.2. Microbial analyses

2.2.1. Experimental microorganisms

On arrival of the blackwater at the laboratory the background levels of total coliforms, *E. coli*, faecal streptococci/enterococcus group, amoxicillin and tetracycline resistant enteric bacteria belonging to the bacterial family Enterobacteriaceae, and somatic coliphages were determined. The two antibiotics used in the experiment were selected from a list of antibiotics being most used in Greenland in 2007/2008, and the selection was based on their different mechanisms of action. During the anaerobic digestion and aerobic storage the content of the microbial groups and microorganisms were analyzed. Sampling was made on days 0, 3, 5, 10, 17, 24, 31 and 38. Subsamples were taken out on each sampling day and transported in a cooling bag to the laboratory where they were stored at 4°C until microbial analyses were done the same day or the next day at the latest. The selected microorganisms and microbial groups, growth media and conditions, as well as detection methods are listed in table 1.

Microorganism/ microbial group	Growth media	Enumeration method	Incubation temp. (°C)	Incubation time (h)
Total coliforms	Lauryl sulphate broth (presumptive phase)	MTF	37	48 ± 3 h
E. coli	Tryptone water + Kovac's reagent (indole test)	MTF	45	24 ±2 h
Faecal streptococci	Azide dextrose broth (presumptive phags)	MTF	37	48 ± 3 h
	Pfizer selective enterococcus agar (confirmed phase)	Brownish-black colonies w/brown halos confirm presence of faecal streptococci	37	24 ±2 h
Enterococcus group	Brain Heart Infusion broth + 6.5% NaCl	MTF	45	Overnight
AR bacteria	MacConkey agar*	Determination of CFU by direct plate count	37	24-72 h
Somatic coliphages	Tryptone top and bottom agar	Determination of PFU by a variant of double-agar-layer method	37	Overnight

*Table 1. Microorganisms and microbial groups determined before and during anaerobic digestion and aerobic storage. Abbreviation: MTF=Multiple tube fermentation method, CFU=Colony Forming Units, PFU=Plaque Forming Units. *Amoxicillin and tetracycline added separately to agar*

Analyses of total coliforms were only performed as an initial step of the *E. coli* analysis, thus results are not shown. *Escherichia coli* (*E. coli*) and faecal streptococcus/enterococcus group were selected to represent gram-negative and -positive indicator bacteria, respectively. Somatic coliphages are bacterial viruses that infect and replicate in *Escherichia coli (E. coli)* and can be used as an indicator of viruses (Eaton et al., 2005). Coliphage assays are easier and less expensive to perform than human virus assays and yield overnight results (Eaton et al., 2005), and were therefore selected as an indicator of viruses in the experiments. The host used in the coliphage assay was *E. coli* C (ATCC 13706), the most commonly used host for detection of coliphages in water environments (Grabow et al., 1998; ISO, 1998). The coliphage ΦX174 was used as a positive control, preparing the stock solution by standard procedure (Eaton et al., 2005). Since spreading of resistance genes is a growing global problem (Kunin, 1993) indigenous antibiotic resistant bacteria were included in the experiments. *S.* Infantis jeo 4208 (78121/5) (fully susceptible) (Aabo et al., 2002) was used as a positive control strain for the agar plates containing amoxicillin and tetracycline (separate plates for each antibiotic).

2.2.2. Enumeration methods

Serial dilution series of the samples from the anaerobic and aerobic mixtures were prepared in 0.9% NaCl by manual shaking and with a mini shaker at medium speed before inoculated into the different growth media. Growth medium used for the presumptive phase of total coliforms was lauryl sulphate broth (LS broth) which was incubated at 37°C for 48 ± 3 h. All presumptive fermentation tubes showing growth or gas formation were submitted to the *E. coli* test where tryptone water was used as growth media with subsequent addition of Kovac's reagent. The incubation temperature and time was 45°C for 24 ± 2 h.

Azide dextrose broth (AD broth) was used as growth media for the presumptive phase of faecal streptococci and pfizer selective eneterococcus (PSE) agar for the confirmed phase. Tubes containing AD broth were incubated for 48 ± 3 h at 37°C while the inverted PSE agar plates were incubated for 24 ± 2 h at 37°C. Brownish-black colonies with brown halos on the PSE agar plates confirmed the presence of faecal streptococci. To confirm if the faecal streptococci belonged to the enterococcus group the colonies were transferred to tubes containing brain-heart infusion (BHI) broth, added 6.5% NaCl, and incubated at 45°C overnight (Eaton et al., 2005). The multiple tube fermentation (MTF) technique (Eaton et al., 2005), performed in triplicate, was used for analyses of total coliforms, *E. coli* and faecal streptococci/enterococcus group, and the most probable number (MPN) values were calculated from the number of positive tubes (Blodgett, 2010).

Triplicate and duplicate analyses of coliphages and antibiotic resistant bacteria, respectively, were performed by plating of two technical replicates. Enumeration of coliphages was performed using a variant of the double-agar-layer method (Eaton et al., 2005), using 1 mL of appropriate dilutions of the samples per petri dish (d=140 mm). The double layer agar plates were incubated at 37°C overnight. The host, *E. coli* C, was prepared according to Eaton et al. (2005).

MacConkey agar, which is selective for bacteria belonging to Enterobacteriaceae, was used for detection of antibiotic resistant bacteria. The concentrations of amoxicillin and tetracycline in the agar were based on available information on MIC values of Enterobacteriaceae. Antibiotic stock solutions were prepared using appropriate solvents and stored at -20 ºC prior to use in the experiment. The stock solutions were inoculated into the fluid sterilized agar at a temperature below 50°C to obtain a concentration of 16 µg/mL for both antibiotics. The inverted plates were incubated at 37°C for 24-72 h until countable colonies had grown.

2.3. Experimental setup

2.3.1. Anaerobic digestion and estimation of methane potentials

The anaerobic digestion of the blackwater and fish offal was carried out in batch tests. Quadruplicate-samples of blackwater (BW), as well as mixtures of BW and Greenlandic Halibut offal (BW+GH) and BW and shrimp offal (BW+S) were distributed in 1000 ml serum bottles in amounts of 0.75g VS (BW) and 1.0 g VS (GH and S). All bottles were inoculated with 100 g anaerobically digested sewage sludge from the wastewater treatment plant "Mølleåværket" in Lyngby, Denmark. The plant's digester operates at 37°C, treating primarily sludge derived from household wastewater. By the addition of tap water, the liquid volume in all bottles was aligned to 287 ml, thereby maintaining an equal headspace for gas collection. To account for background methane production from the inoculum, an additional set of bottles only containing inoculum and water was set up. Prior to incubation, all bottles were flushed with nitrogen and closed with butyl rubber stoppers. The digestion experiment was carried out at 37°C for a period of 38 days. Mixing was done by manually shaking the bottles once a day.

