Norwegian University
of Life Sciences

Master's Thesis 2021 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Compost and N_2O-reducing Microbes as Possible Stabilizers of Biosolids

Lars Johan Eide
Master of Science, Biotechnology

Acknowledgments

This thesis has been a storm of emotions and throughout every wave of emotion, I have had the support of close friends and family. The lab work performed for this thesis was done at the Microbial Ecology and Physiology part of the NMBU nitrogen group, at the faculty of Chemistry, Biotechnology and Food Science

I would like to express my sincerest gratitude and respect for my supervisor Lars Bakken for allowing me the opportunity to write a thesis for the Nitrogen group, providing me with much-needed guidance and feedback and historical metaphors without which,

I thank my co-supervisor Kjell Rune Jonassen. His advice, humor, humility and wealth of knowledge served as an inspiration during the lab work and writing of this thesis.

I thank my good friend Elisabeth Gautefall who showed me a lot of support, kept my spirits high and kept me motivated. Her kindness and humor illuminated me during a particularly dark period of my life.

I would also like to thank my parents and especially my father, who throughout this thesis supplied me with emotional support, a raw sense of humor and subsidies.

Lastly, I extend my full-hearted love and gratitude to my (then?:)) girlfriend Emilie Slettvik, who was there for me emotionally and physically, supporting me through a distressing time of my life that saw me challenge the boundaries of my human psyche. She grounded me, always reminding me of the meaningfulness of actions, even small ones, which is easy to forget

Table of Contents

List of figures

List of tables

List of abbreviations and definitions

VNBC – Viable but nonculturable cells

MPN – Most probable number

THP – Thermal hydrolysis process

TAD – Thermophilic anaerobic digestion (55C)

AD – Anaerobic Digestion

MPN – Most probable number

VS – Volatile Solids

DS – Dry Solids

TS – Total Solids

MUG-EC Broth – 4-Methylumbelliferyl Glucuronide Escherichia Coli Broth

VEAS-SS – An acronym for «Vestfjorden Avløpsselskap Solid Stream» essentially a biosolid produced by the post-anaerobic digestion-thermal hydrolysis process.

Abstract

Biosolids generated from dewatering the end-product of sludge treatment, *digestate*, are rich in nitrogen, phosphorous and recalcitrant carbon, and are often utilized as fertilizers/soil conditioners in agriculture. Biosolids originating from municipal wastewater are governed by strict regulations relating to the presence of human pathogens, which represent a health risk, thus pathogen reduction/sanitation is a crucial treatment step at such plants before the biosolid can be applied to agricultural soil. Svennevik et al. (2020) demonstrated that regrowth of pathogens in post-AD-THP biosolids may be prevented by introducing a diverse microbial community, free of indicator organisms, via compost to the sterile biosolids. In parallel, as demonstrated by Jonassen et al. (2020), digestates enriched with nitrous oxide (N_2O) reducing bacteria have the potential to reduce a significant share of soil N_2O emissions from agriculture, which currently constitutes 30% of total climate forcing.

This thesis expands upon the work of both Svennevik et al. (2020) and Jonassen et al. (2020), and we hypothesized that the two concepts might be integrated and that isolates of promising N_2O reducing strains, growing in pre-sterilized biosolids could limit the growth of *E.coli* introduced as a contaminant, rendering the use of compost obsolete. We used a pure *E. coli* culture, isolated from municipal wastewater, as an indicator organism of pathogen growth and a robotized incubation system to aerobically incubate and measure microbial respiration rates in sterile biosolids for 24 days. Mainly we looked at three different bacteria as potential inhibitors of *E. coli* growth either by antagonism or competition: 1) *Pseudomonas stutzeri*, 2) *Pseudomonas DC1* and 3) *Cloacibacter*, chosen based on their potential for growth in an N_2O atmosphere. All three bacterial strains were inoculated and allowed to adapt to the biosolids for 4 days (compost bacteria were given 9 days), before *E. coli* contamination. We prepared a mixture of all three bacteria to check for potential synergistic effects. To quantify the metabolic activity as affected by the bacteria and compost, and the added *E. coli*, we surveyed the respiration rates (robotized incubation system), and to assess the growth/survival/death of *E. coli*, the abundance of viable *E. coli* was measured (most probable number, MPN) as well as the abundance of *uidA* genes specific for *E. coli* (total *E. coli* cells).

While the compost bacteria were able to effectively suppress *E. coli*, none of the single strains (or the consortium of the three strains) repressed *E. coli*. The respiration kinetics showed that the compost bacteria were able to exploit a much larger fraction of the organic material than *E. coli* alone, or *E. coli* in combination with the N2O-reducing strains.

Sammendrag

Biosolider er avvannet, utråtnet slam (digestat) fra slambehandlingsprosesser. Biosolidene er rike på nitrogen, fosfor og organisk karbon. Dette gjør dem egnet som gjødsel- og jordkondisjoneringsagenter i jordbruk. For biosolider med opprinnelse i kommunalt avløpsvann, er reguleringene knyttet til tilstedeværelse av patogene mikroorganismer, strenge. Dersom antallet patogene mikroorganismer ikke blir tilstrekkelig redusert før tilføring på kultivert mark, vil det medføre en reell helsefare. I en studie av Svennevik et al. (2020) ble det vist hvordan patogen rekolonisering av biosolider kan bli effektivt redusert ved å integrere et spektrum av kompostbakterier i det sterile materialet under lagring. Videre viste Jonassen et al. (2020) at digestater beriket med lystgassreduktanter besitter et potensial til å redusere en signifikant del av lystgassemisjon fra jordbruk, som i skrivende stund står for 30% av forandringer i klimafølsomhet.

I denne studien vil arbeidet til både Svennevik et al. (2020) og Jonassen et al. (2020) utvides ved å evaluere muligheten for å integrere de to konseptene. Lovende denitrifikanter ble testet som mulige stabilisatorer i sterile biosolider, og vurderes som mulige arvtakere til kompost. Vi benyttet en rendyrket *E. coli*-stamme, isolert fra avløpsvann, som en indikatororganisme for patogen vekst, samt et robotisert inkubasjonssystem som målte mikrobielle respirasjonsrater i sterile biosolider over 24 dager under aerobe forhold. Hovedsakelig ble tre ulike bakterier benyttet som potensielle inhibitorer på *E. coli* vekst, enten ved antagonisme eller ressurskonkurranse. De tre artene var 1) *Pseudomonas stutzeri*, 2) *Pseudomonas DC1* og 3) *Cloacibacter*. Samtlige bakterier ble i hovedsak valgt basert på deres evne til respirere i en lystgassberiket atmosfære. Bakteriene ble gitt 4 (*P. stutzeri*, *P. DC1* og *Cloacibacter*) og 9 (kompost) dager til å tilpasse seg biosolidene, før en invasjon av *E. coli* ble iverksatt. Mulige synergiske effekter for de tre denitrifikantene ble undersøkt ved tillagging av en blandingskultur. For å kvantifisere den metabolske aktiviteten i seriene under hele inkubasjonen, ble CO_2- og O_2 -respirasjonsratene overvåket. I tillegg til kvantifisering av totale mengde levedyktige *E. coli*-celler (MPN) og total mengde *uidA* kopier (totalt antall *E. coli*-celler), ga respirasjonsratene en evaluering av *E. colis* livssyklus i behandlingene.

Kompostbakteriene viste klar evne til å motstå og undertrykke *E. coli*-vekst, men ingen av enkeltstammene (eller blandingskulturen) demonstrerte lignende effekt. Respirasjonsratene avdekket at kompostbakteriene utnyttet en betydelig større fraksjon av det tilgjengelige organiske materialet enn både *E. coli* alene, og *E. coli* sammen med N_2O-reduktanter

Aims of this study

This thesis's main objectives were twofold: One was to establish a most probable number (MPN) method for *E. coli* to use in our lab: The *E. coli* was isolated from sewage wastewater on MUG-EC medium and monitored at varying optical density (OD660) levels and compared against MPN numbers. Two methods of cell dispersion for MPN counts were compared (vortexing versus harsh treatment in a “fast prep” shaker without glass beads). The second was to determine the growth/survival of *E. coli* in biosolids as affected by the presence of other bacteria, by comparing the CO2 and O2 kinetics- the ability of organisms to exploit the organic carbon (C) present in the biosolids- and growth/survival of *E. coli* quantified by viable counts (MPN) numbers and gene abundance (ddPCR). Microbial respiration kinetics was measured using a robotized incubation system developed by (Molstad et al., 2007).

1 Introduction

All waste material will be recirculated in a future circular economy, either as feed for animals or as organic fertilizers to agricultural soils. Recirculation at a high trophic level (feed for animals or fish) is much to prefer over recirculation as fertilizer because it most efficiently reduces the environmental footprints of food production, as exemplified by the nitrogen budget of food production (Bleken & Bakken, 1997; Smil, 2002). Nevertheless (and for various practical reasons), the major fraction of food waste is expected to be destined for agricultural soil, together with organic wastes from agriculture (manure and crop residues) and wastewater sludge. For all these materials, a pre-treatment by anaerobic digestion (AD) is desirable for three reasons: 1) the process produces methane (replacing fossil fuel) 2) methane emissions that otherwise occur during storage are eliminated (Miranda et al., 2015), 3) most of the easily degradable organic components are mineralized. (Grimsby et al., 2013) The latter is crucial because it represents a "stabilization" of the organic waste: the microbial activity during storage will be marginal. Additionally, thermophilic AD (> 50 ^{o}C) can eliminate pathogens. (Iranpour et al., 2004; USEPA, 2000)

The residues of anaerobic digestion (called digestates hereafter) contain large amounts of water that must be removed before being transported to agricultural areas and stored. Farmers can only fertilize once or twice a year, while waste is produced continuously,

creating a need for a product that is stable during storage. This stability can be achieved partly through dewatering (see section 1.1)

Recently, novel technologies backboned by thermal hydrolysis processes (THP) after AD (post-AD THP) have emerged. Dewatering of digestates is technically challenging, and thermal hydrolysis of the digestate can enhance the dewaterability of the digestates (Sapkaite et al., 2017; Svensson et al., 2018). The Thermal Hydrolysis Process (THP) is desirable for other reasons as well: Due to the high temperature (134-175 ^{o}C), the violent treatment results in effective sterilization of the final product, and if applied after AD (post-AD THP) the methanogens are killed as well, thus eliminating methanogenesis (biogas production) during storage (Svensson et al., 2018).

Elimination of pathogens (sanitation) is an important issue, both for treating urban organic wastes to avoid contamination of the agricultural products, and authorities have set some standards to allow utilization of organic wastes as fertilizers. For instance, the EU standard for sanitation is 70 ^{O}C for one hour (Törnwall et al., 2017). That may be insufficient for securing 100% elimination of all pathogens, hence THP is preferable.

However, whatever sanitation one uses, there is a risk for recontamination and subsequent growth of pathogens in sanitized materials (Svennevik et al., 2020).

1.1 Biosolids

Fecal coliforms pose a persistent problem in wastewater treatment as high numbers of coliform bacteria are associated with a heightened risk of pathogenic bacteria (Hachich et al., 2012; Sidhu & Toze, 2008). The most common fecal coliform, accounting for over 84,3% thermotolerant coliforms in wastewater, is *E. coli*, a facultatively anaerobic, gram-negative bacterium native to the intestinal tract of humans and most warm-blooded animals (Bartram, 1996; Hachich et al., 2012). Certain strains are anthropogenic pathotypes such as enterohemorrhagic *E. coli*, responsible for diarrhea and dysentery in humans. Other fecal pathogens include *Salmonella sp, Helminth ova,* and enteric viruses. The abundance of fecal coliforms, or *E. coli*, are used by authorities as a criterium for sanitation (Iranpour et al., 2004) and it is desirable to reduce and maintain the number of coliform bacteria below regulated level, which varies between local legislations. Achieving a stable product for use in agricultural sectors requires the wastewater and the resulting sludge to go through a sludge treatment process with two main objectives: 1) reduction of volume and 2) stabilization.

Stabilization in sludge treatment is a removal of odor and pathogens to acceptable levels. This is usually achieved by processing the sludge in a series of steps in which the end products are mainly biogas, digestates, and biosolids from the digestates. (Fane et al., 2021; Sidhu & Toze, 2008)

A technique developed by Arden and Lockett in 1914 known as activated sludge technique has become the standard for wastewater treatment in most industrialized nations (Ardern & Lockett, 1914). Sludge treatment consists of key steps: thickening, anaerobic digestion (AD), and dewatering. Thickening refers to the flocculation of sludge to increase its total solids (TS) and reduce total volume (Brandt et al., 2017). Anaerobic digestion (AD) is the sequential breakdown and conversion of complex organic polymers to methane without the presence of oxygen, facilitated by methanogenic microbial communities, and is a core technology in the treatment of municipal wastewater sludge and other organic wastes. THP is a new process that in most cases has been applied before AD. It lyses the cells through a high-pressure steam explosion at 165^{o}C, resulting in increased biodegradability, and heightened biogas production (Barber, 2016; Svensson et al., 2018). The wastewater treatment involves the removal of suspended solids from the wastewater by adding coagulation agents which cause flocculation and sedimentation of the flocs. The resulting sludge has too high viscosity to allow efficient AD. The viscosity can be reduced by adding water, but the THP process prior to AD is an attractive alternative: it reduces the viscosity without adding water (Barber, 2016)

Applying THP as a step after AD will result in a sterilized product (Barber, 2016; Svensson et al., 2018). Dewaterability of sludge is important owing partly to limitations on handling and storage capacity and its effect on cell growth. The correlation between the osmotic strength of the environment and bacterial growth is apparent. A hyperosmotic environment causes water to exit the bacterial cell and inhibits its ability to reproduce. To achieve complete sterilization of biosolids, the dry weight needs to be 75% of the total weight (USEPA, 2003).

1.1.1 Criteria for use

A set of criteria are used to determine whether a treated sludge (biosolid) can be used on farmland or for other soils (roadsides etc. i.e., not involved in food production). For land application, the United States Environmental Protection Agency classifies biosolids into Class A and Class B, mainly on the solid residues level of thermotolerant coliform bacteria (TCP), *Salmonella*, *Helminth ova,* and enteric viruses (Iranpour et al., 2004; USEPA, 2000). Both Class A and B can be applied to agricultural lands. However, lands treated with class B sludge are restricted from harvesting for at least 30 days after application. The European Union publishes official directives for its member states to regulate the treatment and disposal of wastewater and treated sludge, very similar to the US regulations (Collivignarelli et al., 2019). Local municipal authorities mainly determine the specific limits, which vary across nations. The Norwegian use of treated sludge is regulated by the "forskrift om gjødselvarer mv. Av organisk opphav". In Norway, the maximum acceptable level of fecal coliforms is 2500 per gram dry solids (enumerated by most probable number, MPN), and in the US 1000 MPN/gDS. For *Salmonella,* the limits for Norway and US are 0,75 and 0 MPN/gDS, respectively (Iranpour et al., 2004; USEPA, 2018) Reducing the level of pathogens is just one quality aspect another is the need to reduce the solids attraction to vectors of infectious agents such as rodents, mosquitoes, flies, etc. (Hussong et al., 1985) referred to as Vector Attraction Reduction (VAR) in the US (USEPA, 2003). These criteria must be met for the highest quality biosolid, Class A. Conventional methods for achieving acceptable levels of indicator bacteria include thermal drying, mesophilic AD in series, pasteurization, thermophilic AD in batch or multi-stage, and more recently THP (Collivignarelli et al., 2019; Svennevik et al., 2019; Svensson et al., 2018; Ward et al., 1999).

1.2 Previous Research

In the study by Svennevik et al. (2020), *E. coli* was introduced through wastewater in compost-amended post-AD-THP treated biosolids. Such direct contamination of a sanitized material is a plausible scenario because of the close vicinity of the processes (raw sludge treatment, AD and sanitation) within the same building in a system without THP, the risk for recontamination by cells surviving the AD process as dormant cells is credible. Previous literature (Chen et al., 2011; Higgins et al., 2007) has suggested that fecal coliforms can enter a viable but non-culturable state (VBNC) during AD in response to the extreme

environmental stress present in the tank. This survival technique renders the cell incapable of reproducing, essentially entering a stasis state in anticipation of milieu improvement. After dewatering, the non-culturable cells can resuscitate (become viable) in an extremely nutrient-rich environment with very few to no competitors unless dried to >75%, this however is costly (Fane et al., 2021).

The compost used in Svenneviks (2020) paper was integrated with biosolid 48 hours ahead of wastewater contamination, representing the available maturing time of full-scale systems, before transportation off-site (Svennevik et al., 2020). However, questions arose such as if given more time to adapt to the solids would the resistance of compost communities to invaders improve? Moreover, the results open the possibility of using other microbiological communities than those present in compost for stabilization. Jonassen et al (2020) show the feasibility of digestate-based biofertilizers destined for agriculture, as agents for efficient mitigation of anthropogenic N_2O emissions from agricultural soil by growing N_2O-reducing bacteria in digestates. Similarly, if biosolids were to be stabilized by an N_2O-reducing microbiota community, it would ensure the safe application of post-AD-THP treated sludge on farmland and mitigate nitrous oxide emissions. Jonassen et al (2020) examined this possibility by adapting microorganisms through repeated inoculation in soil amended with enriched digestate containing N_2O microorganisms and complete denitrifiers were selected by genomic and phenotypic characterization based on their ability to thrive in N_2O rich environments. In this paper, we further examine the possibility of using N_2O reducing microorganisms to stabilize biosolids. Using the same methods described by Jonassen et al. (2020) revealed three potential N_2O respiring bacteria: *Pseudomonas stutzeri*, *Pseudomonas DC1*, and *Cloacibacterium*.