Three bottles of each quadruplicate set were used to determine the methane production. The fourth bottle was equipped with a glass tube outlet for taking out samples for microbial analysis. The methane production in the flasks was measured regularly by gas chromatography. Samples of 0.2 ml volume were taken from the bottle headspace gas with a gas-tight syringe. The amount of methane in the samples was measured using a Shimadzu GC-8A, with a 2 m Porepak Q column and flame ionization detector. Measurements were carried out twice a week in the beginning and once a week after the methane production rate had lowered significantly. From the obtained data the specific methane yield (ml $CH_4/gVS_{substrate}$) was computed.

2.3.2. Aerobic storage

The same mixtures of inocula and substrate, previously described for the anaerobic digestion, were placed in glass beakers in duplicates and stored in a water bath (37°C) with water proof magnetic disc. Stirring magnets were inserted into each beaker to ensure constant stirring to avoid formation of anaerobic pockets in the mixtures. The water bath was kept half-open to ensure sufficient oxygen supply of the mixtures and furthermore it was located in a fume hood for protection of the experimental setup. The initial liquid content of the mixtures in the aerobic beakers was withheld by weighing each beaker

approx. every second day and before each sampling and adding sterile water to compensate for evaporation occurring during the experiment.

2.3.3. Recovery experiment

At day 38 in the anaerobic and aerobic experiments a recovery study was carried out. Previous studies have shown that recovery in tryptone soy broth (TSB) for 2 h at 17-25°C of stressed Enterobacteriaceae restores their ability to grow on MacConkey media (Mossel et al., 1980). The time needed for complete recovery is however not always so short, and may for some organisms be 6-8 h (Mossel et al., 1980). TSB was selected as a resuscitation media in attempt to recover possibly injured microorganisms. Serial dilution series in triplicate of the samples were prepared in TSB and incubated at room temperature overnight. After incubation 1 mL from all tubes showing turbidity were transferred to selective growth media for the microbial groups listed in table 1, and standard analytical procedure was followed subsequently. For analyses of the antibiotic resistant bacteria a big loopfull (about 10 µL) from each tube showing growth was streaked out on an agar plate containing one of the four mentioned antibiotics. Plates showing growth after incubation at 37°C for about 2 days were noted as being positive and thus likewise the tubes from where the inocula on the positive plates originated.

3. Results

3.1. Methane yield of the different substrate mixtures

Table 2 shows pH and substrate concentrations of the different mixtures as well as results for the specific CH_4 yield for the waste fractions used in the experiments and results for specific CH_4 yield (mL/g VS) obtained in the anaerobic batch experiments. The specific CH_4 yield of the GH and S offal was determined by calculations based on the effect of the two substrate types when digested together with the BW in the batch experiments. Figure 1 shows the total CH_4 production obtained in the triplicates (mL CH_4).

Substrate	TS/VS (%)	ph		VS load	Specific CH_4 yield
		Initial	End	($gVS\ L^{-1}$)	($mL \cdot g\ VS^{-1}$)
BW	0.65/0.44	7.96	7.97	2.7	438.6 ± 33.6
BW + GH	-	7.66	8.02	6.3	619.7 ± 13.5
BW + S	-	7.87	7.73	6.3	346.4 ± 25.3
GH*	35.7/33.5	-	-	-	755.5 ± 30.4
S*	18.1/10.0	-	-	-	277.3 ± 42.1

*Table 2. Total solids (TS) and volatile solids (VS) for the separate fractions, and pH, VS load and specific CH_4 yield of the different mixtures. *Yield calculation based on the effect of GH/S when digested together with BW.*

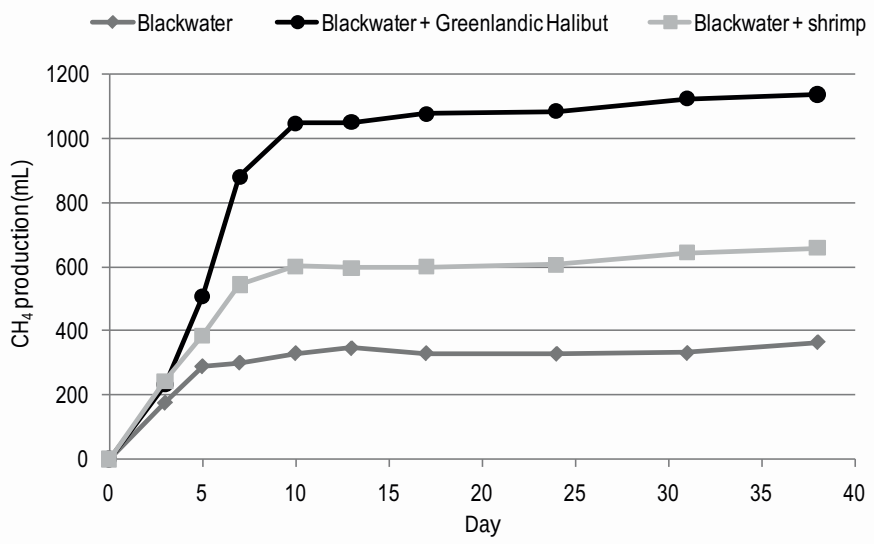


Figure 1. Accumulative CH_4 production in the anaerobic bottles obtained in a batch trial using three different substrate mixtures. CH_4 production from the inoculum has been subtracted from the total CH_4 production.