1.2.1 *Pseudomonas stutzeri, Cloacibacter* and *Pseudomonas DC1*

Pseudomonas stutzeri is a motile, Gram-negative bacteria belonging to the genus *Pseudomonas,* indigenous to soil and wastewater. It is an aerobic denitrifier preferring oxygen as a terminal electron acceptor when present but capable of reducing N_2O to N_2 in the presence of oxygen (Lalucat et al., 2006). *P. stutzeri* is of interest owing to its nutritional versatility, capable of utilizing a large spectrum of distinct substrates including, but not limited to: maltose, starch, ethylene glycol, amino acids, and volatile fatty acids (Jonassen et al., 2020; Lalucat et al., 2006). Furthermore, it contains the enzymatic pathway for complete denitrification: NO_3^- (nitrate) ($\rightarrow NO_2^-$ (nitrite reductase) $\rightarrow$NO (nitric oxide reductase) $\rightarrow N_2O$ (nitrous oxide reductase) $-N_2$, but owing to its aerobic preference and oxygens irreversible

inhibition of nitrous oxide reductase (nosZ) (Miyahara et al., 2010), it is impossible to reduce N_2O to N_2 unless anaerobic conditions can be invented either in the cell or its environment. Such conditions are often found in sludge treatment processes and wastewater, whereby *Stutzeri* can reduce nitrous oxide to N_2 and research has demonstrated the ability of *Stutzeri* to reduce N_2O in microaerobic conditions in wastewater (Miyahara et al., 2010). This is of importance as biosolids are not stored at anaerobic conditions, nor is the environment in agricultural fields, making oxygen-tolerant N_2O reductants attractive candidates for pathogen suppression in the biosolids.

All three bacterial strains were chosen owing to their ability to survive in and reduce an N_2O atmosphere. They were cultivated by successive inoculations in an autoclaved sludge mixed with digestate, and sludge mixed with soil, under N_2O atmospheric conditions. A 16S amplicon sequencing of the complete bacterial soil profile revealed *Cloacibacter* as a dominant bacterial species by gene abundance. Similarly, *P. DC1* was cultivated from just sludge injected with N_2O and identified by 16s sequencing. They used the robotized incubation system described in Molstad et al. (2007) for monitoring the nitrous oxide reducing rates for each of the bacteria, which can also be used for

However, how well these three bacterial strains do in an aerobic nutrient-rich environment with competitors is unknown.

1.3 MPN Method

A recurring issue with the methods of enumeration is the lack of standardized methods across the paucity of research papers on the quantification of pathogens and indicator organisms in biosolids. But perhaps exactly because of the limited research that has been performed on the subject and owing to the general immensity of various diverse metabolical niches and ecological interactions existing between organisms of fecal matter, has led to a diversification in methodology between laboratories and a need to develop specialized local methods for specific strains. This issue is compounded by the lack of detection limits documentation for the methods used by the authors. (Sidhu & Toze, 2008). Rectifying the issue would require the consecration of a viable method for accurately and precisely assessing the level of fecal pollution in the biosolids. One way to achieve this could be combining methods for quantification. Ideally, methods for assessing the level of fecal pollution in biosolids should be sensitive, rapid, accurate and easy to use (Sidhu & Toze, 2008). Traditional methods have involved the cultivation of indicator organisms on media or cell

lines (hemocytometer) and for liquid cultures the process usually involves the creation of a standard curve to establish a linear relationship between cells in medium with colony-forming units (CFU). Other methods such as multiple tube fermentation use gas production and a dilution gradient to enumerate coliforms. The multiple tube fermentation technique is the standard approved method for enumeration of fecal coliforms in biosolids (Feng & Hartman, 1982; Garthright & Blodgett, 2003; USEPA, 2003)

As already mentioned, organisms can enter a dormant stage, often called viable but nonculturable (VBNC) stage, when existing in an unfavorable environment. Such cells are physically intact, with intact DNA, but metabolically "offended" to such a degree that they become dormant, unable to grow in artificial laboratory media, but theoretically they can be resuscitated if the right conditions are invented. Hence, cells in this stage will not be enumerated with traditional viable counting methods such as dilution plating or dilution-to-extinction MPN method. They can be enumerated, however, by measuring the abundance of their genomes, since DNA is still intact. This is feasible if suitable primers can be found, which secure PCR amplification selectively for the organism in question.

By combining enumeration of *E. coli* by viable count (MPN) with the enumeration of genes specific for *E. coli*, it should be possible to assess the fraction of the population entering a dormant (VBNC) stage.

The principle of the MPN method is stepwise dilution in a set of parallel dilution lines and calculation of the most probable number based on the number of tubes showing growth at each dilution level. A prerequisite for the method is that dilution is extended far enough to secure tubes with no viable cells (hence the term dilution to extinction). A detection limit for MPN can be easily included by using the lowest dilution level of sterile control. The dilution limit would then equate to the MPN of one positive tube for the lowest dilution level. For the purposes described in this paper, "FDA's preferred MPN methods for standard, large or unusual tests" will be tested with an associated "BAM-MPN" spreadsheet to quantify viable *E. coli* numbers in the biosolids. (Garthright & Blodgett, 2003).

1.3.1 Selective medium: 4-Methyl-umbelliferyl-β-D-glucuronide (MUG)

Svennevik et al. (2020) demonstrated how stabilization of biosolids can be achieved through soil-amendment with compost, free of indicator organisms. The introduction of *E. coli* was done by using wastewater as a vector for contamination. Hence, this invariably introduces non-target microbes and other possible metabolic factors in the solids. Using

laboratory *E. coli* cultures usually result in a *domesticated* strain, selected for its ability to grow in laboratory conditions with abundant food, few competitors, and optimal environmental conditions. This is akin to using a docile strain and is inadequate to represent actual conditions in a high-species diverse competitive environment and so obtaining a "wild" strain could be important. Using a native strain of *E. coli* would not only be more representative of plausible interactions between bacteria in solids but also provide a challenge for the compost community. If they are able to suppress a wild *E. coli* strain then it would further enhance the prestige and viability of compost-microbiota as biosolid-stabilizers.

To isolate and enumerate *E. coli* in environmental materials, the growth medium incubation conditions should give *E. coli* a selective advantage over other organisms. Traditionally, this has been achieved by incubation at 45 ^{o}C (which is lethal to many indigenous bacteria in the environment) (Cheeptham & Lal, 2010), and a medium containing bile salts and lactose (Odonkor & Ampofo, 2013). The bile salt has antimicrobial effects by causing protein aggregation in the periplasm leading to cell lysis in gram-positive bacteria such as *bacilli* and *streptococci* but *E. coli* is more resistant than others (Merritt & Donaldson, 2009). The reason is speculated to be an activation of cytosolic chaperons called Hsp33 that respond to protein aggregation forming disulfide bonds with unfolded proteins and preventing aggregation, reducing their impact (Cremers et al., 2014). Lactose is used as a C source because *E. coli* produce β – galactosidase which hydrolyzes lactose (some organisms lack this enzyme). Growth under these conditions indicates that the organism is a fecal coliform of some kind but is no proof. Lately, an additional technique has been introduced to discriminate more precisely between *E. coli* and other organisms (be it in MPN tubes or on agar plates): 97% of all tested *E. coli* strains produced β–D- glucuronidase, which cleaves the nonfluorescent compound 4-methyl-umbelliferyl-β-D-glucuronide (MUG) to the fluorescent product 4-methyl-umbelliferone. Thus, by growing organisms in a medium with MUG, *E. coli* will produce blue fluorescent colonies on agar, and fluorescent MPN-tubes, which are both easily detected by exposure to UV light. (Cremers et al., 2014). Some other enteric bacteria also possess the β-glucuronidase enzyme including specific strains of *Shigella* and *Salmonella* in the *Enterobacteriaceae* family (Cheeptham & Lal, 2010).

The general issue with cultivation techniques is that the act of cultivating in of itself can lead to recovery and multiplication of inured cells which can cause an overestimation of pathogen numbers especially when present in low numbers initially (Sidhu & Toze, 2008). Caution should therefore be employed when choosing a selective medium as it can aggravate this issue essentially creating an enrichment culture. MUG-EC has been shown to not be

conducive or inhibitory for *E. coli* growth (Cheeptham & Lal, 2010). However, if forced to choose between underestimation or overestimation of pathogens, the natural inclination should be towards overestimation with respect to human health risk.

1.3.2 Homogenization method

Effective dispersion is essential for correct enumeration of viable cells (be it *E. coli* or any other organism) because each free cell is counted as one *E. coli*, but so is the case for an aggregate of many *E. coli* cells. Hence, inefficient disruption of aggregates of *E. coli* cells will result in erroneously low estimates of the number of viable cells present. This argues for using strong shear forces when dispersing the ecological materials. On the other hand, strong shear forces (be it by homogenization in mixers or by vigorous shaking) can disrupt the cells, as demonstrated by Lindahl and Bakken (1995), who found that the number of viable *E. coli* declined with a first-order rate of 0.02 min^{-1} during dispersion in a mixer (Waring blender).

An attractive alternative to using mixers is to use vigorous shaking by the equipment used to lyse cells by shaking cells in a slurry of glass beads (Fast prep 24). The lysis in this procedure is achieved by collisions between the glass beads, not by the shear forces themselves. Using the same instrument without glass beads would theoretically lead to efficient dispersion without lysing the cells.

1.4 DdPCR for enumeration of total *E. coli*

Selective culturing mediums are by their very nature *selective* and therefore underestimate the number of bacteria in the source (Ben-David & Davidson, 2014). Outside of laboratory environments, most organisms exist in ecosystems consisting of complex networks of biotic and abiotic factors such as symbiotic relations with other organisms, nutrient niches, specific local atmospheric conditions, etc. Naturally, environmental stress when introduced to artificial habitats affects less adaptable cells misconstruing the actual number present in the native habitat.

A population of *E. coli* that is dying out, be it by starvation of antagonistic effects or other organisms, is expected to differentiate into a viable fraction, which can be quantified by viable counts, and a fraction of dormant and moribund cells, which are unable to grow in the laboratory media despite the presence of an intact genome. This calls for a method to quantify the total number of cells with an intact genome. This is achievable by quantitative PCR,

provided that the genome of *E. coli* possesses genes that are unique in the sense that their abundance in the pristine environment (biosolid without invasion of *E. coli*) is negligible. Such a gene is *uidA* which encodes the β-glucuronidase enzyme explained in section 1.2.1 and is around 600 base pairs in length. It belongs to the glycoside hydrolase family of enzymes in which β-galactosidase, encoded by *lacZ,* is also a member, as *lacZ* and *uidA* are paralogs (Molina et al., 2015)

Quantitative PCR can also be used to enumerate the total bacterial abundance, by using "universal 16s rDNA primers", i.e. primers that secure amplification of all bacterial 16SrDNA in a sample.

2 Methodology

Water used in the experiments was either Milli-Q-water (MilliQ Reference water Purification systems, Merck) or Rho-water produced by reverse osmosis (RO) (Synergy Water purification System, Merck KGaA, Darmstadt Germany).

2.1 Production of biosolids using digestate from VEAS

The experiment was a follow up of the experiments performed by Svennevik et al. (2020), who measured the re-growth of *E. coli* in digestates (from an Anaerobic Digester, AD) which had been sterilized by the Thermal Hydrolysis Process (THP), in which the material is heated to 165^0C followed by a rapid release of pressure ("explosion"). Unfortunately, the Post-AD THP-treated material used in those experiments was not available, and we had to produce an imitation of this material, called "biosolids" hereafter.

All experiments were conducted with a sample of pre-AD THP-treated digestate provided by VEAS (Norway). Time constraints limited the availability of the thermal hydrolysis machine, so the imitation was not thermally hydrolyzed post-AD. In the following experiments, the produced biosolid is also referred to as "VEAS-SS" an acronym for Vestfjordens Avløpsselskap-SolidStream a reference to the product developed by Cambi SolidStream®.

Digestates are not sterile after AD, and in addition to the organisms present in the anaerobic digester, the digestates are exposed to possible contamination sources during transportation, hence autoclaving was necessary. Autoclaving releases CO_2 from the sludge resulting in increased pH (Bajón Fernández et al., 2014), usually around 1 pH increase.

For the sludge to attain conducive growth conditions, a neutral pH around 7-8 is desirable. The pH in 6 independent digestate plastic bottles was adjusted to 6.5 by titration of 4M HCl. All 6 bottles were pooled in a bucket and total pH was measured at 6.7 and autoclaved at 121^{o}C for 20 minutes.

Autoclaved digestate pH was measured at 7,49. The digestate was left overnight to cool down and the new pH was measured at 7,42 (Final pH of biosolids). We added polymers to the bucket of digestate until flocculation occurred. Filtration of flocs was performed and finally, the filtrated flocs were dewatered using an improvised filter made by folding a mosquito net and desiccated overnight under UV light. The material was stored in one blue cap bottle. The total imitated biosolid produced was 320g.

The dry weight of the material was measured by weighing three samples (in aluminum trays) before and after drying overnight at 110^{o}C. The measured % dry weight ranged from 53.6 to 55.6 %, average = 54.7 % (st. dev.= 0.9).

The biosolids were tested for sterility by monitoring microbial respiration. For this, four 2g samples were placed in sterilized 120 mL serum vials, which were crimp sealed with butyl rubber septa, He-washed, provided with a minimum of O_2 (injection of 5 mL O_2 to each vial), and monitored for O_2 consumption and CO_2 production by the incubation robot which takes headspace-samples at intervals, measuring O_2, CO_2, N_2; NO and N_2O (Molstad et al 2007). The experiment included sterilized biosolids, with and without yeast extract (0,5mL of sterile yeast extract solution, 10g L^{-1}), as well as unsterilized material as a positive control. While the unsterilized material showed high O_2 consumption rates, increasing exponentially when provided with yeast extract, the sterilized material showed minuscule O_2 consumption which was not stimulated by the addition of yeast extract (results not shown). The minuscule oxygen consumption in the sterilized material was ascribed to abiotic reactions, and the experiment confirmed the sterility of the material.

2.2 Isolation and cultivation of *E. coli*

The *E. coli* used in our experiments was isolated from sewage water sampled from the primary sedimentation tank at VEAS (Norway) also called primary effluent. Isolation was performed by plate spread technique with a dilution series ranging from 10^{-2} - 10^{-7}. Seven Eppendorf tubes were prepared with 900 µl of sterile water in each. 100µl of sewage water was added to the first Eppendorf tube homogenized and serial diluted. From each dilution, 50µL was added to individual MUG-agar plates and evenly dispersed with a Drigalski spatula. The MUG-agar plates were sealed with parafilm and incubated overnight at 37 ^{0}C for

optimal conditions. The following day the plates were inspected for fluorogenic colonies under Trans-UV light, produced by a "Molecular Imager® Gel Doc™ XR System. One of eight colonies showed fluorescence and a single fluorogenic colony was transferred to a new 100 ml bottle of MUG medium to be used as a stock culture for the survivability experiment. The plates were photographed under Trans-UV and the agar-plate used for isolation is shown in Figure 3.1.1. Also, a streak culture on MUG-agar, from a fluorescent colony was prepared utilizing standard methods (Eyler, 2013). Subsequent cultivations were created from the original isolation by transferal to new tubes and replicating the conditions.

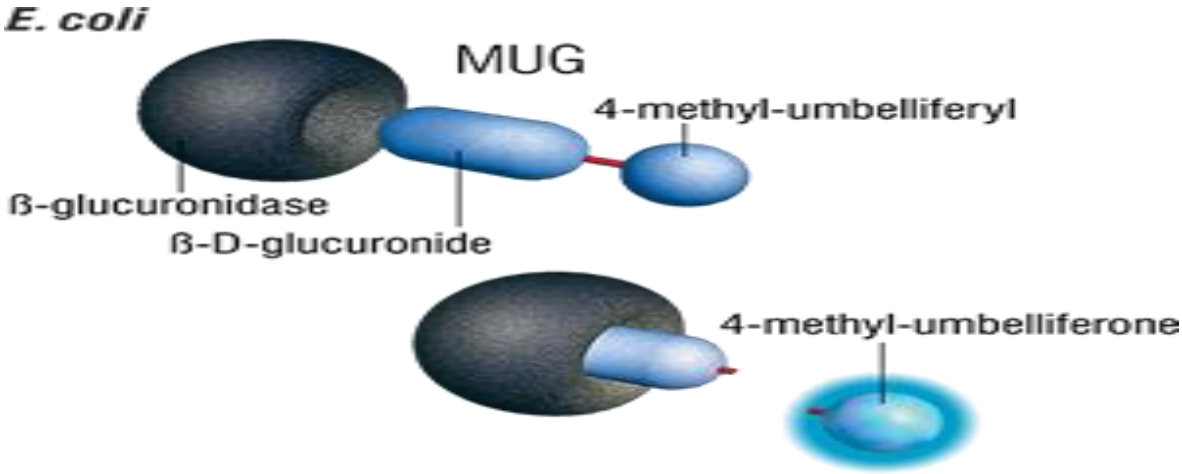


Figure 2.2.1 - The panel shows how *E. coli* becomes fluorescent by cleaving (β-glucuronidase) the non-fluorescent methyl-umbelliferyl-β-D-glucuronide to fluorescent 4-methyl-umbelliferone.

2.2.1 Confirmation of *E. coli* isolates by 16s sequencing

Verification of successful *E. coli* isolation using Sanger Sequencing targeting the 16S ribosomal RNA gene sequence universal for all prokaryotes was performed on streaked fluorescent colonies of *E. coli.* A culture of *E. coli* with proven fluorescence (see Figure 3.1.1) was streaked out on a MUG-agar plate, incubated overnight and three colonies were transferred to three independent PCR tubes containing master mix with contents shown in table 2.2.1, using an inoculation loop sterilized with heat. Negative and positive control was included containing only master mix (negative) or template DNA (Lars kultur 10.06.18)

For the amplification of 16s rRNA gene universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 1492R (5'GGTTACCTTGTTACGACTT-3') targeting the 27F and 1492R positions on the small-subunit of prokaryotic ribosomal RNA (SSU) (Frank et al., 2008). The PCR program used is shown in Table 2.2.2

Table 2.2-1 - Reagents used in each PCR tube for *E. Coli* sequencing. A master mix was prepared for the number of samples plus one, to account for sampling errors.

Reagents	Per reaction (µL)	Total (5 + 1 reactions)
GreenTaq	24	24*6 = 144
27F 10Mm	1	1*6 = 6
1492R 10mM	1	1*6 = 6
Nuclease free water	23	23*6 = 138
Template	1/or small fraction of colony	

To determine the size and successful amplification of our DNA, gel electrophoresis was performed. An agarose gel was produced using standard methods with specific reagents shown in Table 2.2.3 The amplified DNA fragments (PCR products) were injected in individual wells on the agarose gel. For our DNA fragments to visualize under UV light, peqGreen was added to the gel solution before casting, producing a green fluorescence. A 100bp ladder, colored blue, was also added to the gel. For our experiment, we maintained a current of 80V for 45min. After electrophoresis, the gel was exposed to UV light so visualization of the DNA fragments, and subsequent excision, could be performed. Excised DNA fragments were transferred to three individual Eppendorf tubes containing buffer equal to the weight of the excised fragment.

The three DNA samples were split into two Eppendorf tubes, one for the 27F primer and one for the 1492R primer for each sample corresponding to the primers attached to anti-sense and sense-strands. 5µL of respective primers in a 10µM concentration were added to the associated tube. The tubes were marked with a barcode to be identifiable during sequence analysis. The tubes were sent to Eurofins Genomics for sequencing.

The raw sequencing results were presented in 6 independent samples barcoded for user identification and were analyzed using Serial Cloner v2.6.1. Forward primers need to be read in a 5'-3' direction and reverse primers in an opposite 3'-5' direction necessitating alignment to be performed anti-parallel to the forward primer. Sequence chromatograms were truncated by trimming away "noisy" chromatograms or low-quality bases such as very low-intensity peaks or broad peaks (double peaks). The trimmed sequences were coupled with a complementary primer for each DNA sample and a consensus sequence built by aligning forward and reverse sequences. The consensus sequence was compared against a database of

genomes using the NCBI Blast tool and the highest percentage identity, 97% or higher, was used as criteria for identification.

Table 2.2-2 - PCR program used in the amplification of *E. coli* rDNA

Time (s)	Temp (^{O}C)	Cycles
10	98	30
30	55	30
60	72	30
∞	4	

Table 2.2-3 - Reagents used in the gel-electrophoresis

Reagents:	Amount	Note
1x TAE Buffer	200mL	Also used in covering the gel
Ultrapure™ Agarose	4g	
PeqGreen Dye	8µL	Fluorescent dye
DNA (PCR product)	49µL	
100 bp ladder	5µL	Ladder for gel electrophoresis

2.3 Enumeration of *E. coli* using MPN

2.3.1 Survival of *E. coli* in live soil versus gamma-sterilized soil

To optimize the method for dispersion of *E. coli* for enumeration by MPN, and to perform the first test of survival of *E. coli* we added cells to a clay loam soil taken from a long-term liming experiment described by (Bakken et al., 2020). The soil used was from the calcite treated plots, with pH =6.6 (measured in 10 mM $CaCl_2$). Fresh soil samples were used, as well as soil that had been sterilized by gamma-radiation. The soils were portioned into 50 mL Falcon tubes (12 with gamma-sterilized soil and 12 with live soil). Each tube contained 7.1 g dry soil (93% dry weight) to which 2.5 mL sterile water was added containing an unknown number of *E. coli* cells. The tubes were capped loosely to allow diffusion of oxygen, and incubated at room temperature, in a closed container at room temperature with moisturized paper to avoid desiccation of the samples. At intervals, tubes were removed to enumerate viable *E. coli.*

To enumerate viable *E. coli*, 1g of soil was mixed with 9mL of PBS (pH 7.4) and vortexed for 4 minutes to disperse the cells. From this suspension, 20µl was transferred to each well on the first column of a 96 well microtiter plate, filled with 180µl of MUG EC-

Broth. Further dilutions to the adjacent columns were done by multichannel pipette, resulting in a dilution range of 10^{-2} to 10^{-13}. The plates were incubated at 37ºC overnight and inspected for positive wells (fluorescence) by taking photographs under trans-UV light. The Most Probable number of cells per mL was calculated using the BAM-MPN spreadsheet developed by (Garthright & Blodgett, 2003).

From each sampling point, frozen samples were taken containing 2g of calcite and stored at -80ºC for later determination of *E. coli* by PCR.

To test if the cells were effectively dispersed by the vortexing, the efficiency was compared with that achieved by shaking the suspensions in a FastPrep-24® Sample preparation system (MP-Biomedicals), which is normally used to disrupt cells by bead beating (shaking a slurry of cells and glass beads). The very fast shaking (frequency 4 m/s) should secure more efficient dispersion than vortexing, but could also result in some cell disruption, despite the absence of glass beads. We homogenized each sample for 1, 2, 3, 4, and 5 minutes in Fast prep (intermittently, 1 min on and 1 min off, to minimize heating), and performed serial dilutions on microtiter plates for MPN as described in section 2.2.2.

2.3.2 Standard Curve for MPN/CFU

Shimadzu – uv1280 spectrophotometer was used to monitor optical density in the creation of our "MPN - CFU standard".

Optical density (OD) is a powerful tool for measuring the light attenuated by particles in the medium, while absorbance is measured in light absorbed by the liquid measured. To obtain an accurate reading, one must account for the absorbance by calibrating the instrument with a cuvette containing only the medium used for cultivation. This is also called baselining. The absorbance is equal to the light received by the medium. The light attenuated means the light transmitted by the sample. It is well known that light with a wavelength of around 600nm (orange/yellow part of the spectrum) is easily scattered by particles the size of bacteria. Regular bacteria used for research such as *E. coli* are incapable of producing pigments that can absorb energy from the 600nm part of the electromagnetic spectrum, and as such OD around 600 is commonly used when measuring OD of bacterial cultures. However, in cases where noticeable coloration of a medium is present, an increase or decrease in wavelength, away from the observed color, will improve precision in measurements. MUG-medium in liquid form is a transparent orangey color, on this basis the use of OD660 wavelength was decided for the creation of the MPN-CFU standard. OD550 was used in all measurements using a PBS suspension (preparation of *E. coli* inoculate).

For the creation of a standard curve showing CFU/mL to OD660 level and MPN, a growth experiment using the isolated *E. coli* strain was performed. A colony of *E. coli* isolate was inoculated in 10mL MUG-medium and OD660 monitored. For OD660 0.1, 0.2, 0.3, 0.4 and 0.6, serial dilutions (10^{-1}-10^{-12}) in 96-wells microtiter plates using 20µL of bacterial growth culture, was performed. From the 10^{-4}, 10^{-5}, 10^{-6} dilutions 50µL was transferred to MUG-agar plates and plate spread. Triplicate agar plates were prepared for each OD level totaling nine for each target OD. The agar- and microtiter plates were incubated overnight at 37ºC. CFU/mL were obtained from the resulting plates the following day using standard microbiological procedures and MPN/mL as described previously. Also, each agar-plate was sealed with parafilm ahead of incubation.

Lastly, from each OD level, 900µL of bacterial culture was transferred into three individual Eppendorf tubes containing 100µL formalin for fixation and stored at room temperature. Formalin fixated sample cells were quantified by cell counting utilizing a Neubauer hemocytometer, a counting-chamber device allowing for enumeration of cells in a

liquid sample through a Leica DM1000 microscope, and cells/mL calculated using standard methods.

2.4 Respiration kinetics.

Gas kinetics for the microbial communities were obtained using a "robotized incubation system connected to a gas chromatograph and an "NO analyzer" allowing for automated time incremental sampling of the headspace in each vial. (Molstad et al., 2007). A total of 42 serum vials can be monitored simultaneously while submerged in a thermostated water bath. The system enables measurement of O_2, N_2, NO, N_2O, CO_2, and CH_4 levels, however only O_2 and CO_2 will be evaluated in this experiment. The system samples gas in the vials using an autosampler, with a needle attached, connected to a peristaltic pump, and returns an equal volume of helium to maintain the gas pressure at ~1 atm. (Molstad et al., 2016). To ensure aerobic conditions in the vials, oxygen was manually added using a 5 ml syringe, when oxygen concentrations fell below 10 vol%. After oxygen injection, the overpressure was released by piercing the septum with a needle coupled to a syringe with no plunger and filled with 1mL of 70% ethanol.

The robot-incubations for measurement of respiration kinetics in biosolids with and without *E. coli* and other bacteria (see 2.5) were run in parallel with incubations of the same material in falcon tubes at the same temperature.

2.5 Suppression experiments

A series of experiments were set up to test if single cultures or a mix of compost bacteria were able to suppress the growth of *E. coli* in biosolids. The three strains tested (*Cloacibacter* (CB), *P. DC1* (PD), *P. Stutzeri* (PS)) were previously isolated from N_2O-reducing enrichment cultures in digestates from VEAS (Jonassen et al., 2020). These experiments were conducted in 50 mL Falcon tubes with sterile biosolids which were first inoculated with "suppressor candidates" (single strains, a mix of single strains, and compost) and incubated at 20 ^{0}C. The compost was obtained from Lindum AS industrial facility for organic waste in Drammen and was mixed with biosolids in a 1/5 ratio (20% compost, 80% biosolid) in a plastic container 5 days (-96 hours) before transfer to falcon tubes and serum vials. Each experiment included controls that were not inoculated. After 96 hours, *E. coli* was introduced, and the abundance of viable *E. coli* was monitored over 22 days, using the MPN method described above (2.3.1). Each time, samples were taken for MPN measurements, duplicate 1 g samples were frozen in cryotubes at -80^{o}C for later quantification of *E. coli* by

ddPCR. Experimental details are shown in Table 2.5.1. Parallel samples were incubated for monitoring the respiration in each of the treatments.

The tubes were placed in plastic containers with wetted paper tissues covering the bottom and their caps loosely fastened, allowing air diffusion into the tubes. Only during sampling were the containers opened and resealed afterward.

Table 2.5-1 - Treatments used in the robotized incubation system.

Vial	Treatment name	VEAS-SS (g)	*Cloacibacter* µL	*P. DC1* µL	*P. Stutzeri* µL	*E.Coli* uL	H2O uL	Compost g
1	VS (Control (No *E. coli*)(water)	2	-	-	-	-	100	-
2	VS (Control (No *E. coli*)(water)	2	-	-	-	-	100	-
3	VS (Control (No *E. coli*)(water)	2	-	-	-	-	100	-
4	VSE (Control)	2	-	-	-	100	100	-
5	VSE (*E. coli*)	2	-	-	-	100	100	-
6	VSE (*E. coli*)	2	-	-	-	100	100	-
7	VSCE (Compost + *E. coli*)	1,6	-	-	-	100	100	0,4
8	VSCE (Compost + *E. coli*)	1,6	-	-	-	100	100	0,4
9	VSCE (Compost + *E. coli*)	1,6	-	-	-	100	100	0,4
10	VSME (Mix + *E. coli)*	2	33	33	33	100	-	-
11	VSME (Mix + *E. coli)*	2	33	33	33	100	-	-
12	VSME (Mix + *E. coli)*	2	33	33	33	100	-	-
13	VSPSE (*P. stutzeri* + *E. coli)*	2	-	-	100	100	-	-
14	VSPSE (*P. stutzeri* + *E. coli)*	2	-	-	100	100	-	-
15	VSPSE (*P. stutzeri* + *E. coli)*	2	-	-	100	100	-	-
16	VSPDE (*P. DC1* + *E. coli*)	2	-	100	-	100	-	-
17	VSPDE (*P. DC1* + *E. coli*)	2	-	100	-	100	-	-
18	VSPDE (*P. DC1* + *E. coli*)	2	-	100	-	100	-	-
19	VSCBE (*Cloacibacter* + *E. coli*)	2	100	-	-	100	-	-
20	VSCBE (*Cloacibacter* + *E. coli*)	2	100	-	-	100	-	-
21	VSCBE (*Cloacibacter* + *E. coli*)	2	100	-	-	100	-	-

* OD_{550} for the bacterial suspensions were *Cloacibacter*: 0,75, DC1: 0,75, *Ps stutzeri*: 0,75 *E coli* 0.096.

2.6 ddPCR for enumeration of *E. coli*

Digital droplet PCR (ddPCR) is a novel technology for quantifying genomic material such as cDNA, rDNA and DNA. Private developers including QIAGEN and Bio-Rad have developed instruments utilizing a droplet generation step, performed ahead of PCR-amplification. A Bio-Rad QLX100™ Droplet Digital PCR system partitions the samples into approximately 20 000 evenly sized droplets, each 1nL in volume. Each droplet undergoes PCR amplification of its genomic material, however depending on the concentration of template DNA, it can be saturated or hold no template at all (negative droplet). To avoid saturation, a test run using a dilutions series of the genomic material is performed. The results from this initial test will determine the dilution level to use for each series in subsequent runs. This is important for increasing sensitivity and specificity in estimating the number of copies per sample. Downstream application of PCR will amplify the target nucleic acids in each droplet.

During PCR, the primers used in the reaction mix activates and binds to the primer binding sites on the target sequence. The number of PCR cycles determines the theoretical number of copies you will produce in a 2^n where "n" is the number of PCR cycles. A problem with PCR is that the exponential growth eventually planes after an uncertain number of cycles. Another issue is that PCR sensitivity can be affected by residues remaining from DNA isolation such as proteins, isopropanol, fats, humic acid, etc called PCR inhibitors (Acharya et al., 2017). PCR inhibitors function by binding to the template DNA, making it unavailable for the enzymes to replicate the sequence. They can also directly inhibit the DNA polymerase or its cofactor magnesium (Rossen et al., 1992). The sample partitioning in ddPCR and the initial dilution series reduces these particular issues (Quan et al., 2018).

Partitioning creates the possibility of multiple target sequences existing within a single droplet. ddPCR accounts for this by assuming that the molecule population in each droplet follows a Poisson distribution predicting that as the number of target molecules increases the likelihood of droplets containing two or more target molecules increases. (Quan et al., 2018)

After amplification, ddPCR can be performed where Taq-polymerase binds to the amplified sequence and issues a fluorescent signal for each successful amplification per droplet. Each reaction (droplet) is quantified for each sample using the absolute fluorescence issued by the total number of droplets in each sample. The analysis in the ddPCR is performed by applying Poisson distribution (Quan et al., 2018). Advantages of ddPCR include precision, high tolerance of PCR inhibitors, its high reproducibility rate and it does

not need to rely on references or standards (Quan et al., 2018). All of which will be employed to further our understanding of microbial growth rates.

2.6.1 DNA extraction

DNA extractions were performed on frozen solid samples attained during the incubation experiments (not for calcite). The DNA was extracted from 0,25g per solid sample using Qiagen's DNeasy PowerLyzer PowerSoil Kit according to the manufacturer's protocol. Lysis of cells was done by bead beating in a FastPrep 24 at 4m/s for 45seconds. DNA concentrations were measured using NanoDrop (µg/mL).

2.6.2 ddPCR for 16s and uidA gene abundance

DdPCR was used to enumerate duplicated genomic extracts from one replicate of the suppression experiments frozen samples. DdPCR is based on a "dilution to single genome method" and a dilution series of the DNA must be prepared. To find the correct dilution level, a preliminary test was performed on all DNA samples immediately following spiking (115 hours/5 days), and individual dilution levels were decided for each series based on this test (Quan et al., 2018). To target the *uidA* gene, primer pairs ECF-*uidA*/ECR-*uidA* (5′-CGGAAGCAACGCGTAAACTC-3′, 5′-TGAGCGTCGCAGAACATTACA-3′) distinct for *E. coli* was used (Feng & Hartman, 1982). For quantification of the total 16s gene abundance, 10nM concentration of primer pairs PRK341F/PRK806R (5'-CCTACGGGRBGCASCAG-3', 5'-GGACTACYVGGGTATCT-3') (Eurofins Genomics) targeting the V3-V4 hypervariable region of prokaryotic 16s rDNA (Svennevik et al., 2020; Yu et al., 2005) was used.

2.6.2.1 PCR amplification and quantification

For each sample, a PCR mix was made consisting of 20.7µL of the reaction mixture, and 2.3µL of DNA template. The reaction mixture contained 11.5µL of QX200 ddPCR EvaGreen supermix (Bio-Rad), 10nM concentration 0.46µL of each primer pairs, 16s or *uidA*, and 8.28µL nuclease-free water.

Oil droplet generation was performed using a QX200 droplet generator (Bio-Rad). 20µL PCR mix was loaded onto a disposable plastic DG8 cartridge (Bio-Rad) with 70µL of droplet generation oil for EvaGreen (Bio-Rad). 40µL of the resulting oil suspensions were transferred to a 96 well twin.tec PCR plate (Eppendorf) and heat-sealed with aluminum foil using a PX1™PCR plate sealer (Bio-Rad).