No lag phase was observed in the methane production of all substrate mixtures. The conversion was relatively fast in all cases since more than 90% of the total CH_4 production was obtained within the first 10 days, more specifically 91, 92 and 91% for BW, BW+GH and BW+S, respectively (figure 1). The pH in BW and BW+GH was measured at each sampling day and remained constant throughout the experimental period, indicating no acidification of the mixtures during the experimental period. However,

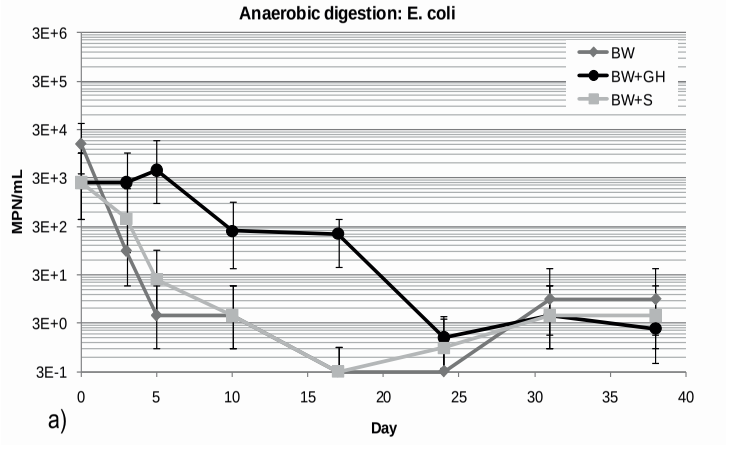
the pH in BW+S decreased by 0.14 in the course of the experiment. The initial and final pH values for each substrate mixture are shown in table 2.

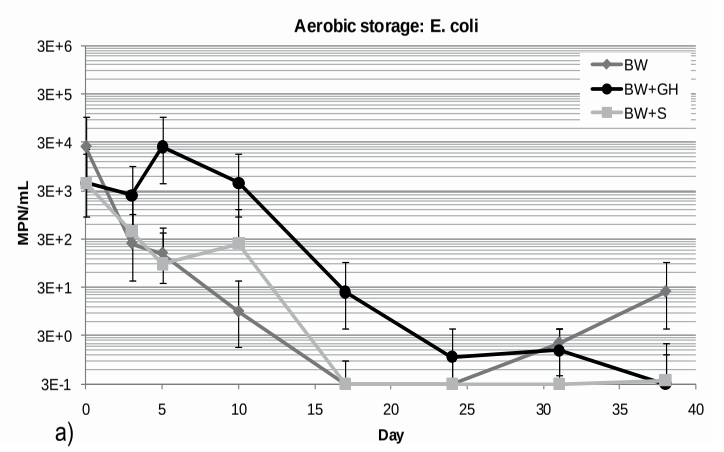
3.2. The impact of anaerobic digestion and aerobic storage on the microorganisms followed by recovery treatment

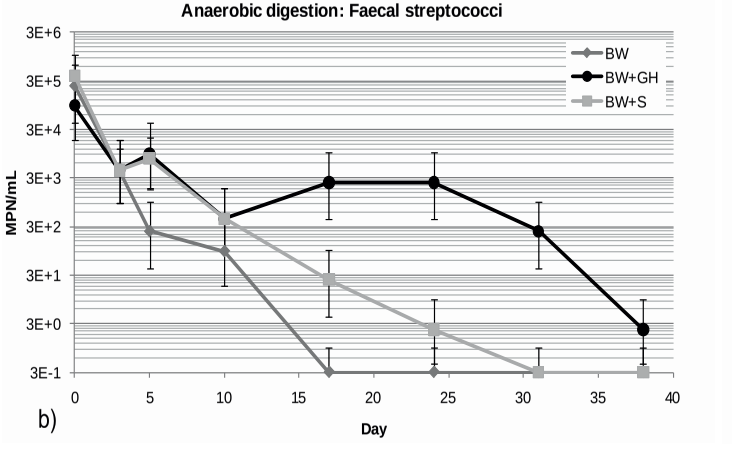
The background levels of the microbial groups in the different substrate mixtures are shown in table 3. Figures 2a-e shows the results for the different bacterial groups.

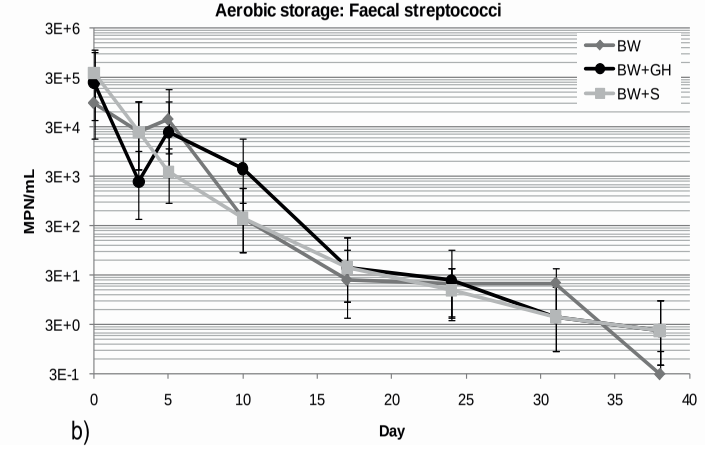
Biomass mixture	*E. coli* (MPN/mL)	Faecal streptococci/ enterococcus group (MPN/mL)	Ccoliphages (PFU/mL)	Amox resistant bacteria (CFU/mL)	Tet resistant bacteria (CFU/mL)
BW (an)	$1.5 \cdot 10^4$	$2.4 \cdot 10^5/2.4 \cdot 10^4$	$1.8 \cdot 10^3$	$2.93 \cdot 10^5$	$2.93 \cdot 10^4$
BW (aer)	$2.4 \cdot 10^4$	$9.3 \cdot 10^4/2.4 \cdot 10^4$	$1.8 \cdot 10^3$	$4.98 \cdot 10^5$	$2.28 \cdot 10^4$
BW+GH (an)	$2.4 \cdot 10^3$	$9.3 \cdot 10^4/2.4 \cdot 10^4$	$3.0 \cdot 10^3$	$6.95 \cdot 10^5$	$4.05 \cdot 10^4$
BW+GH (aer)	$4.3 \cdot 10^3$	$2.4 \cdot 10^5/9.3 \cdot 10^4$	$2.8 \cdot 10^3$	$4.63 \cdot 10^5$	$4.08 \cdot 10^4$
BW+S (an)	$2.4 \cdot 10^3$	$3.8 \cdot 10^5/1.4 \cdot 10^5$	$3.17 \cdot 10^3$	$1.83 \cdot 10^5$	$3.10 \cdot 10^4$
BW+S (aer)	$4.3 \cdot 10^3$	$3.8 \cdot 10^5/2.4 \cdot 10^5$	$2.83 \cdot 10^3$	$4.23 \cdot 10^5$	$3.13 \cdot 10^4$