PCR profile when amplifying primers were specific to primer pairs. For *uidA* amplification, PCR conditions conformed to Svennevik's paper. For amplification of 16s rDNA, conditions were denaturation/enzyme activation at 95^{O}C for 5 min. 40 cycles at 95^{o}C (denaturation) for 30s , 55^{o}C for 30s (annealing) and 72^{O}C for 45s (extension) ending with signal stabilization at 4^{o}C- and 90^{o}C for 5 min each. All PCR products were produced from a 2720 Thermal Cycler (Applied biosystems) with a lid temperature of 110^{O}C and. The amplified plates were inserted in a droplet reader (Bio-Rad) and analyzed in a Quantasoft™ Analysis Pro software (Bio-Rad) to calculate the concentration of target DNA in copes/µL.

Table 2.6.2 - PCR protocol for the amplification of prokaryotic 16srRNA (left) and *E. coli* specific gene; uidA (right)

PCR Protocol for ddPCR			Reaction Mixtures	PCR protocol for *E. coli* primers		
Temp ^{o}C	Time	Cycles		Temp ^{o}C	Time	Cycles
95	5min			95	5min	
95	30s			95	30s	40
55	30s	40		63	1min	
72	45s			4	5min	
4	5min			90	5min	
90	5min					
4	indef			4	indef	

3 Results

3.1 Isolation and cultivation of *E. Coli*

The isolate of an *E. coli* strain from sewage water is presented in Figure 3.1.1. The fluorescent colony in Figure 3.1.1 was streaked out on a new agar plate shown to the right together with dilution level in the top left of both pictures. The dilution gradient was -1 to- 6. Non-fluorescent colonies can be seen in the same figure. Two plates showed growth: 10^{-1} and 10^{-2} with 41 colonies and 6 colonies respectively. Out of a total of 47 colonies, only one colony from the 10^{-2} plate (out of 6) emitted fluorescence. The percentage of colonies exhibiting fluorescence was only 2,13% which is considered very low.

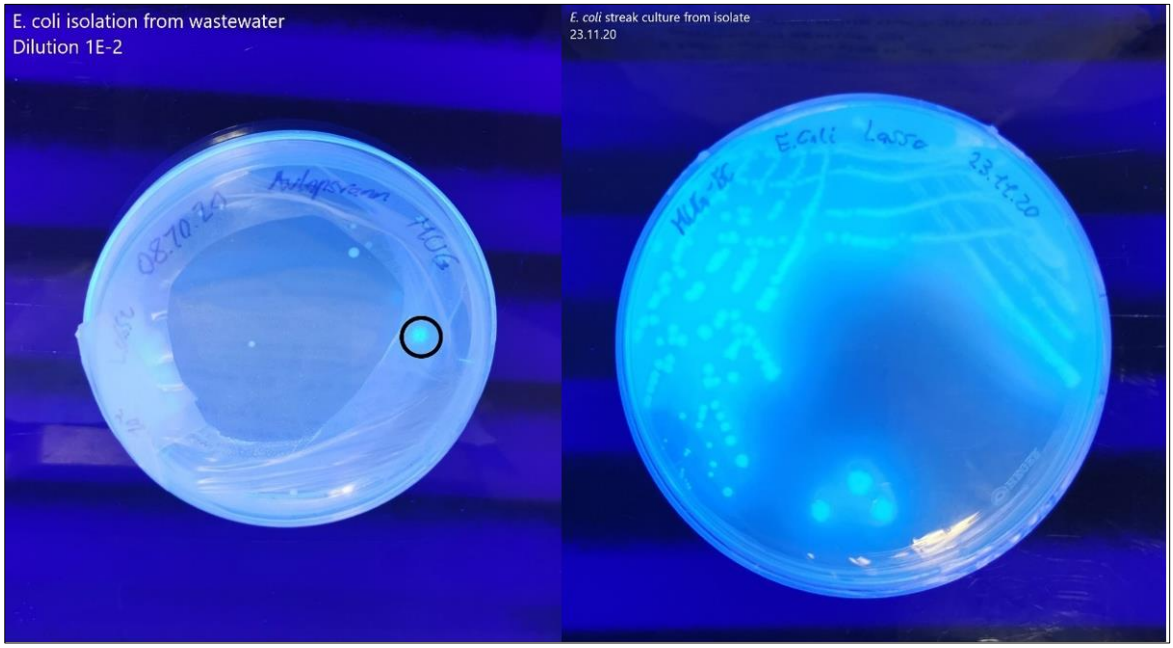


Figure 3.1.1 - Isolation of *E. coli* by dilution plating on agar with MUG-EC. Left: a dilution plate with several colonies, of which one was fluorescent. Right: cells from the fluorescent colony were streaked out on MUG-EC agar, giving rise to only fluorescent colonies

To establish a method for estimating the viable counts of an *E. coli* wild type by MPN, the method was compared to MUG-agar plated colonies (colony forming units, CFU) and microscopic counts (in a hemocytometer). All results for the three methods were obtained from two separately grown *E. coli* cultures at OD660 1) 0.2, 0.4, and 0.6 and 2) 0.1. Formalin fixated bacterial sample for OD 0.1 was degraded and could not be used.

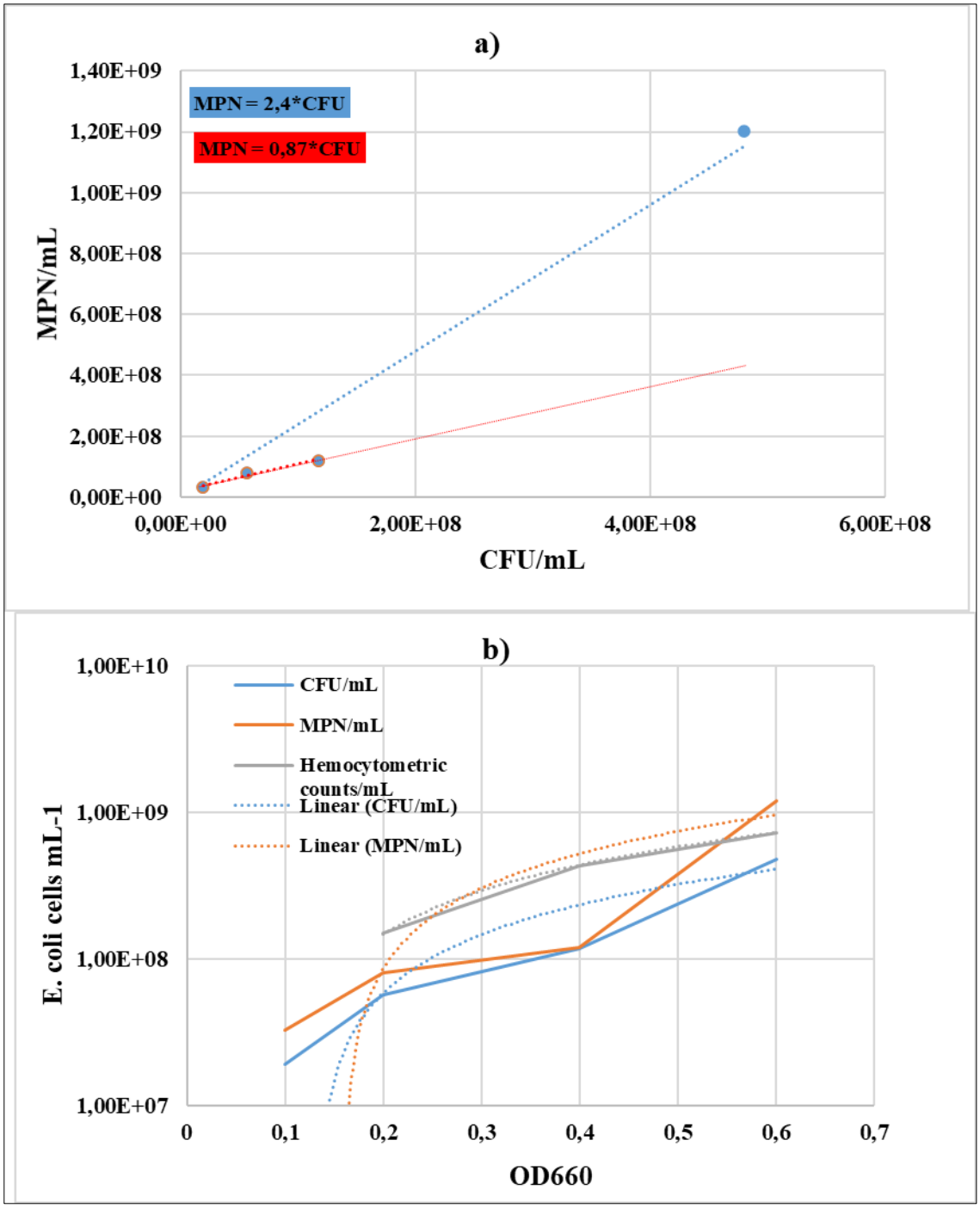


Figure 3.1.2 - *E. coli* viable counts by MPN and CFU. Panel a) shows MPN/mL plotted against CFU/mL for OD660 levels 0.1, 0.2, 0.4, and 0.6, and Panel b) shows the *E. coli* cells/mL for each of the three-enumeration methods Hemocytometer, MPN and CFU, plotted against OD. In panel a the linear regression line suggests that MPN exceed CFU by a factor of 2.4, but the 1:1 line (dashed red line) shows that the deviation is due to a single sample (OD 0.6), and excluding this measure the factor is only 0,87 showing that there is a reasonable linear agreement for the three other samples (OD 0.1-0.4).

3.1.1 Sequencing of *E. Coli*

Sequence analysis of wastewater *E. coli* was performed using “Serial Cloner v2.6.1”, a software for annotating genomic sequences to confirm the success of *E. coli* isolation. A consensus sequence was compared against a database of genomes using the NCBI Blast tool. In 16S rRNA Sanger sequencing, standard procedure is identification by a percentage threshold of equal to or higher than 97%, for the same species. The 16S rRNA consensus sequence showed a 100% identity with *E. coli* for DNA samples 1 and 2 (results not shown). DNA sample 3 contained generally lower quality sequencing chromatograms and was not used in identification.

3.2 Enumeration of *E. coli* by MPN

In quantification of viable counts of *E. coli*, it was apparent that some treatments had a more suppressive effect on *E. coli* growth than others see figure 3.2.1.

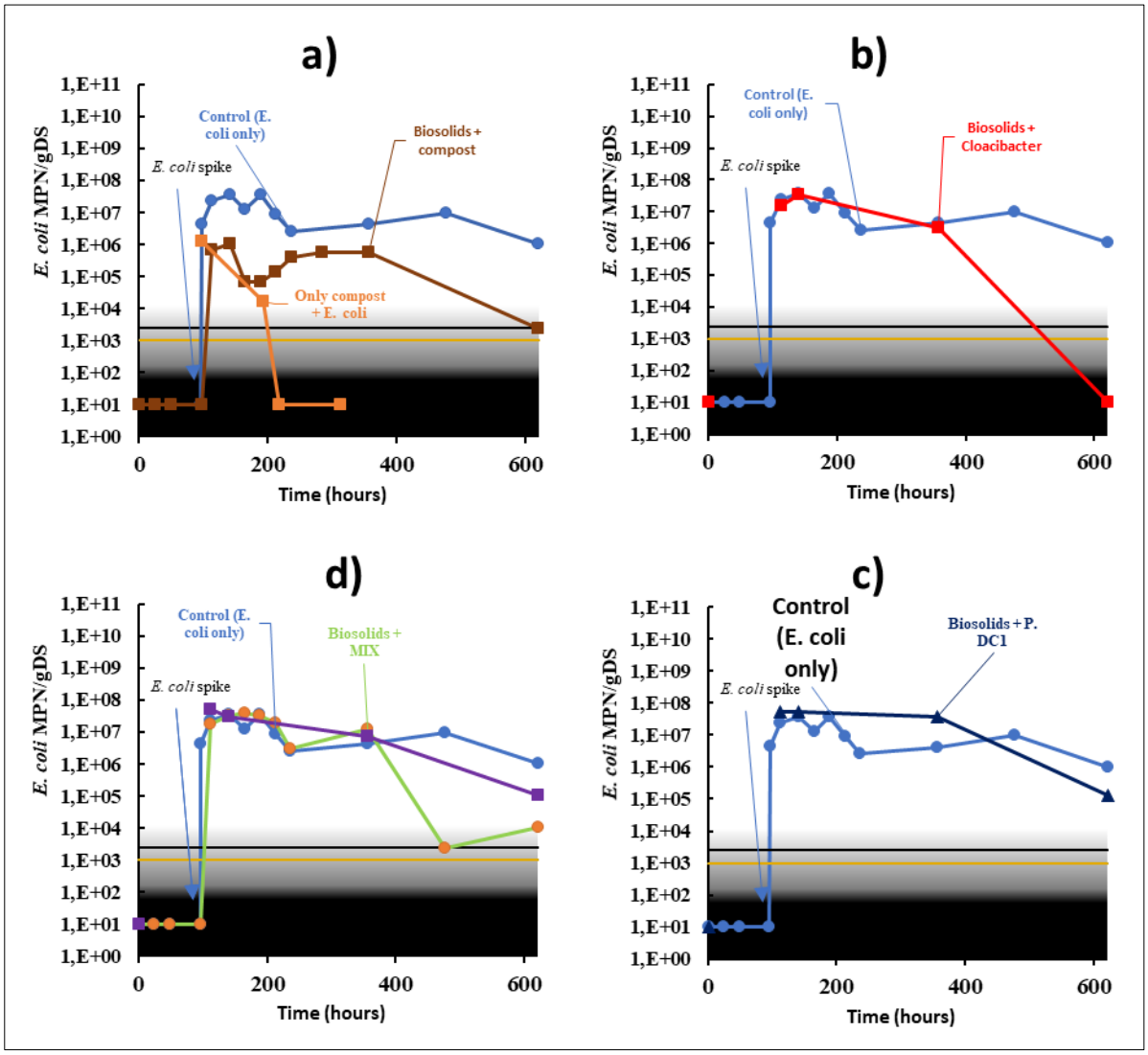


Figure 3.2.1 - Viable counts of *E. coli* throughout incubations, suppression by compost bacteria versus single strains.

The panels show the MPN of *E. coli* plotted against time for the various treatments. The treatments are a) biosolids inoculated with compost (compost alone, spiked with *E. coli* is shown for comparison), b) biosolids inoculated with Cloacibacter, c) biosolids inoculated with strain DC1, d) biosolids inoculated with Ps. stutzeri and with a mixture of the three strains. The result for the control (i.e., *E. coli* in sterile biosolids, panel a) is plotted in all panels, for comparison. The orange line is the US regulation limit (1000MPN/gDS) and the

black is the Norwegian (2500MPN/gDS). Black and grey areas indicate MPN and ddPCR detection limits, respectively. Introduction of the various strains to the solids was done at time = 0 while *E. coli* was added at t = 97, indicated by a blue arrow in the panels. Panel a show that *E. coli* survived well in sterile biosolids, but not in biosolids with compost bacteria: the MPN values were ~1 order of magnitude lower than in biosolids during the first 400 hours, declining to ~3 orders of magnitude lower thereafter. Panel b shows that *Cloacibacter* had no suppressive effects initially, except for the very low number at the very end of the incubation. Panel c and d suggested minor/no suppressive effects of strain *DC1*, but some apparent effects of *P. stutzeri* and the mixture towards the end of the incubation.

3.2.1 Dispersion for viable counts of *E coli*; Fast Prep vs Vortexing

The effect of dispersion by vortexing versus dispersion by FastPrep on the viable counts of *E. coli* (MPN) is shown in Figure 3.2.2. As expected, dispersion by FastPrep resulted in higher viable counts than vortexing. In theory, the strong shear forces during shaking in the FastPrep could result in physical disruption of *E. coli* cells, but the results suggest that this is not the case: The viable counts did not decrease with time of FastPrep dispersion. Based on these results, FastPrep dispersion for 1 min was used for subsequent experiments, testing the survival of *E. coli* in biosolids.

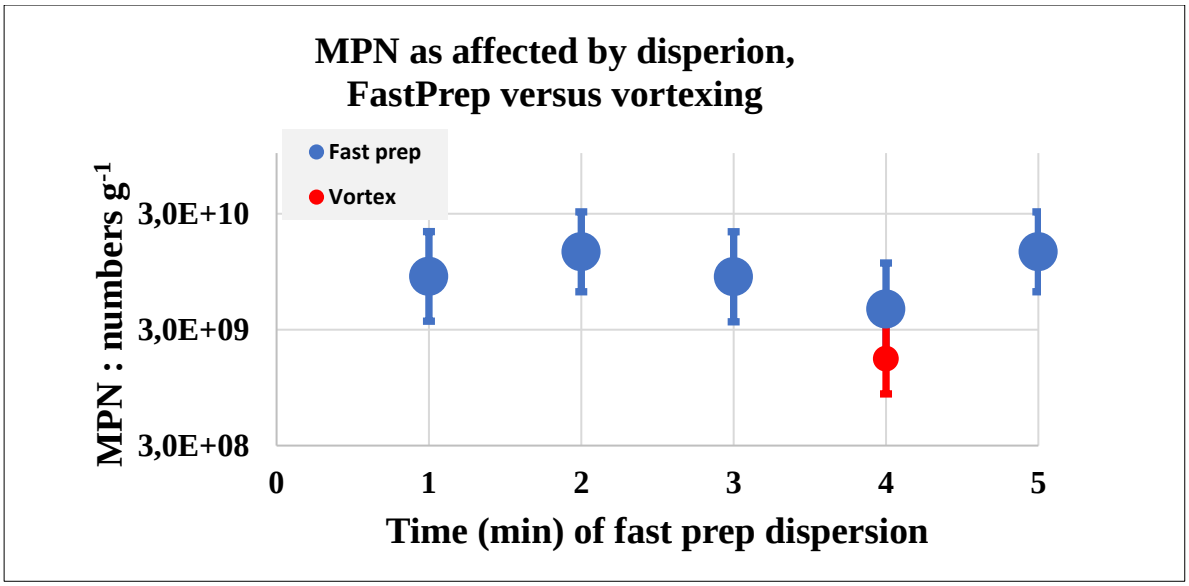


Figure 3.2.2 - Viable counts of *E. coli* as affected by agitation method. *E. coli* was added to gamma-sterilized calcite soil 5 days before the experiment. The biosolids were dispersed in PBS (1/10 g/mL) and dispersed for 4 minutes by vortexing, and by 1, 2, 3 4, and 5 minutes of shaking in the FastPrep machine, and each sample was then diluted on MPN plates to quantify the number of viable cells. The results are shown with standard deviations as vertical lines. Vortexing for 4 minutes (red symbol) was clearly inferior to FastPrep (blue symbol).