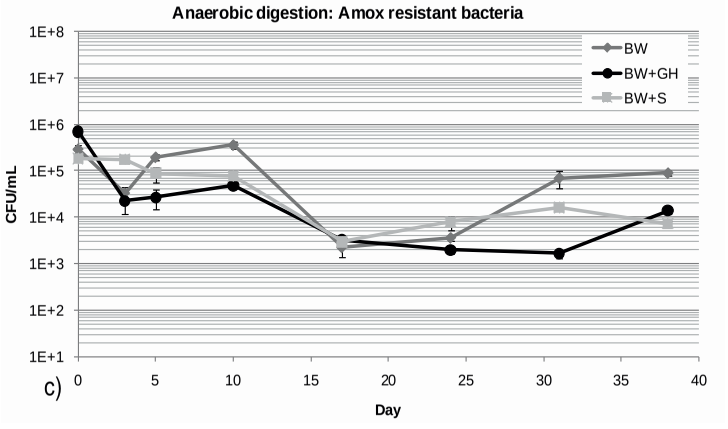
Table 3. Background levels of the selected microorganisms and microbial groups in each substrate mixture (both anaerobic and aerobic). Standard deviations are shown in figures 3a-e. Abbreviations: BW=Blackwater, GH=Greenlandic Halibut, S=Shrimp, an=anaerobic mixtures, aer=aerobic mixtures, Amox=Amoxicillin, Tet=Tetracycline.


Anaerobic digestion: E. coli
BW
BW+GH
BW+S
MPN/mL
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
3E-1
0
5
10
15
20
25
30
35
40
Day
a)


Aerobic storage: E. coli
BW
BW+GH
BW+S
MPN/mL
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
3E-1
0
5
10
15
20
25
30
35
40
Day
a)


Anaerobic digestion: Faecal streptococci
BW
BW+GH
BW+S
MPN/mL
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
3E-1
0
5
10
15
20
25
30
35
40
Day
b)


Aerobic storage: Faecal streptococci
BW
BW+GH
BW+S
MPN/mL
3E+6
3E+5
3E+4
3E+3
3E+2
3E+1
3E+0
3E-1
0
5
10
15
20
25
30
35
40
Day
b)


Anaerobic digestion: Amox resistant bacteria
BW
BW+GH
BW+S
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
0
5
10
15
20
25
30
35
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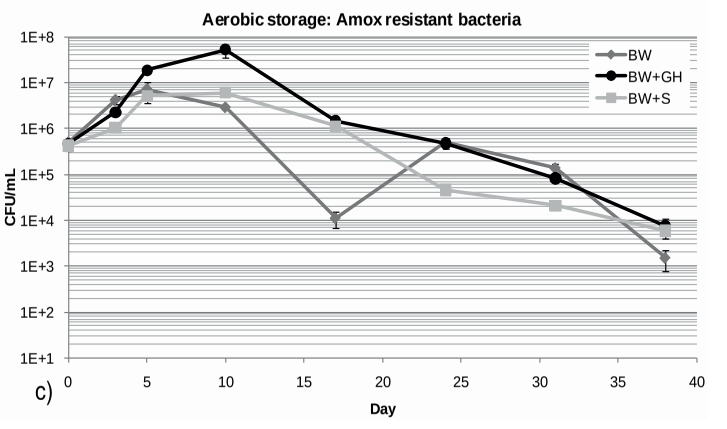
Day
c)


Aerobic storage: Amox resistant bacteria
BW
BW+GH
BW+S
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
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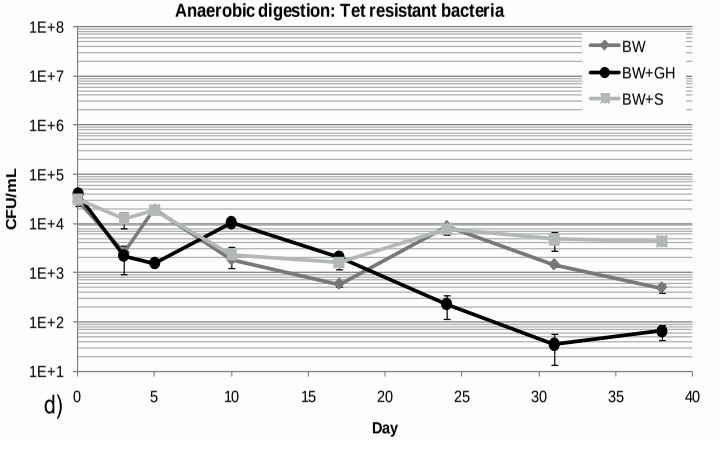
Anaerobic digestion: Tet resistant bacteria
BW
BW+GH
BW+S
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
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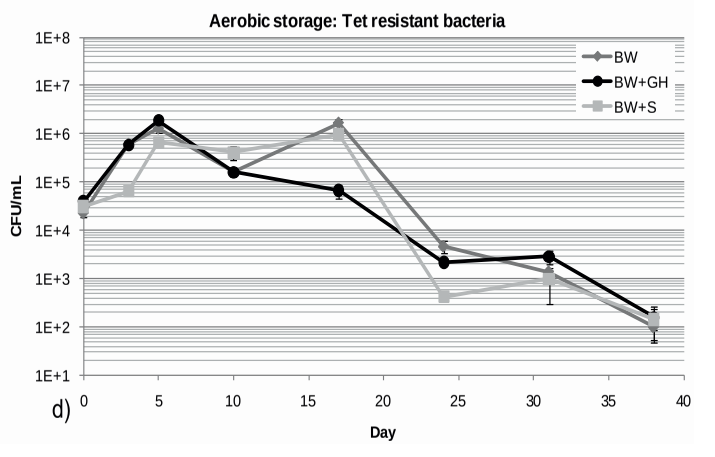
Day
d)