3.3 Respiration kinetics

To judge the growth of the various strains versus that of the compost bacteria, 2 g Biosolids with and without bacteria were incubated in vials which were monitored for O_2 and CO_2 in the headspace. The primary data on measured O_2 consumption is shown in Figure 3.3.1, demonstrating that compost bacteria were able to exploit a larger share of the organic material in the biosolids than the single strains. One could suspect that the high respiration in the biosolids with compost could be due to consumption of organic C in the added compost (0.4 g compost per vial), but the measured O_2 consumption in vials with compost only showed that this is not the case (see inserted panel a Figure 3.3.1a). Inspection of the O_2 consumption rate during the first 100 hours (before spiking with *E. coli*) showed that all strains were able to respire in the biosolids (and presumably grow), but to different degrees: *P. stutzeri* was evidently more active than the two other strains. A closer inspection of the respiration rate of the three strains during the first 100 hours is shown in Figure 3.3.2, where their net O_2 consumption, corrected for abiotic O_2 consumption is plotted. The remarkable similarity between the measured O_2-consumption by the mixed culture (all three added together) and the sum of the O_2-consumption by the three strains when added alone can suggest that the three strains exploited different fractions of the available organic compounds in the biosolid.

Figure 3.3.3 shows the CO_2 production, which largely corroborates the patterns observed for O_2-consumption, although the molar amounts of CO2 produced were somewhat lower than the molar amounts of O_2 consumed. The molar ratio CO_2/O_2 (i.e., mol CO_2 produced per mol O_2 consumed) should be ~ 1 for microbes respiring carbohydrates ($C_m(H_2O)_n$) and lower if respiring more reduced C-sources. The CO_2/O_2 ratio for the treatment with compost was ~0.75, to begin with, but declined to 0.5 towards the end of the incubation (result not shown). Although this could suggest that the substrates utilized changed gradually towards more reduced C-compounds, the result could also reflect carbonate formation, not accounted for by the simple routine for the carbonate chemistry used (see Molstad et al 2007, and the available Kincalc spreadsheet at ResearchGate (See Appendix B))

Compost and the single strains were added to biosolids to test their ability to suppress *E. coli*, and the meticulous analysis of the respiration in the various treatments was done to assess their ability to grow by scavenging the available C in the biosolids. The complex community added with the compost outcompeted the single strains, utilizing 2-3 times more of the organic C than any of the single strains. The net O_2 consumption of the single strains

during the first 100 hours (i.e., before spiking with *E. coli*) ranged from 50 to 150 µmol O_2 $vial^{-1}$. If assuming the same growth yield per mol O_2 as that measured for aerobic growth of *Paracoccus denitrificans* = $15*10^{13}$ cells mol^{-1} O_2, 50-150 µmol O_2 $vial^{-1}$ is equivalent to 7.5-$23*10^9$ cells $vial^{-1}$, or 3.8-$11*10^9$ cells g^{-1} biosolid.

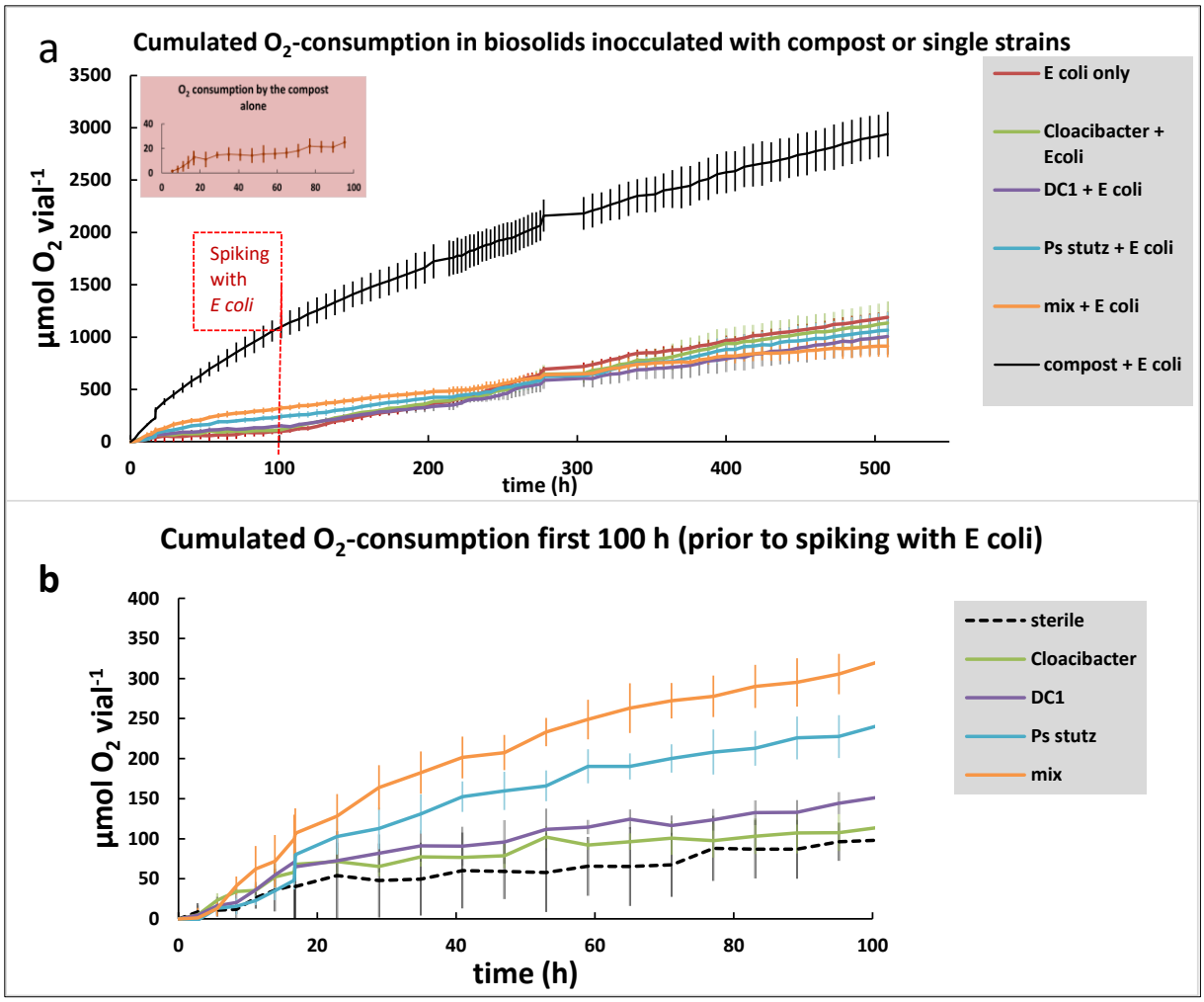


Figure 3.3.1 - Measured oxygen consumption. Panel a shows the cumulated O2 consumption in vials with 2 g biosolids throughout the entire incubation. There were three replicate vials for each treatment. *E. coli* was added to the biosolid after 100 hours, while the organisms intended to suppress *E. coli* (called suppressors hereafter) were added to the biosolids at the very beginning of the incubation. The suppressors tested were three N2O-respiring strains isolated from digestate enrichment by Jonassen et al (2020), as well as compost (0.4 g compost added to 2 g biosolid), see legend in the panel. The cumulated O2 consumption for each treatment is shown with standard deviation as vertical bars. The incubation experiment included vials with 2 g compost alone, to assess the respiration of the compost itself, i.e., without access to the organic C in the biosolids). The inserted panel shows the predicted respiration by the 0.4 g compost added to biosolids (=1/5 of that measured in the vials with compost only). This shows that the very high respiration rate in biosolids with 0.4 g compost added is sustained primarily by the consumption of organic compounds in the biosolid. Panel b shows the O2 consumption during the first 100

hours, i.e., before spiking with *E. coli*, which included a sterile control (see legend in panel "a"). The O2 consumption rate in the sterile control was significant initially but declined after 20 h.

Since abiotic O_2 consumption was significant (see Figure 3.3.1b), the net respiration of each strain was calculated by subtracting the abiotic O2 consumption rate as measured in the sterile biosolid (See Figure 3.3.1b). The resulting net O_2-consumption by the three strains, and by the mixture of the three strains (mix) are shown. The dashed line is the sum of the respiration by the three strains as measured in vials with the strains added alone. The similarity of this (sum of all) and the measured respiration by the mixed culture (mix) is remarkable, suggesting that the three strains utilize different fractions of the available organic compounds.

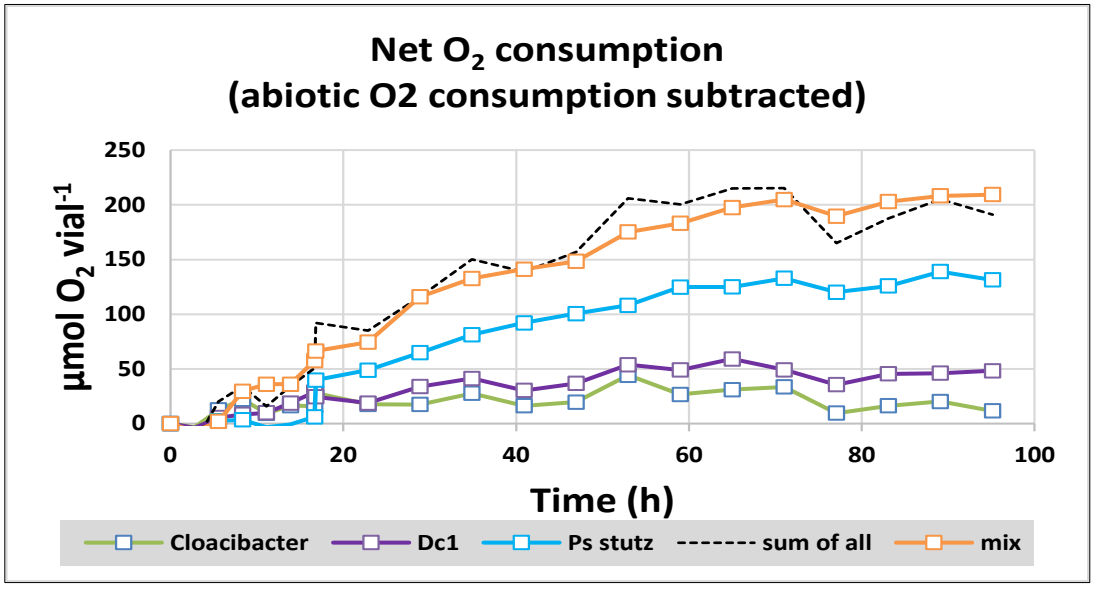


Figure 3.3.2 - Oxygen consumption by single strains during the first 100 hours.

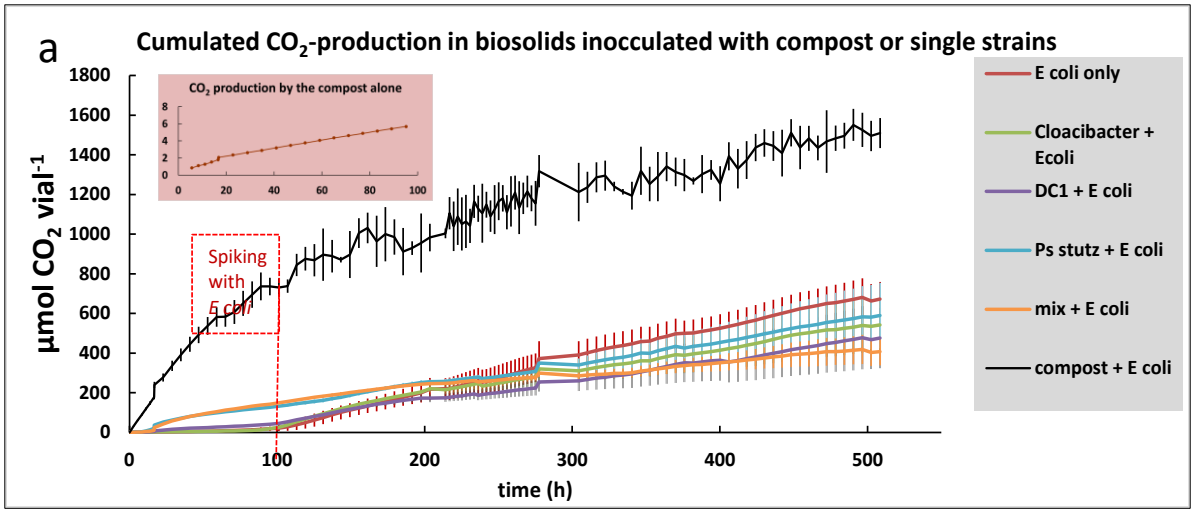


Figure 3.3.3 - Measured CO_2 production. The panel shows the cumulated CO_2 production for the same experiment as in Figure 3.3.1.

Although the single strains respired much less than the compost, a close inspection of the rates of CO_2 production during the first 200 hours of incubation (3.3.4) suggests that they do compete with *E. coli* for a common pool of substrates, and their competitive edge is proportional with their ability to respire: *Pseudomonas stutzeri* (and *P. stutzeri* together with the two other strains) had high respiration during the first 100 hours (prior to spiking with *E. coli*), and marginal respiration after spiking with *E. coli* compared to that in the biosolid which was sterile until spiking with *E. coli*. In contrast, *DC1* and *Cloacibacter* respired very little during the first 100 hours, and in these vials, the spiking with *E. coli* resulted in a peak of CO_2 production likening the control. Assuming that the peak of respiration after spiking with *E. coli* is a result of respiration by *E. coli*, its available pool of substrate was surely diminished by *P. stutzeri*.

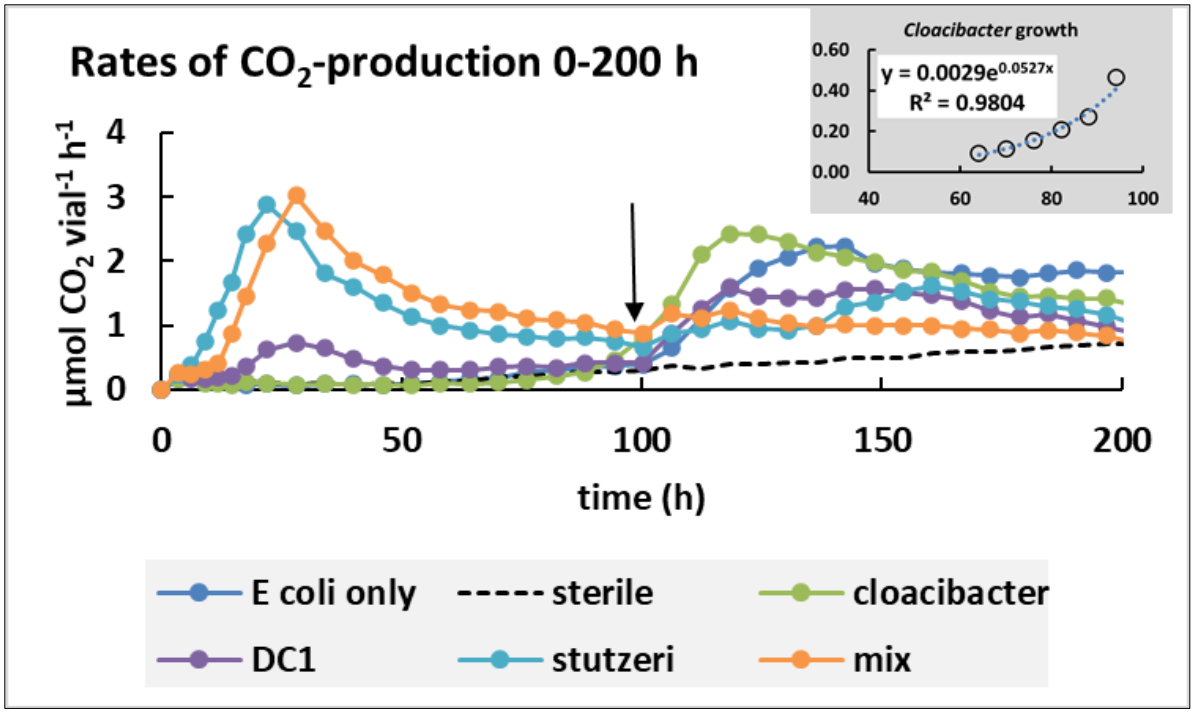


Figure 3.3.4 - Competition for substrates between single strains and *E. coli*?. The panel shows the rate of CO2 production in vials with sterile biosolids, sterile solids in which *E. coli* was added after 97h (*E. coli* only), and in vials with sterile biosolids inoculated with the three different strains (and the mixture of them) at time 0, and with *E. coli* at time = 97h. The inserted panel shows the exponential increase in CO2 production between 60-100 hours for *Cloacibacter*. This increase shows *Cloacibacters* survival and proliferation ability albeit rather small with a generation time = ln(2)/0,0527 = 13,15.