Aerobic storage: Tet resistant bacteria
BW
BW+GH
BW+S
CFU/mL
1E+8
1E+7
1E+6
1E+5
1E+4
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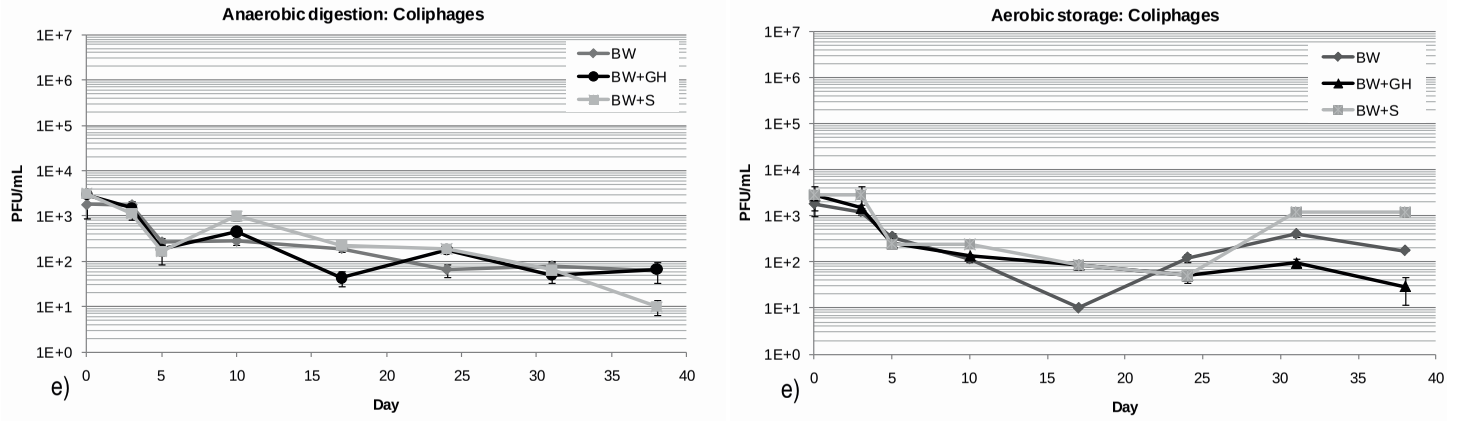

Figure 2. Effect of anaerobic digestion and aerobic storage on a) E. coli, b) faecal streptococci, c) total count of amoxicillin resistant enteric bacteria, d) total count of tetracycline resistant enteric bacteria, e) somatic coliphages. The lower limit on the y-axis is the detection limit in all cases. Standard deviation is indicated by error bars.

The background levels (day 0) of *E. coli* in the different biomass mixtures ranged from $2.4 \cdot 10^3$ to $2.4 \cdot 10^4$ MPN/ml (table 3 and figure 2a). The largest reduction in levels of *E. coli* in both anaerobic and aerobic BW mixtures occurred between day 0 and 3; 2.21 and 2.0 log, respectively. *E. coli* was under detection limit at days 17 and 24 in both anaerobic and aerobic mixtures containing BW and BW+S. In both the anaerobic and aerobic mixtures of BW *E. coli* was though detected again at day 31 and 38, indicating that the concentration has been just under the detection limit at day 17 and 24. This was also the case in the anaerobic BW+S mixture where *E. coli* was detected just above the detection limit on day 38. In the anaerobic and aerobic mixtures of BW+GH the reduction of *E. coli* was limited for the first 5 and 10 days, respectively, but at day 24 the level was down to 1.5 and 1.1 MPN/mL, respectively. The levels of *E. coli* in the anaerobic BW and BW+S samples as well as the aerobic mixture of BW showed an increase in number between days 24 and 31, 17 and 31, and 24 and 38, respectively. This could be an indication of regrowth. The survival of *E. coli* seems to have been better for the first 24 days in BW+GH in both anaerobic and aerobic samples, even though the final levels were approximately the same as in the other anaerobic and aerobic samples.

The results from the last step of the faceal streptococci analyses, which confirm whether or not the detected faecal streptococci belong to the enterococcus group, are not shown in figure 2b in order to make interpretation of the graphs easier. The results revealed that the faecal streptococci belonged to the enterococcus group in all cases except for streptococci detected in the anaerobic mixture of BW+GH, where the level of streptococci belonging to the enterococcus group was 0.5-1 log lower than the confirmed step of the faceal streptococci analyses had shown. This was the case for all sampling days except for day 38 where the level was the same; 2.3 MPN/mL. The faecal streptococci in the anaerobic and aerobic mixtures of BW were under the detection limit at day 17 and 38, respectively (figure 2b). In the anaerobic BW+S faecal streptococci were under the detection limit at day 31 but they were over the detection limit at all days in the anaerobic BW+GH. There was a slower reduction in the in the anaerobic mixture of BW+GH than the aerobic one, but the final level was the same; 2.3 MPN/mL. Addition of fish offal seems to have had a different effect on survival of the faecal streptococci in the anaerobic samples. Comparing the anaerobic and aerobic results, it can be seen that the survival of faecal streptococci seems to have been better under aerobic circumstances, indicating that the anaerobic environment has had an inhibiting effect on growth of faecal streptococci, possibly due to competition with methanogenic bacteria and lack of available carbon.

The antibiotic resistant (AR) enteric bacteria showed a difference in survival in the anaerobic and aerobic samples (figure 2c and d). Comparing the survival of antibiotic resistant bacteria in the anaerobic and aerobic samples the anaerobic environment seems to have had a limiting effect on growth of both amoxicillin and tetracycline resistant bacteria in the first part of the experiment, possibly due to competition with active methanogenic bacteria. In the anaerobic mixture of BW+GH there was an overall reduction in number of amoxicillin resistant bacteria until day 31, but an increase between days 31 and 38. For the other anaerobic substrate mixtures (BW and BW+S) there was an overall reduction until day 17. After that there was a slight increase in number of bacteria in BW+S and almost a 2 log increase in the mixture containing BW. In the aerobic mixtures of BW the amoxicillin resistant bacteria showed growth for the first 5 days and in the mixtures

containing BW+GH and BW+S for the first 10 days. After that the levels of bacteria declined. The overall reduction of amoxicillin resistant bacteria in the mixtures of BW+GH and BW+S was similar under anaerobic and aerobic conditions. In the aerobic sample of BW the reduction was 2 log higher than in the anaerobic BW mixture.

For the tetracycline resistant bacteria a similar pattern can be seen (figure 2d), with an increase of bacterial number in the beginning in the aerobic mixtures, but at the same time a larger overall reduction in those mixtures. There was no clear effect of fish offal addition on reduction of amoxicillin resistant bacteria but the reduction of tetracycline resistant bacteria seems to have been greatest in the anaerobic sample of BW+GH, followed by BW, and finally BW+S; 2.79 log, 1.78 and 0.53 log, respectively. It can therefore be seen that the reduction of the tetracycline resistant bacteria followed the same order as the degree of CH_4 production, that is, it was greatest in the sample showing the highest CH_4 production, and so on.