3.4 Enumeration of *E. coli* by digital PCR

The results of the gene abundance measurements are shown in Figure 3.4.1. The abundance of 16S in the sterile biosolids was generally stable around 10^9 g^{-1} in the sterile biosolids (panel a) and increased to 10^{10} g^{-1} in response to inoculation with *E. coli* after 100 days (panel b) and remained stable thereafter. The abundance of the *E. coli* gene *uidA* was negligible in biosolids before spiking with *E. coli* after 100 hours and remained negligible in the materials that were not spiked with *E. coli* (Sterile biosolids, panel b, compost, panel f). In response to the spiking with *E. coli*, the *uidA* abundance increased to 10^8 g^{-1} in the control treatment, i.e., without any other bacteria present (panel a), and declined gradually to 10^7 g^{-1} towards the end of the incubation. For the treatments in which the biosolids had been inoculated with single strains 100 hours before spiking with *E. coli* (panels c-f), the *uidA* – abundance showed very similar trajectories as that in the control, which corroborate the results of the viable counts (Figure 3.2.1): the three strains tested (*DC1*, *Cloacibacter*, and *P. stutzeri*) were essentially unable to reduce the abundance of *E. coli*, be it by interference competition or exploitation competition. The microbiota of the compost, on the other hand,

showed competence, not only in reducing the number of viable *E. coli* (Figure 3.2.1), but also by destroying its DNA (panel g), either by inducing cell lysis or by protozoal grazing.

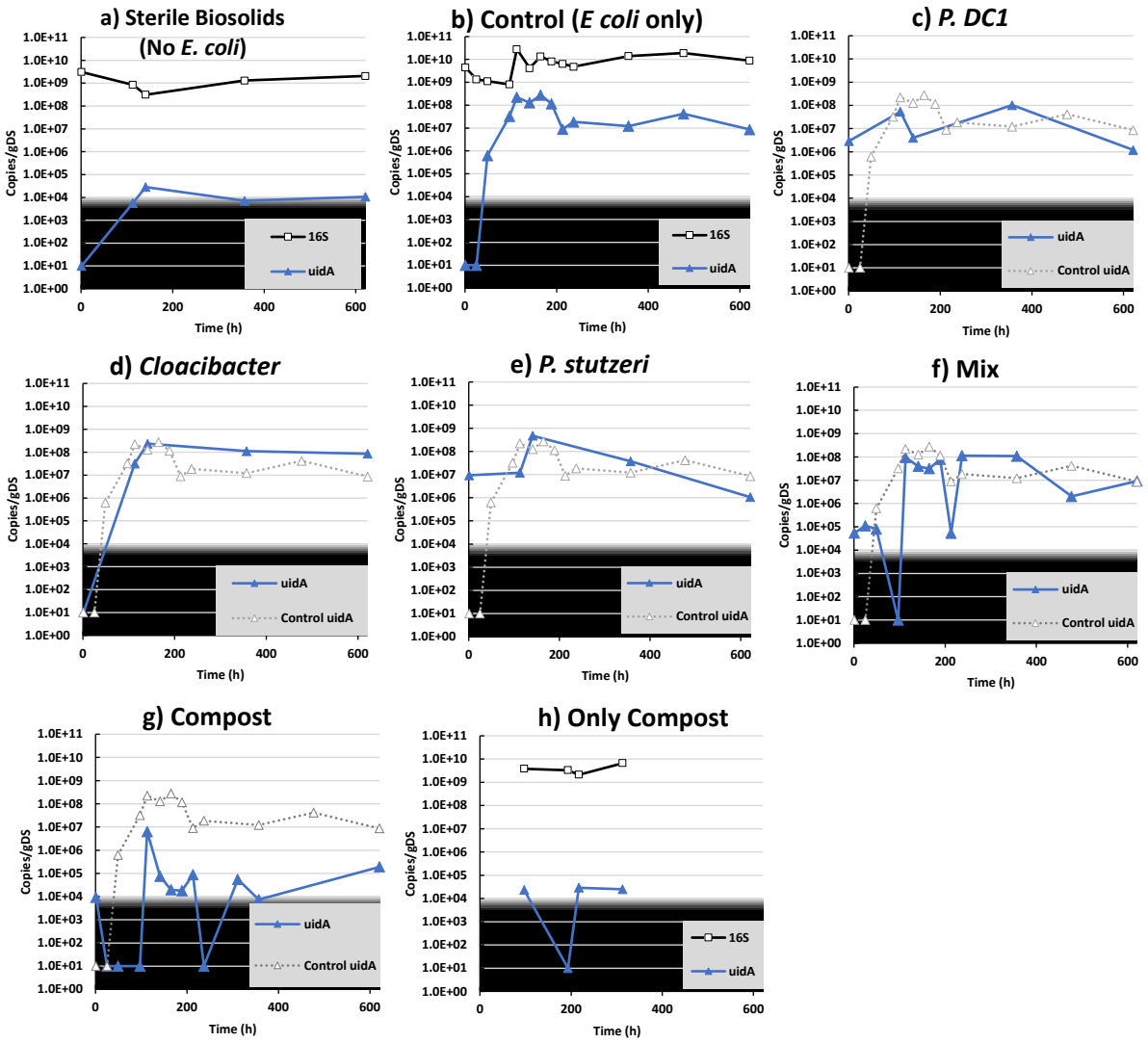


Figure 3.4.1 - Quantification of gene abundance. Panel a shows the abundance of 16SrDNA and the *E. coli* gene uidA for sterile biosolids (i.e., without any bacteria added). Panel b shows the result for sterile biosolids that were spiked with *E. coli* after 100h, but without any other bacteria present. This is the Control treatment for comparing the suppressive effects of the various bacteria, and the uidA abundance in this treatment is shown as grey triangles (dashed lines) in the panels c-g. Panels c-f show the result for biosolids inoculated at time 0 with single strains, and with *E. coli* at time 100 h. Panel g shows the result for biosolid inoculated with compost. Panel h shows the result for the compost material used to inoculate the "compost" treatment (panel g)

Stutzeri and *DC1* seem to have a minuscule effect on *E. coli* growth with results almost mirroring the control for both *uidA* and MPN, Figure 3.4.2a and e. In the *Cloacibacter* no viable counts of *E. coli* were detected after 600 hours (Figure 3.2.1), although *uidA* abundance remained stable. The slow growth of *Cloacibacter*, with generation time close to

13 hours (calculated from the carbon respiration) could be the reason for this staggered effect. The effect seems to persist in the mixture, with notably lowered viable counts after 400 hours, suggesting a correlation with the presence of *Cloacibacter* as the other single strain treatments do not demonstrate a similar impact on *E. coli* growth.

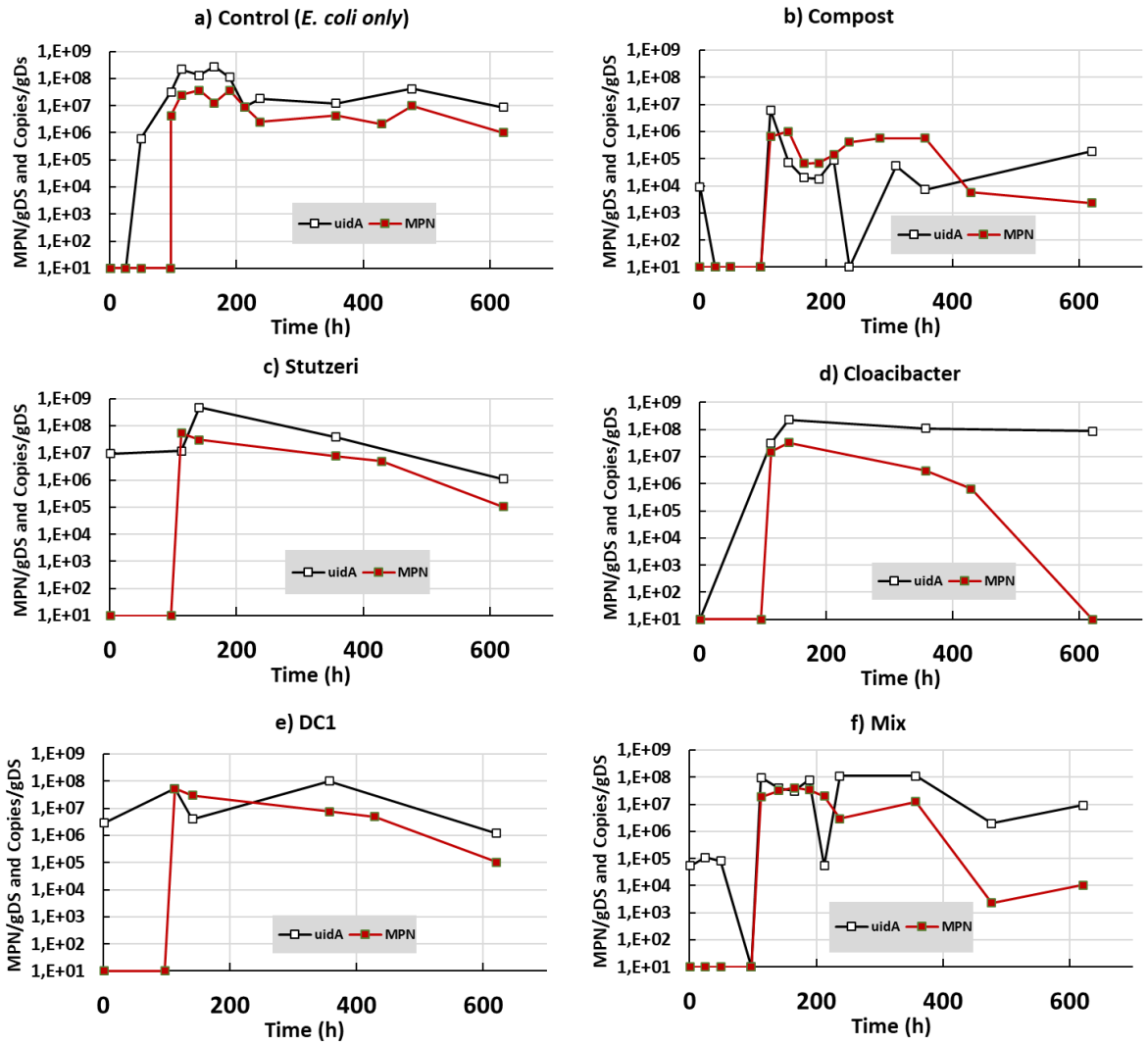


Figure 3.4.2 - Viable counts compared to uidA gene abundance, for every treatment. On the log-transformed y-axis, MPN/gDS and Copies/gDS are plotted. Panel a shows the abundance of uidA and the number of viable counts (MPN) for the biosolids with only *E. coli* added. This is the control treatment used to measure the suppressive effect of the various bacterial strains and the compost community. Panel b shows the results for the compost-enriched biosolids demonstrating a clear suppressive effect on MPN and uidA abundance for *E. coli*, with both metrics lowered by around one order of magnitude compared to the control between 100-200 hours reaching regulation limits after 400 hours. Panel c-f shows the results for the three bacterial strains and the mixture of all three.

4 Discussion

4.1 Imitated post-AD-THP treated Biosolid

The decision of using an imitated variant of the post-AD-THP treated biosolid was made based on time constraints. This limitation is apparent in many ways but mainly that THP treatment fractionates larger particles in the digestate material making the organic compounds more accessible to microbial organisms and reduction of VNBC caused by the high-pressure steam explosion in the process (Barber, 2016). However, autoclaving proved to be effective at sterilizing the material (Figure 3.2.1), containing no culturable cells throughout the incubations, and respiration rates remaining very low even when compared with single strain treatments (Figure 3.3.1, 3.3.4). From these results, it is clear that while the imitated biosolid does not replicate the physiochemical conditions of post-AD-THP treated biosolids, it can be used for comparative purposes.

4.2 Choosing model strain

The first obstacle was choosing which strain to use for the suppression of pathogens in biosolids. *E. coli* is an indicator organism encompassing the vast majority of fecal coliforms in waste products and its presence is closely correlated to a heightened risk of infection to humans, not only in and of itself but with other fecal pathotypes such as *Salmonella sp, Helminth ova,* and enteric viruses. *E. coli*s metabolic patterns, virotypes, and genealogy are also well documented strengthening its position as a model strain. The decision to use a wild *E. coli* was rooted in the well-funded suspicion that strains kept in laboratory cultures tend to become domesticated as cultivation selects for traits that secure survival in the laboratory, and against traits that ensure survival in natural/complex environments. Isolating *E. coli* from a relevant complex environment- such as one found in sewage water- would more accurately represent conditions *in vivo*.

4.3 Choice of enumeration methods and testing

The next challenge was to find valid methods for quantifying the number of viable (culturable) *E. coli* cells and the absolute number of *E. coli* cells, in the environment, which in this case, was biosolids. Luckily, *E. coli* possess a gene, *uidA,* encoding the beta-glucuronidase enzyme, for which suitable primers have been identified, which are sufficiently specific for selective amplification of *E. coli uidA* in heterogeneous samples. Thus, quantitative PCR could be used to estimate the number of intact *E. coli* in a sample, assuming

each intact cell has a single *uidA* sequence, and that the entire genome, including the *uidA* gene, is quickly degraded if the cells lyse.

The enzyme encoded by *uidA*, beta-glucuronidase is also the key to an effective quantification of the number of viable *E. coli* cells in an environment: this enzyme cleaves the non-fluorescent 4-methyl-umbelliferyl molecule to the fluorescent 4-methylumbelliferone, hence fluorescence (or lack of fluorescence) in an MPN-tube can be used to assess if it contains metabolically active *E. coli* cells or not.

These enumeration methods were tested in several ways: 1) checking the calculated MPN number from positive wells on the 96-wells microtiter plates dilution gradient, with CFU from select dilutions: 10^{-4}, 10^{-5} and 10^{-6} at varying OD levels (Figure 3.1.2a). 2) Dispersion methods' effect on MPN showed that FastPrep is superior to vortexing in addition to being non-disruptive to viable *E. coli* cells (Figure 3.2.1.1). 3) Quantification by ddPCR of *uidA* gene and 16S rRNA showing that there is clear effect on *uidA* gene abundance from treatments and a reasonably similar prokaryotic abundance across samples and finally the negligibility of *uidA* gene quantity in sterile biosolids and compost.

4.4 Choosing respiration kinetics

Using respiration measurements as a method to gauge the growth of the various microbial single strains and *E. coli* was motivated by 1) its ability to accurately assess the materials sterility pre-inoculation. 2) the method allows for a quantification of the metabolic activity of the organisms over a given period of time. Unlocking this metric allows us to investigate to which degree the pure cultures added to the sterile biosolids, what the metabolic activity of *E. coli* was when added alone to the biosolids and compare the capacity of the pure cultures utilization of organic C in the biosolids with that of the compost microbiota. Measuring both O_2 consumption and CO_2 production is important as both metrics are susceptible to abiotic influence, thereby using them in different phases of the incubation will give a clearer picture of biotic and abiotic factors. Initial O_2 consumption showed significant abiotic influence making it difficult to study the organisms' O_2 consumption. Consequently, CO_2 was used for inspection of early phase respiration kinetics. However, the long-term accumulation of CO_2 was an uncertain measure of the cumulated respiration owing to the difficulty in assessing the formation of carbonate in the material (H_2CO3) (and possibly oxidation of iron Fe_2O_3). Hence, cumulated O_2 was probably a better variable to assess the long-term respiration kinetics.

4.5 Recapitulation of aims

To refine the analyses of Svennevik et al (2020), in which some evidence that compost bacteria could suppress *E. coli* was found, we aimed to design an experiment with a higher resolution and a more accurate assessment of viable cells. Additionally, we wanted to see if we could solve two problems in one: reducing N_2O emissions by anthropogenic agriculture, and stabilizing recirculated sewage waste for safe application in agricultural fields using N_2O reductase-producing microorganisms.

Approach

To provide the compost microbiota and the N_2O reducing strains a better chance to suppress *E. coli* than in the experiments by Svennevik et al, they were given a head-start by adding them to the biosolids a total of 5 days (9 days for the compost bacteria) before spiking with *E. coli*. Evaluating their growth during their "head-start" phase, respiration (CO_2 production and O_2 consumption) was measured from the very beginning.

4.6 Isolation of *E. coli*

The isolation of *E. coli* using MUG-EC and identification by Sanger sequencing proved effective in obtaining a wild strain. Based on the sequencing results for DNA samples 1 and 2 and the emitting of clear blue fluorescence from MUG-EC-agar streak-plated colonies shown in Figure 3.1.1, there remains no doubt that the strain is in fact *E. coli*.

As can be seen in Figure 3.1.1, only one fluorescent colony was observed at dilution level 10^{-2} translating to 2.13% of total isolated colonies. This was surprising as in previously stated literature (Cheeptham & Lal, 2010) 97% of *E. coli* produced fluorescence when cultivated on MUG. The issue is concerning, as the enumeration method for *E. coli* should not underestimate the total number present in the biosolids nor drive a heavy selective force upon the sewage *E. coli*. MUG is neither conducive nor inhibitory for *E. coli* growth and contains tryptose and lactose as nutrient and carbon sources, respectively, both of which are readily available for *E. coli* to reduce, so it seems unlikely that the medium is at fault. The presence of indicator organisms is associated with high levels of turbidity and total solids suspended in the water (Liu et al., 2018) the inoculate sewage water might therefore be suspect in holding very few *E. coli* cells and that the growth of non-fluorescent colonies is attributed to non-target β-glucuronidase producing wastewater contaminants such as *Salmonella* or *Shigella*. It follows that the wastewater sampled could have a different microbiota composition than the

literature suggests, and the specific *E. coli* strain in the sampled wastewater had reduced competence relative to conventional wastewater *E. coli*.

Virulence

The possibility of the isolate being a virotype exists, and owing to the variety of *E. coli* strains showing diverse physiochemical, genetic and physiological profiles, identification can be difficult. Determining the *E. coli* virulence could be of importance as virulent factors, such as Shiga toxin production, has been shown to negatively influence survival in complex soil communities (Xing et al., 2019) and their growth patterns are different showing staggered growth at elevated temperatures (44-45^{o}C) compared to their non-virulent counterparts. Further distinguishing factors for virulence is the lack of acid and gas production from lactose fermentation in *Escherichia Coli* broths (EC broth) and for enterohaemorrhagic *E. coli* only, the absence of functional β-glucuronidase enzymes, meaning no fluorescence from axenic colonies or cultures of this serotype. (Odonkor & Ampofo, 2013). Luckily, all MPN results demonstrated clear fluorescence from positive wells and observations made during sampling of MPN-plates saw elevated sealing adhesive above positive wells, providing proof of the bacterias' ability to produce gas from the EC broth. Taking these results into account, one can assume that the isolate of *E. coli* while not conclusive for the absence of virulent factors, at least the impact of *E. coli*s state of virulence or not, would most likely be irrelevant for the purposes described in this paper.

4.7 Suppression experiments

MPN with ddPCR for evaluating total *E. coli* numbers

MPN with MUG substrates proved to be a reasonable method to quantify the number of *E. coli* (Figure 3.1.2). The microbiota of the compost prior to invasion by *E. coli*, had negligible abundance of viable *E. coli* as quantified with this method (Figure 3.2.1).

Likewise, the quantification of *E. coli* genes, using quantitative PCR with specific primers for *E. coli uidA,* confirmed that the abundance of this gene was insignificant for the compost environment and the sterile biosolid (Figure 3.4.1). Thus, the fate of the *E. coli* cells introduced at 100 hours could be effectively trailed using this method.

Suppressive effect on *E. coli*

The viable counts (MPN) demonstrated that 1) *E. coli* was able to grow and survive reasonably well in sterilized biosolids (Figure 3.2.1) 2) that the presence of a compost microbiota severely suppressed *E. coli*, but the isolated strains demonstrated minuscule suppressive effect, except for some very low MPN values towards the end of the incubation

both for the *Cloacibacter* treated biosolids and biosolids with a mixture of the three strains (Figure 3.2.1). Although interesting, the result cannot be taken as a proof, but rather as a hypothesis-generating observation that warrants further investigation of the suppressive effects of *Cloacibacter*.

The respiration measurements show convincingly that the compost microbiota was able to utilize a much larger share of the organic carbon than the pure strains (Figure 3.3.1), probably due to a broad repertoire of enzymes degrading polymers in the compost, of which the pure cultures had a limited set of (Jonassen et al. paper in preparation) Hence, the single strains could only degrade a small fraction of the organic C in the biosolids thus leaving nutrient niches open. This is important from a practical point of view: the biosolids inoculated with single strains (as those tested here) are open for invasion by microbes with broader sets of catalytic enzymes, which will inevitably happen if stored for prolonged periods. Anaerobically digested sludge contains quantities of volatile sulfur compounds, byproducts of methionine and cysteine degradation which *E. coli* can catalyze or possibly other microbes, and if such nutrient niches cannot be sufficiently occupied by the suppressor organisms the biosolids will not be stabilized (Chen et al., 2011). Although such regrowth may not include pathogens, the invasion itself is a problem because it may produce unwanted odor and increased release of N_2O (Chen et al., 2011). In contrast, biosolids inoculated with a complex microbiota such as compost are likely to resist invasive growth during storage because most of the enzymatically available organic C is already mineralized.

The respiration data suggest the nutrient pool utilized by *E. coli* overlaps (but not entirely) with the substrate pool utilized by the isolates as evidenced by the respiration kinetics before and after spiking with *E. coli* (Figure 3.3.4): while spiking sterile biosolids with *E. coli* resulted in a transient peak of respiration ("*E. col*i only", Figure 3.3.4), no such respiration peak was observed after spiking the biosolids in which *P. stutzeri* had been actively respiring a lot of organic C during the preceding 100 h. Further evidence for this is that *DC1* and *Cloacibacter* grew poorly and consumed a marginal fraction of the nutrient pool, and here we see that the spiking with *E. coli* resulted in a flush of respiration, comparable to that for the treatment that was sterile until spiking with *E. coli*

Given that the organisms and *E. coli* apparently compete for the same substrate, one would expect a suppression of *E. coli* by the strains with the highest initial respiration rate, but this was clearly not the case. A plausible explanation could be that the biosolids were spiked with so many *E. coli* cells that growth by *E. coli* in the biosolid (after spiking) play a minor role. Nevertheless, the available C for *E. coli* during the 500 hours should be lower in

the treatments with *P. stutzeri* than in the sterile control (with *E. coli* alone). This can be taken to suggest that the severity of starvation plays a minor role for the survival of *E. coli* in the biosolids.

The measured abundance of *uidA* genes was approximately 10 times higher than viable counts (MPN), (Figure 3.4.2), with two remarkable exceptions: 1) In compost, the measured *uidA* abundance equaled (or was even somewhat lower than) the viable counts, and both declined sharply during the first 100 hours after spiking 2) In *Cloacibacter*, the measured *uidA* abundance remained stable throughout, while viable counts declined to very low values. The very low respiration rate for *Cloacibacter* in the early phase could be an adaptation to the environment or the time needed to start growing. Most likely it is a mix of the two because the growth phase is exponential which decreases as the available CO_2 is consumed seen as a fall in CO_2 rates rather than staying constant. The generation time of *Cloacibacter* is quite small, only duplicating every 13 hours (Figure 3.3.4) and the exponential phase commences late (60 hours) leaving it with only 3 duplication events before invasion by *E. coli*. Invasion impact is seen almost immediately with a marked increase in both O_2 consumption (3.3.1) and CO_2 production rate (Figure 3.3.3) for subsequent measurements (100-200). Following this pattern and comparing it to the DC1 reveals a striking similarity.

These results suggest that while *Cloacibacter* effectively "killed" *E. coli* without causing cell lysis (leaving the genome more or less intact), the microbiota of the compost accomplished both. The lysis of *E. coli* cells (causing degradation of its genome, hence *uidA*) by the compost microbiota is probably the result of lysis within the biosolids as well as predation by protozoa introduced with the compost (as we do not see similar reductions in *uidA* for the other treatments).

5 Conclusion

In establishing a most probable number enumeration method for use on the isolated *E. coli*, proved relatively successful showing that there was a relatively linear relationship between MPN and CFU (0,87:1).

In evaluating the homogenization methods' effect on MPN, FastPrep shows a higher extraction of viable cells, as quantified by MPN from calcite soil, than vortexing.

The main purpose of this study was to assess the suppressive effects of complex compost microbiota in *E. coli* invaded sterile biosolids as demonstrated by Svennevik et al. (2020) and expand upon the experimental design by using single strains of select N_2O reducing microorganisms and a mixture of the three; *Pseudomonas stutzeri, Pseudomonas DC1* and *Cloacibacter*, as suppressors of the fecal indicator organism *E. coli*. As expected the results convincingly demonstrate the viability of compost bacteria in resisting *E. coli* invasion based on viable counts (MPN) total *uidA* gene abundance and metabolic activity, showing several orders of magnitude reduction in both viable counts and *uidA* as well as the ability to metabolize the available organic carbon in the biosolids leaving few substrates for the invading *E. coli* cells to degrade. This ability was not evidenced for the N_2O reductant organisms nor for the consortium, showing a limited impact on either of the metrics used for quantifying the survival of *E. coli* albeit *Cloacibacter* showed prospect as a suppressor with endpoint measurements for the series possessing no detectable viable counts of *E. coli* after 30 days, a result only achieved by this particular strain.

Despite the alterations and increased resolution in the experimental design from those presented in Svennevik et al. (2020), the compost-enriched biosolid only reached Norwegian regulation limit (2500MPN/gDS) after 30 days. As for the N_2O reductants, none of the strains successfully suppressed *E. coli*, and only one strain: *Cloacibacter* showed any significant impact on *E. coli* growth.

Limitations and future work

The final experimental results were obtained shortly before completion of this paper, leading to a paucity of supplementary metrics such as chemical oxygen demand in the biosolids (COD), which is of importance in the evaluation of available substrate for microbial growth and would reveal more of the metabolic patterns and possibly a link to substrate-depletion by the compost microbial community as the major factor inhibiting *E. coli* growth.

Duplicate frozen samples were taken during the incubation but only one replicate was used, ddPCR proved problematic with some samples showing a large deviation from other series in 16s rRNA especially *P. DC1*, the unused frozen samples could therefore be used to rectify the deviations.

Owing to a lack of time and stock-biosolids, the material used was an imitation of the post-AD-THP treated biosolids used in Svenneviks paper. There was a general lack of the material, which led to a lower resolution of the falcon tubes for the single strains, having only 5 sampling points. The issue of biosolid material shortage was compounded by the discovery of *E. coli* in the PBS after 357 hours, which was used to mix the biosolids during destructive sampling of falcon tube samples causing an overestimation of viable *E. coli* by an amount equal to the contaminated sterile control, which was luckily only around 10^6 representing around 1% of the total *E. coli* quantified in the various falcon tube series in the time between last negative sterile control (day 124) and first positive sterile control (day 357).

Cloacibacter is of interest for two reasons its potential as an N_2O sink carrying the gene for nitrous-oxide reductase and results indicating an effect on *E. coli* viability. This warrants further research as a possible suppressor of *E. coli* in biosolids.

References

Acharya, K. R., Dhand, N. K., Whittington, R. J. & Plain, K. M. (2017). PCR Inhibition of a Quantitative PCR for Detection of Mycobacterium avium Subspecies Paratuberculosis DNA in Feces: Diagnostic Implications and Potential Solutions. *Front Microbiol*, 8: 115. doi: 10.3389/fmicb.2017.00115.

Ardern, E. & Lockett, W. T. (1914). Experiments on the oxidation of sewage without the aid of filters. *Journal of the Society of Chemical Industry*, 33 (10): 523-539. doi: https://doi.org/10.1002/jctb.5000331005.

Bajón Fernández, Y., Soares, A., Villa, R., Vale, P. & Cartmell, E. (2014). Carbon capture and biogas enhancement by carbon dioxide enrichment of anaerobic digesters treating sewage sludge or food waste. *Bioresource Technology*, 159: 1-7. doi: https://doi.org/10.1016/j.biortech.2014.02.010.

Bakken, L., Nadeem, S., Frostegard, A., Gaby, J. & Dörsch, P. (2020). Contingent Effects of Liming on N 2 O-Emissions Driven by Autotrophic Nitrification. *Frontiers in Environmental Science*, 8. doi: 10.3389/fenvs.2020.598513.

Barber, W. P. F. (2016). Thermal hydrolysis for sewage treatment: A critical review. *Water Research*, 104: 53-71. doi: https://doi.org/10.1016/j.watres.2016.07.069.

Bartram, J. (1996). *Water quality monitoring: a practical guide to the design and implementation of freshwater quality studies and monitoring programmes*.

Ben-David, A. & Davidson, C. E. (2014). Estimation method for serial dilution experiments. *Journal of Microbiological Methods*, 107: 214-221. doi: 10.1016/j.mimet.2014.08.023.

Bleken, M. & Bakken, L. (1997). The nitrogen cost of food production: Norwegian society. *Ambio*, 26.

Brandt, M. J., Johnson, K. M., Elphinston, A. J. & Ratnayaka, D. D. (2017). Chapter 13 - Energy Use, Sustainability and Waste Treatment. In Brandt, M. J., Johnson, K. M., Elphinston, A. J. & Ratnayaka, D. D. (eds) *Twort's Water Supply (Seventh Edition)*, pp. 553-580. Boston: Butterworth-Heinemann.

Cheeptham, N. & Lal, A. (2010). *Use of EC-MUG Media to Confirm Escherichia coli Contamination in Water Samples Protocol*.

Chen, Y.-C., Higgins, M. J., Beightol, S. M., Murthy, S. N. & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research*, 45 (8): 2616-2626. doi: https://doi.org/10.1016/j.watres.2011.02.014.

Collivignarelli, M., Abbà, A., Frattarola, A., Carnevale Miino, M., Padovani, S., Katsoyiannis, I. & Torretta, V. (2019). Legislation for the Reuse of Biosolids on Agricultural Land in Europe: Overview. *Sustainability*, 11: 6015. doi: 10.3390/su11216015.

Cremers, C. M., Knoefler, D., Vitvitsky, V., Banerjee, R. & Jakob, U. (2014). Bile salts act as effective protein-unfolding agents and instigators of disulfide stress in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (16): E1610-E1619. doi: 10.1073/pnas.1401941111.

Eyler, E. (2013). Pouring Agar Plates and Streaking or Spreading to Isolate Individual Colonies. *Laboratory Methods in Enzymology: Cell, Lipid and Carbohydrate*, 533: 3-14.

Fane, S., Nocker, A., Vale, P., Casado, M. R., Cartmell, E., Harris, J., Fernández, Y. B. & Tyrrel, S. (2021). Characterisation and control of the biosolids storage environment: Implications for E. coli dynamics. *Science of The Total Environment*, 752: 141705. doi: https://doi.org/10.1016/j.scitotenv.2020.141705.

Feng, P. C. & Hartman, P. A. (1982). Fluorogenic assays for immediate confirmation of Escherichia coli. *Applied and environmental microbiology*, 43 (6): 1320-1329. doi: 10.1128/AEM.43.6.1320-1329.1982.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and environmental microbiology*, 74 (8): 2461-2470. doi: 10.1128/AEM.02272-07.

Garthright, W. E. & Blodgett, R. J. (2003). FDA's preferred MPN methods for standard, large or unusual tests, with a spreadsheet. *Food Microbiology*, 20 (4): 439-445.

Grimsby, L. K., Fjørtoft, K. & Aune, J. B. (2013). Nitrogen mineralization and energy from anaerobic digestion of jatropha press cake. *Energy for Sustainable Development*, 17 (1): 35-39. doi: https://doi.org/10.1016/j.esd.2012.10.009.

Hachich, E., Di, M., Ana, B., Christ, A., Lamparelli, C., Lamparelli, S., Ramos, M., Sato, M. & Sato. (2012). Comparison of thermotolerant coliforms and Escherichia coli densities in freshwater bodies. *Brazilian Journal of Microbiology*, 43: 675. doi: 10.1590/S1517-83822012000200032.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Hendrickson, D., Farrel, J. & Schafer, P. (2007). Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Research*, 41 (3): 665-673. doi: https://doi.org/10.1016/j.watres.2006.09.017.

Hussong, D., Burge, W. D. & Enkiri, N. K. (1985). Occurrence, growth, and suppression of salmonellae in composted sewage sludge. *Applied and environmental microbiology*, 50 (4): 887-893. doi: 10.1128/AEM.50.4.887-893.1985.

Iranpour, R., Cox, H., Kearney, R., Clark, J., Pincince, A. & Daigger, G. (2004). Regulations for Biosolids Land Application in U.S. and European Union. *J Res Sci Technol*, 1.

Jonassen, K. R., Hagen, L. H., Vick, S., Arntzen, M., Eijsink, V. G. H., Frostegard, A., Lycus, P., Molstad, L. & Bakken, L. R. (2020). Bacteria in biogas digestates for reduced climate forcing. *bioRxiv*: 2020.11.16.384990. doi: 10.1101/2020.11.16.384990.

Lalucat, J., Bennasar, A., Bosch, R., García-Valdés, E. & Palleroni, N. J. (2006). Biology of Pseudomonas stutzeri. *Microbiology and molecular biology reviews : MMBR*, 70 (2): 510-547. doi: 10.1128/MMBR.00047-05.

Lindahl, V. & Bakken, L. R. (1995). Evaluation of methods for extraction of bacteria from soil. *FEMS Microbiology Ecology*, 16 (2): 135-142. doi: https://doi.org/10.1111/j.1574-6941.1995.tb00277.x.

Liu, L., Hall, G. & Champagne, P. (2018). Disinfection processes and mechanisms in wastewater stabilization ponds: a review. *Environmental Reviews*, 26 (4): 417-429. doi: 10.1139/er-2018-0006.

Merritt, M. & Donaldson, J. (2009). Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *Journal of medical microbiology*, 58: 1533-41. doi: 10.1099/jmm.0.014092-0.

Miranda, N. D., Tuomisto, H. L. & McCulloch, M. D. (2015). Meta-analysis of greenhouse gas emissions from anaerobic digestion processes in dairy farms. *Environ Sci Technol*, 49 (8): 5211-9. doi: 10.1021/acs.est.5b00018.

Miyahara, M., Kim, S.-W., Fushinobu, S., Takaki, K., Yamada, T., Watanabe, A., Miyauchi, K., Endo, G., Wakagi, T. & Shoun, H. (2010). Potential of aerobic denitrification by Pseudomonas stutzeri TR2 to reduce nitrous oxide emissions from wastewater treatment plants. *Applied and environmental microbiology*, 76 (14): 4619-4625. doi: 10.1128/AEM.01983-09.

Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A. & Rebollo, J. E. (2015). Improved detection of Escherichia coli and coliform bacteria by multiplex PCR. *BMC biotechnology*, 15: 48-48. doi: 10.1186/s12896-015-0168-2.