The somatic coliphages (figure 2e) showed a similar reduction rate in the anaerobic and aerobic mixtures in the beginning but at days 17 and 24 the number of phages in the aerobic samples containing BW and BW+S, respectively, started to increase; in the BW sample there was an increase between day 17 and 31 and then the number declined again between day 31 and 38, and in the BW+S sample there was an increase between day 24 and 31 and the number of coliphages was steady between day 31 and 38. On the other hand the number of phages in the anaerobic samples continued to decline throughout the experiment. Addition of fish offal did though not seem to have a specific effect on the die-off rate of coliphages since it was similar for all three mixtures. Lack of available carbon, affecting the host bacteria, is therefore likely to have caused the reduction in number of coliphages.

The initial concentration of the microbial groups used in the recovery study corresponded to the concentration of the last sampling day (day 38) of the anaerobic/aerobic batch trials. The results of the recovery study, where an MTF method was applied, had the unit MPN/mL. Antibiotic resistant bacteria where determined by direct plate count during the anaerobic/aerobic batch trials, and thus had the unit CFU/mL. In order to compare the results to each other it was estimated in which dilutions the plate counts would have shown growth if an MTF method would have been applied. This was compared to the dilutions where growth was detected in the recovery study. No recovery of antibiotic resistant bacteria or coliphages (that is, host bacteria for the coliphages) was observed. On the other hand there was an indication of recovery of *E. coli* in one sample and faecal streptococci in two samples. The results can be seen in table 4.

Microorganism	Sample	MPN value before recovery (day 38) (MPN/mL)	MPN value after recovery (MPN/mL)	Recovery (log)
E. coli	BW aer	24 (4.2/100)	2900 (870/9400)	2.08
Faecal streptococci	BW+GH an	2.3 (0.46/9.4)	2400 (420/10000)	3.02
Faecal streptococci	BW+GH aer	2.3 (0.46/9.4)	24 (4.2/100)	1.02

Table 4. Results of recovery treatment on all samples performed in TSB at room temperature overnight. Lower and higher confidence limits for the MPN values are given in parentheses.

As table 4 shows recovery of *E. coli* was only observed in one of the aerobic samples (BW aer) and for faecal streptococci in the anaerobic and aerobic samples containing BW+GH, indicating that a certain fraction of microorganisms in those samples has not been killed but injured during anaerobic digestion and aerobic storage. It is though uncertain whether or not recovery of injured streptococci has found place for the aerobic BW+GH sample, since there is an overlap of the upper and lower confidence limit for the MPN values before and after recovery, respectively. Regarding the faecal streptococci, it was confirmed that they belonged to the enterococcus group in both samples.

4. Discussion

4.1. Anaerobic digestion of the different substrate mixtures

The high specific CH_4 yield of BW+GH (table 1) was in accordance with what would be expected, taking into consideration the high lipid content of the halibut measured to be over 80% of the VS content. Lipid degradation during anaerobic digestion can lead to the accumulation of ammonia and long chain fatty acids (LCFAs), which are important inhibitors of anaerobic microorganisms (Salminen and Rintala, 2004). However, with the applied amount of added GH, gas production was high, indicating no inhibition during digestion of BW+GH. Furthermore the pH measurements revealed a stable pH throughout the experiment, indicating that the high fat content in the GH did not cause acidification. Earlier studies on anaerobic digestion of fish offal have given promising results (Ahring, 2003; Mshandete et al., 2004) and it has also been shown that co-digestion of fish offal and cattle slurry result in a higher specific CH_4 yield than digestion of cattle slurry alone (Callaghan et al., 1999). This is in line with the results of the present study that showed a higher specific CH_4 yield from BW+GH than BW alone. Sludge and BW have a very low dry matter content which makes co-digestion with other organic waste fractions , such as food waste or fish offal, an attractive treatment option (Ahring, 2003). Co-digestion of source-sorted food waste with sewage sludge has for instance given good results in Denmark (Ahring, 2003). Furthermore it has been shown that the gas production can be increased

significantly by addition of even small amounts of organic industrial wastes, especially fatty or oily as well as carbohydrates and protein rich wastes that have a much higher gas potential than manure and/or BW (Ahring, 2003). E.g. digestion of sewage sludge will result in a biogas yield of about 1-2 m^3 biogas/m^3 reactor volume per day, while this number would be increased to 4-10 m^3 biogas/m^3 reactor volume when adding approx. 20% fatty waste (Ahring, 2003).

The digestion of BW resulted in a specific CH_4 yield of 439 L/kg VS, with a VS loading of 2.7 g VS/L. Earlier studies on digestion of brownwater (source separated toilet wastewater, exclusive urine), using a VS loading of 2.5 g VS/L, resulted in a CH_4 yield of 587 L/kg VS (Lim, 2011). Based on the high protein content in shrimps (Ravichandran et al., 2009) the specific CH_4 yield of BW+S (346.4 L/kg) was expected to be higher than that of BW alone, which was not the case. However, the study performed by Lim (2010) showed inhibiting effect of adding food waste to the digesters, which resulted in lower biogas yields than those obtained from brownwater or food waste alone. This is similar to the results for BW+S in the present experiments, and it seems reasonable to interpret the low CH_4 yield as a result of inhibitory effects brought about by the shrimps offal. Inhibition and/or process imbalance could have occurred due to different factors, for instance the high protein concentrations in shrimps which can lead to the development of inhibitory concentrations of ammonia and sulphide (Ahring, 2003). Another reason could be that fish processing industries, such as the shrimp industry, require a large amount of salt (NaCl) for fish conservation and thus the wastewater from such industries is rich in salts (Chowdhury et al., 2010). It is known that anaerobic treatment of wastewater is inhibited by high sodium or chloride concentrations, and furthermore that methanogenesis is strongly inhibited by a sodium concentration of more than 10 g/L (Lefebvre and Moletta, 2006).

Overall the BW seems to have a fairly good CH_4 potential but due to the very low TS/VS content it would be profitable to utilize the digestion process for co-digestion with other organic waste fractions, such as fish offal. Care must though be taken when digesting such fat- and protein rich wastes because of risk of accumulation of LCFAs and VFA which inhibit anaerobic digestion. Regarding the shrimps offal it might be valuable to conduct further studies on different mixing ratios of BW+S to investigate possible negative effects when mixing BW and S. That kind of studies should be more comprehensive than the ones conducted in the present study where the main focus has been the hygienic effect of the process.

4.2. The impact of anaerobic digestion and aerobic storage on the microorganisms

There was an increase in number of *E. coli* in the later part of both the anaerobic and aerobic experiment, that is, in the anaerobic BW and BW+S samples as well as the aerobic mixture of BW, which could be an indication of regrowth. As CH_4 production decreases significantly towards the end, competition with methanogenic bacteria is presumably reduced when compared to the beginning of the experiments, where the methane production rate – and methanogenic activity - was high. The reduction of *E. coli* was similar under anaerobic and aerobic circumstances, which is in accordance with the results of Farrah and Bitton (1982) that compared the effect of anaerobic and aerobic digestion on various microorganisms, e.g. *E. coli*, *S. faecalis* and *Salmonella typhimurium*. The digesters in their study were operated at 28°C, adding wastewater sludge daily

to provide a retention time of 15 days. The results showed that inactivation rates (measured as daily change) of the three bacteria were similar under anaerobic and aerobic conditions. Competition with active methanogenic bacteria from the inoculum in the anaerobic samples in the present study, as well as carbon limitation, is likely to have had an inhibiting effect on the analyzed microorganisms. It has also been assumed that lack of carbon is the most common limiting factor for bacterial growth in soil (Demoling et al., 2007). Kumar et al. (1999) studied the removal of different microorganisms during anaerobic digestion of cattle dung in batch digesters and found a complete removal of *Escherichia coli* (*E. coli*) after 15 days at 35°C which is similar to the results for the anaerobic BW and BW+S samples in the present study. Others have studied the reduction of coliforms in continuous mesophilic reactors, e.g. Bonjoch and Blanch (2009) who found a 1-1.5 log reduction of faecal coliforms during digestion of raw wastewater sludge at an average residence time of 20 days, and Berg and Berman (1980) who under the same conditions found a similar reduction (98%) of faecal coliforms. Furthermore Watcharasukarn et al. (2009) found a 2.6 log reduction of *E. coli* after 5 days of anaerobic digestion at 37°C. Abdul and Lloyd (1985) found that counts of defined strains of *E-coli* were rapidly reduced during anaerobic digestion at 37°C, but antibiotic resistant *E-coli* strains showed a better survival than the sensitive ones. They also found that some of the *E. coli* strains isolated from anaerobic digesters had the ability to grow anaerobically, meaning that anaerobiosis was found not to be the only cause of rapid die-off of *E. coli* in their study which is in agreement with the results of the present study (Abdul and Lloyd, 1985). This could also partly explain the indication of growth of *E. coli* in the two anaerobic samples in the present study. Regarding addition of fish offal, the survival of *E. coli* seems to have been better for the first 24 days in both anaerobic and aerobic BW+GH samples, presumambly due to more available carbon than in the BW samples.

The addition of fish offal seemed to have had a different effect on survival of the faecal streptococci in the anaerobic samples. There was a strong reduction of faecal streptococci under both anaerobic and aerobic circumstances, but it was faster in the aerobic samples. Competition with organisms like protozoans might have played a role in the faster reduction under the aerobic circumstances. It has been shown that if the aeration capacity is sufficient a community of protozoa and other grazing animals will be maintained, resulting in bacteria being progressively removed (Bruce, 1982). It has earlier been stated that the faecal enterococci reduction is rarely more that 1-2 log units in mesophilic biogas plants, continuously fed with fresh biomass (Bendixen, 1994). Berg and Berman (1980) found more than 1 log reduction under mesophilic conditions (about 35°C) with an average residence time of 20 days. Others have observed similar reduction, e.g. Bonjoch and Blanch (2009) who found a 1 log unit reduction for enterococci populations in sludges and biosolids used in mesophilic anaerobic digestion for a period of 20 days. Many have found the survival of faecal streptococci being better than coliforms (Berg and Berman, 1980; Bonjoch and Blanch, 2009) and it has been suggested that faecal streptococci may be used as an indicator microorganism in biogas plants at temperatures up to 60°C (Bendixen, 1994). Furthermore, Kumar et al. (1999) found a complete reduction of faecal streptococci in an anaerobic batch experiment after 20 days at 35°C. The overall reduction was higher in that particular study because the initial level was approx. 3 orders of magnitude higher than in the present study. This was also the case for survival of *E. coli* which in their study had an initial level of approx. 10^6-10^7 CFU/mL whereas it ranged from $2.4 \cdot 10^3$ to $2.4 \cdot 10^4$ MPN/ml in the present study. Another reason for higher

reduction in the study performed by Kumar et al. (1999) may be that they used inoculated laboratory strains (both *E. coli* and *S. faecalis*) while indigenous microorganisms were studied in the present work. Laboratory strains may be more vulnerable against different kinds of stress, such as heat, and thus it would be expected that the survival of the latter one during different kind of treatment would be better. Comparing the survival of *E. coli* and faecal streptococci under the anaerobic conditions the reduction of the streptococci was larger than that of *E. coli* which is in contradiction with many earlier studies (Berg and Berman, 1980; Bendixen, 1994; Bonjoch and Blanch, 2009).

The anaerobic environment seemed to have a limiting effect on growth of both amoxicillin and tetracycline resistant bacteria in the first part of the experiment which could possibly be due to competition with methanogenic bacteria. Addition of fish offal did not seem to have an effect on the amoxicillin resistant bacteria, but the tetracycline resistant bacteria seem to have survived better in the anaerobic mixture of BW+S, which was the mixture having the lowest CH_4 yield. The decrease of tetracycline resistant bacteria was greatest in the BW+GH sample which had the highest CH_4 yield. Lund et al. (1996) studied the inactivation effect of the environment in a mesophilic continuous biogas reactor with an active biomass on bovine enterovirus and porcine parvovirus as well as faecal enterococci, by following their survival in batch experiments using physiological saline incubated at 35°C. They found a significant difference in reduction in the saline and the anaerobic digesters, indicating a strong inactivating effect of the environment in a biogas reactor with an active biomass. This may have been the case for the tetracycline resistant bacteria in the present study, indicated by their increased die-off in the more active anaerobic mixtures, possibly due to increased competition with e.g. methanogens, acetogens and hydrolyzing bacteria.

There was an overall reduction of amoxicillin resistant bacteria until day 17 in the BW and BW+S samples and until day 31 in the BW+GH sample. After that there was an overall increase in number of bacteria. This could suggest either that the organisms harbouring genes encoding for resistance have multiplied within the anaerobic bottles, or that the quantities of resistance genes have increased via lateral gene transfer (Ghosh et al., 2009). High densities of biomass are a known requirement for lateral gene transfer (Snyder and Champness, 2007), thus it seems reasonable that it has occurred within the anaerobic bottles.

Diehl and Lapara (2010) compared the efficiency of anaerobic and aerobic digesters to reduce the quantity of selected genes encoding tetracycline resistance in wastewater solids. They found that statistically significant reductions of the genes at 37, 46 and 55°C in the anaerobic digesters with the removal rates increasing as a function of temperature (Diehl and Lapara, 2010). On the other hand the aerobic digesters in their study were generally found to be incapable of significantly decrease the gene quantities, which is in contrast with the results of the present study since the overall reduction of AR bacteria was not larger in the anaerobic digesters, which is in line with the results of Farrah and Bitton (1982).

The somatic coliphages showed a similar reduction rate in the anaerobic and aerobic mixtures for the first 17-24 days but after that the survival pattern changed and the number of coliphages in the aerobic mixtures of BW and BW+S started to increase while it continued to decline in the anaerobic mixtures throughout the

experiment. Somatic coliphages infect *E. coli* but also certain closely related members of the bacterial family Enterobacteriacea (Hayes, 1968). Some of these hosts may multiply or metabolize in water environments to the extent that they support the replication of somatic coliphages (Grabow, 2004) which means that counts of coliphages may increase in certain water environments (Grabow 1990; Grabow et al., 1998). Even though an increase in numbers of resistant Enterobacteriacae and *E. coli* in the aerobic samples does not seem to have occurred in the present study there may still have been an increase of certain bacteria being outside the scope of the present analyses, supporting reproduction of coliphages in the aerobic BW and BW+S samples. This could for instance be Enterobacteriacae sensitive to amoxicillin and tetracycline. Furthermore the results for the antibiotic resistant Enterobacteriacae indicated that the anaerobic environment seemed to have had a limiting effect on growth in the beginning of the experiment whereas the aerobic environment has been more suitable for growth. The aerobic environment may therefore have supported growth of bacterial hosts for the somatic coliphages. Addition of fish offal did not seem to have an effect on the coliphages.

The temperature dependency of the different microorganisms and microbial groups was not studied in the present experiments, so the temperature effect itself on the microorganisms in our study cannot be evaluated. The temperature has though been found to be one of the most important factors in reduction of microorganisms (Dumontet et al., 1999). In the study done by Kumar et al. (1999) the removal of, among others, *E. coli* and faecal streptococci in an anaerobic batch experiment at room temperature and 35°C was compared. They found that a complete removal of *E. coli* and faecal streptococci at 35°C took 15 and 20 days, respectively while it took 10 and 20 days longer, respectively, at room temperature.

The results of the present study showed that a considerable reduction of the selected microorganisms and microbial groups can be expected under mesophilic treatment conditions. However, anaerobic batch experiments are commonly used to determine CH_4 potentials of various organic waste fractions. In big scale anaerobic digesters there is a continuous flow into the digesters of fresh substrate and thus microorganisms, meaning that their survival in big scale plants is quite different from what can be expected in batch experiments where e.g. lack of available carbon at a certain point becomes one of the inhibiting factors for the microorganisms' survival. The batch experiments can however give a good overall picture and comparison of the survival of different microorganisms and microbial groups, and in the case of the present study, their survival patterns under anaerobic versus aerobic conditions.

4.3. Recovery treatment after anaerobic digestion and aerobic storage

The results of the recovery study indicated that a certain fraction of *E. coli* in the aerobic BW sample and faecal streptococci in the anaerobic and aerobic samples containing BW+GH has been injured and able to resuscitate during recovery. It is likely that lack of available carbon by the end of the experiments has had a limiting effect on microbial growth. The temperature in the recovery experiment was the same as in the anaerobic/aerobic experiments, but what may have supported recovery of the injured cells was the nutritious growth media, TSB. Even though recovery was only observed for a part of the samples, it emphasises that care should be taken if reuse of treated wastewater, such as digestate from anaerobic digesters, is

considered. The requirements and regulations for wastewater treatment differ between regions and also depend on intentions for reuse of the treated wastewater, or the nature and vulnerability of the recipient in question. If reuse of the digestate is intended post-treatment would be required in many regions. In Arctic climate it can be expected that a considerable amount of the produced biogas would be used for heating of the plant. If there is a surplus of biogas it could be used for thermal post-treatment of the digestate. The anaerobic digestate is rich in plant nutrients and can be utilized as fertilizer, giving an additional benefit from the treatment. The digestate can also be dewatered, followed by further treatement, e.g. filtration of the liquid fraction and composting of the fibre part.

5. Conclusions

The reduction of *E. coli* was similar under anaerobic and aerobic circumstances, and the survival of *E. coli* was better for the first 24 days in both anaerobic and aerobic BW+GH samples. Addition of Greenlandic halibut and shrimp offal had a different effect on survival of the faecal streptococci in the anaerobic samples. The reduction of faecal streptococci was strong under both anaerobic and aerobic circumstances, but was faster in the aerobic samples. The anaerobic environment seemed to have a limiting effect on growth of both amoxicillin and tetracycline resistant bacteria in the first part of the experiment. Addition of fish offal did not seem to have an effect on the amoxicillin resistant bacteria, but the tetracycline resistant bacteria survived better in the anaerobic mixture of BW+S, which was the mixture having the lowest CH_4 yield whereas the decrease was greatest in the BW+GH sample which had the highest CH_4 yield. The coliphages in the present study showed a better survival and even an increase in numbers under aerobic conditions, indicating that anaerobic digestion could be a more effective treatment method for viruses and phages than aerobic treatment methods. Addition of fish offal did not seem to have an effect on the coliphages' survival. The results of the recovery study indicated that a certain fraction of *E. coli* in the aerobic BW sample and faecal streptococci in the anaerobic and aerobic samples containing BW+GH was injured and able to resuscitate during recovery. This underlines that care should be taken if reuse of treated wastewater, such as digestate from anaerobic digesters, is considered.

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Today all wastewater in Greenland is discharged untreated to the recipients. However, wastewater treatment in the Arctic is challenging due to e.g. the cold climate and settlement pattern. Decentralized solutions, e.g. composting or anaerobic digestion of the blackwater, may be well suited for Greenland. This thesis presents results from laboratory experiments where the hygienic effect on blackwater of those processes, as well as freezing, was analyzed. None of the processes had the ability to completely hygienize the blackwater, but some of the microorganisms were reduced strongly. Combining the processes might enhance the microbial reduction. Based on those results recommendations are given in the thesis concerning choices of wastewater treatment methods for towns and settlements in Greenland.

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ISBN: 9788778773487
ISSN: 1601-2917