Molstad, L., Dorsch, P. & Bakken, L. R. (2007). Robotized incubation system for monitoring gases (O2, NO, N2O N2) in denitrifying cultures. *J Microbiol Methods*, 71 (3): 202-11. doi: 10.1016/j.mimet.2007.08.011.

Molstad, L., Dörsch, P. & Bakken, L. (2016). *Improved robotized incubation system for gas kinetics in batch cultures*.

Odonkor, S. T. & Ampofo, J. K. (2013). Escherichia coli as An Indicator of Bacteriological Quality of Water: An Overview. *Microbiology Research*, 4 (1). doi: 10.4081/mr.2013.e2.

Quan, P.-L., Sauzade, M. & Brouzes, E. (2018). dPCR: A Technology Review. *Sensors (Basel, Switzerland)*, 18 (4): 1271. doi: 10.3390/s18041271.

Rossen, L., Nørskov, P., Holmstrøm, K. & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int J Food Microbiol*, 17 (1): 37-45. doi: 10.1016/0168-1605(92)90017-w.

Sapkaite, I., Barrado, E., Fdz-Polanco, F. & Pérez-Elvira, S. I. (2017). Optimization of a thermal hydrolysis process for sludge pre-treatment. *Journal of Environmental Management*, 192: 25-30. doi: https://doi.org/10.1016/j.jenvman.2017.01.043.

Sidhu, J. & Toze, S. (2008). Human pathogens and their indicators in biosolids: A literature review. *Environment international*, 35: 187-201. doi: 10.1016/j.envint.2008.07.006.

Smil, V. (2002). Nitrogen and food production: proteins for human diets. *Ambio*, 31 (2): 126-31. doi: 10.1579/0044-7447-31.2.126.

Svennevik, O. K., Solheim, O. E., Beck, G., Sørland, G. H., Jonassen, K. R., Rus, E., Westereng, B., Horn, S. J., Higgins, M. J. & Nilsen, P. J. (2019). Effects of post anaerobic digestion thermal hydrolysis on dewaterability and moisture distribution in digestates. *Water Science and Technology*, 80 (7): 1338-1346. doi: 10.2166/wst.2019.379.

Svennevik, O. K., Jonassen, K. R., Svensson, K., Hagen, L. H., Westereng, B., Solheim, O. E., Nilsen, P. J., Horn, S. J. & Bakken, L. (2020). Protecting Thermally Hydrolyzed Biosolids from Pathogenic Bacterial Growth by Addition of Compost. *Waste and Biomass Valorization*. doi: 10.1007/s12649-020-01300-1.

Svensson, K., Kjørlaug, O., Higgins, M. J., Linjordet, R. & Horn, S. J. (2018). Post-anaerobic digestion thermal hydrolysis of sewage sludge and food waste: Effect on methane yields, dewaterability and solids reduction. *Water Research*, 132: 158-166. doi: 10.1016/j.watres.2018.01.008.

Törnwall, E., Pettersson, H., Thorin, E. & Schwede, S. (2017). Post-treatment of biogas digestate – An evaluation of ammonium recovery, energy use and sanitation. *Energy Procedia*, 142: 957-963. doi: https://doi.org/10.1016/j.egypro.2017.12.153.

USEPA. (2000). Biosolids Technology Fact Sheet - Land Application of Biosolids *United States Environmental Protection Agency, Washington DC*: 9.

USEPA. (2003). Control of Pathogens and Vector Attraction in Sewage Sludge. *Environmental Regulations and Technology* 186.

USEPA. (2018). *PART 503 - Standards for the use or disposal of sewage sludge*. https://www.govinfo.gov/content/pkg/CFR-2018-title40-vol32/xml/CFR-2018-title40-vol32-part503.xml#seqnum503.32.

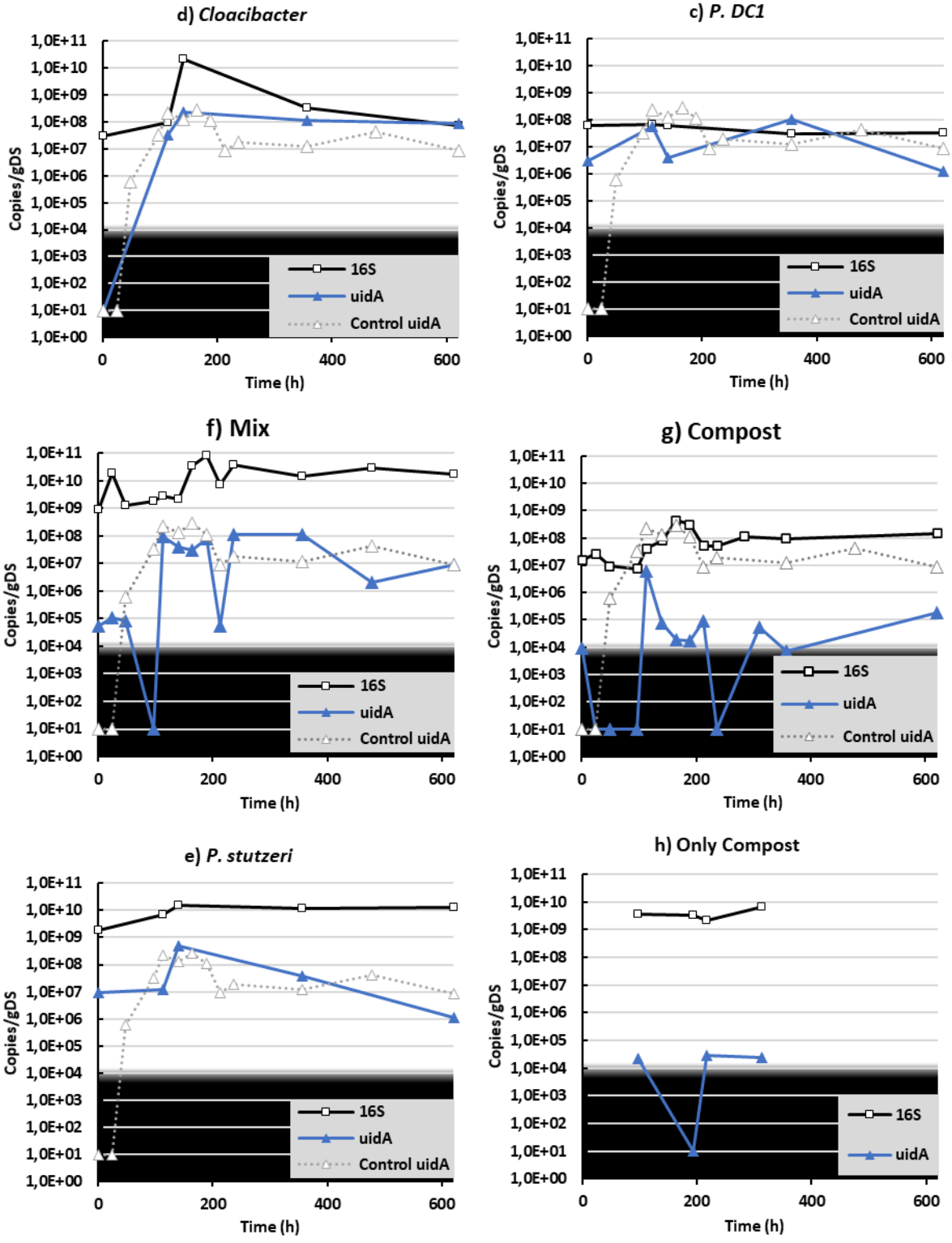
Ward, A., Stensel, H. D., Ferguson, J. F., Ma, G. & Hummel, S. (1999). Preventing Growth of Pathogens in Pasteurized Digester Solids. *Water Environment Research*, 71 (2): 176-182. doi: https://doi.org/10.2175/106143098X121932.

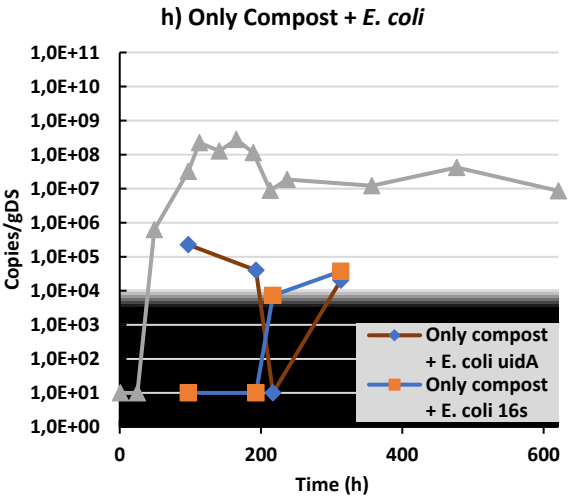
Xing, J., Wang, H., Brookes, P. C., Salles, J. F. & Xu, J. (2019). Soil pH and microbial diversity constrain the survival of E. coli in soil. *Soil Biology and Biochemistry*, 128: 139-149. doi: https://doi.org/10.1016/j.soilbio.2018.10.013.

Yu, Y., Lee, C., Kim, J. & Hwang, S. (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*, 89 (6): 670-9. doi: 10.1002/bit.20347.

Appendix

A. ddPCR Results 16s and uidA for all treatments

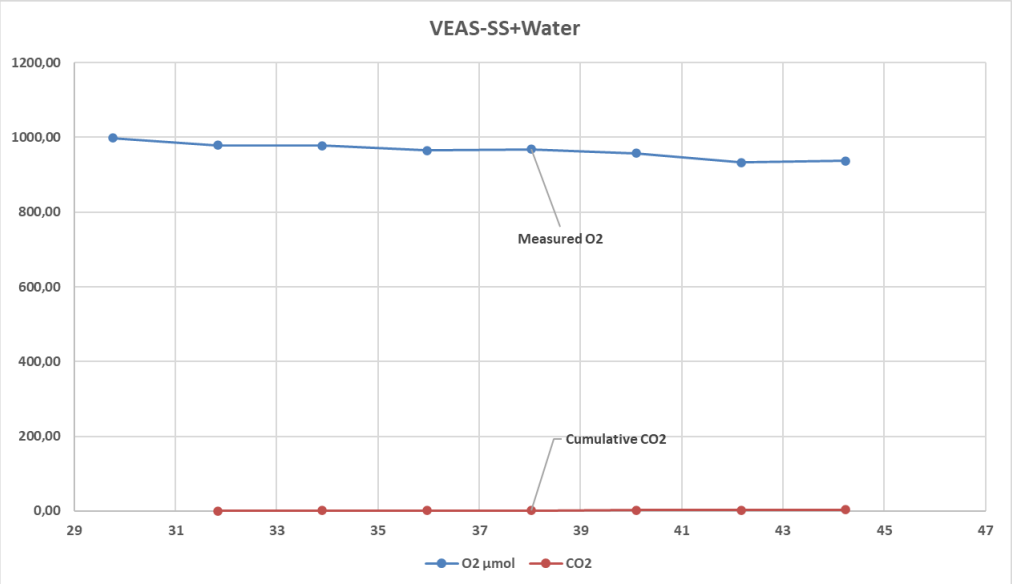


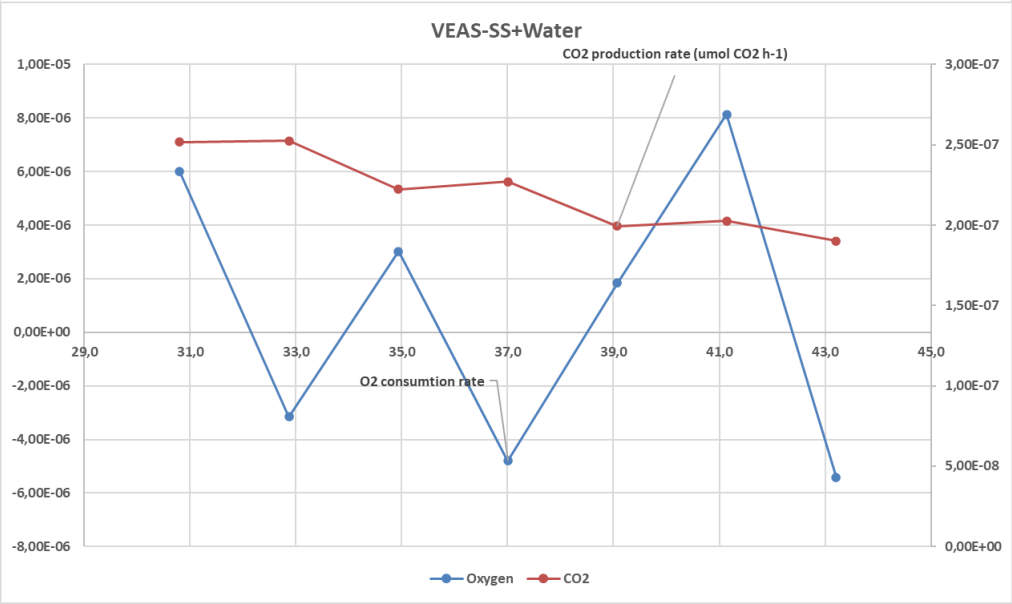


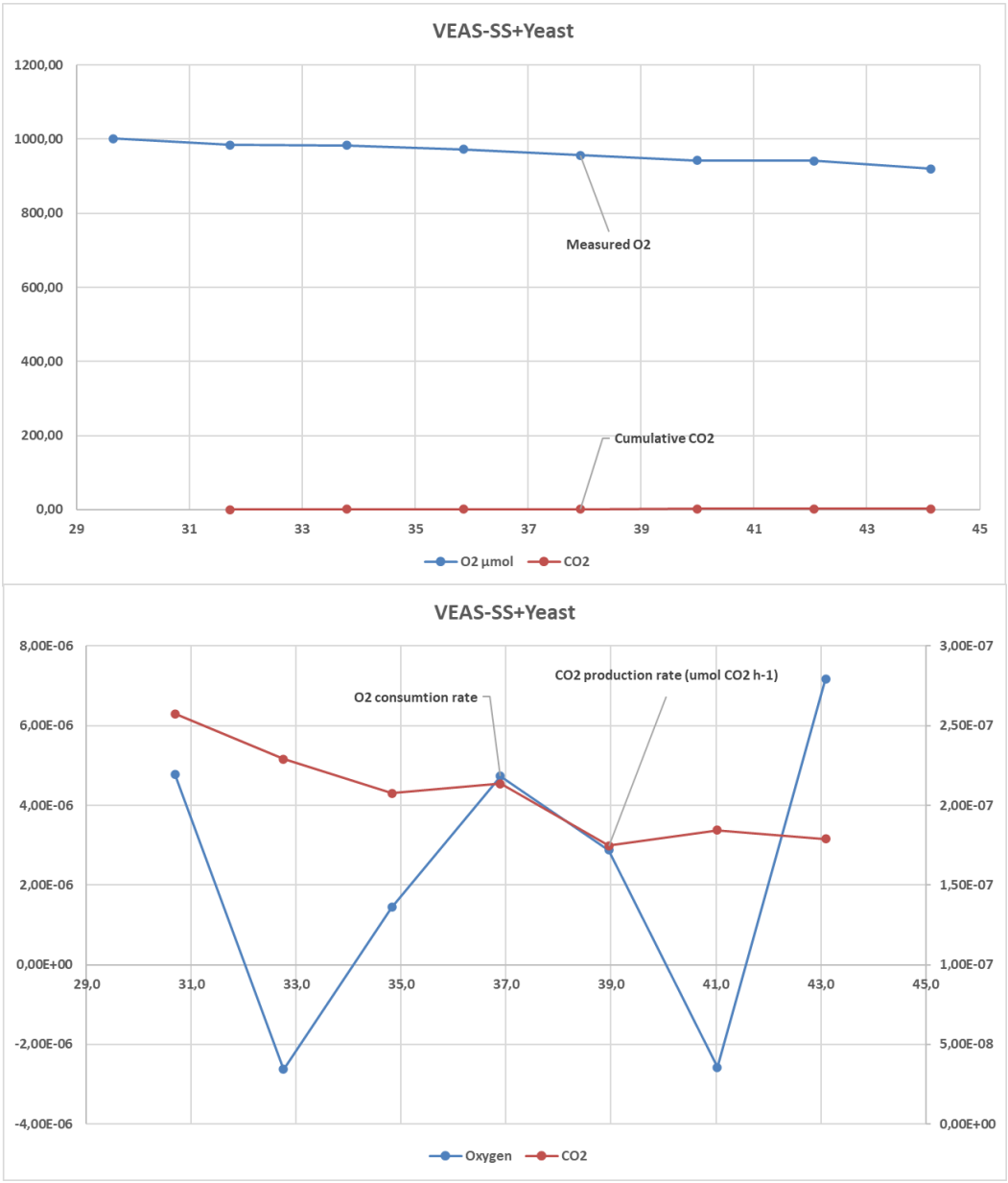
B. Link to kincalc spreadsheet

https://www.researchgate.net/publication/348383243_spreadsheet_for_gas_kinetics_in_batch_cultures_KINCALC

C. Kincalc Sterility test of Biosolids






VEAS-SS+Yeast
1200,00
1000,00
800,00
600,00
400,00
200,00
0,00
29
31
33
35
37
39
41
43
45
Measured O2
Cumulative CO2
O2 µmol
CO2
VEAS-SS+Yeast
8,00E-06
6,00E-06
4,00E-06
2,00E-06
0,00E+00
-2,00E-06
-4,00E-06
3,00E-07
2,50E-07
2,00E-07
1,50E-07
1,00E-07
5,00E-08
0,00E+00
29,0
31,0
33,0
35,0
37,0
39,0
41,0
43,0
45,0
O2 consumtion rate
CO2 production rate (umol CO2 h-1)
Oxygen
CO2

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway