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# THE EFFECT OF ACUTE AMOXICILLIN EXPOSURE ON ANAEROBIC MICROBIAL COMMUNITIES - ACTIVITY, ECOLOGY AND RESISTOME

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SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
*DOCTOR OF PHILOSOPHY*

SCHOOL OF ENGINEERING
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# ABSTRACT

Antimicrobial resistant (AMR) genes in water treatment technologies are an issue of global concern. However, the impact of antibiotics on the anaerobic microbial communities within the decentralised wastewater treatment systems such as septic tanks is not well researched. This thesis aims to quantify the effects of an acute shock exposure of amoxicillin on the methanogenic activity, treatment efficacy, and structure of the microbial community in anaerobic granules. The work was conducted using an adapted specific methanogenic assay (SMA) to measure the rate of methane production with additional analytical methods including soluble chemical oxygen demand (sCOD) quantification, next generation sequencing (NGS), and AMR detection using Smart-Chip Real time PCR (quantitative PCR, qPCR) array. This work found that the presence of amoxicillin within the anaerobic microbial communities had measurable effects on the rate of methane production, community assembly, and the concentration of AMR genes within the effluent. However, the influence of amoxicillin on sCOD removal was limited. An additional study was conducted to determine the abiotic decay of amoxicillin within anaerobic culture medium. This study found that the oxygen scavenger L-cysteine has a strong catalytic effect on the decay of amoxicillin. As such, studies assessing the sensitivity of amoxicillin in lab grown cultures should take into account the abiotic decay within the medium used. Overall, the research demonstrates that the impact of pharmaceuticals, such as amoxicillin within decentralised waste treatment systems like septic tanks should be more widely investigated. Although septic tanks are rarely monitored, this work demonstrates that they have the potential to be point sources for the spread of AMR. Furthermore, changes to the microbial community caused by shock exposure to amoxicillin and other pharmaceuticals could lead to septic tank failure and discharge of untreated sewage into the water environment.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-binding cassette |
| AD | Anaerobic digestion |
| AMG | Antimicrobial genes |
| AMR | Antimicrobial resistance |
| ANOVA | Analysis of variance |
| ARA | Average relative abundance |
| ARG | Antimicrobial resistance gene |
| ASV | Amplicon sequence variant |
| ATP | Adenosine triphosphate |
| $CH_4$ | Methane |
| $CO_2$ | Carbon dioxide |
| COD | Carbon Oxygen Demand |
| CT | Threshold cycle |
| DNA | Deoxyribonucleic acid |
| g | Gram |
| GC-FID | Gas chromatograph flame ionising detector |
| GMA | General methanogenic assay |
| gVSS | Grams volatile solids |
| $H_2$ | Hydrogen gas |
| $H_2S$ | Hydrogen disulphide |
| HCl | Hydrogen chloride |
| HPLC | High-performance liquid chromatography |
| KOH | Potassium hydroxide |
| L | Litre |
| LC-UV | Liquid chromatography ultraviolet |
| M | Moles/litre |
| MATE | Multidrug and toxic compound extrusion |
| MBL | Metallo beta-lactamase |
| MDR | Multi drug resistant |
| MFS | Major facilitator superfamily |
| MGE | Mobile genetic elements |
| ml | Millilitre |
| NAG | N-acetyl glucosamine |
| NAM | N-acetyl muramic acid |
| NGS | Next generation sequencing |
| $O_2$ | Oxygen gas |

| | |
|---|---|
| PBP | Penicillin binding protein |
| PCR | Polymerase chain reaction |
| psi | Pound per square inch |
| RND | Resistance-nodulation-division protein |
| rRNA | Ribosomal ribonucleic acid |
| sCOD | Soluble Chemical oxygen demand |
| SMA | Specific methanogenic assay |
| SMR | Small multidrug resistance |
| tCOD | Total chemical oxygen detection |
| UNICEF | United nations children's fund |
| UV Detector | Ultraviolet detector |
| VFA | Volatile fatty acid |
| WHO | World health organisation |
| WWT | Wastewater treatment |
| WWTP | Wastewater treatment plant |

# Chapter 1
# **Introduction**

Antimicrobial resistant genes in the water environment is an issue of global pressing concern (Torres *et al.*, 2021). Whilst water infrastructure is recognised as key to limiting the spread of AMR, it is increasingly recognised that wastewater treatment facilities can act as point sources for the proliferation and spread of resistant microbes (Kümmerer, 2009a; Nguyen *et al.*, 2021). However, much of the world is reliant on decentralised water treatment systems such as the septic tanks (Zhou *et al.*, 2014; Harada and Strande, 2016). Unlike centralised water treatment, biological treatment within decentralised systems often utilises anaerobic microbial communities to treat wastewater (Diaz-Valbuena *et al.*, 2011). Therefore, to understand the influence of antibiotics on decentralised water treatment and septic systems, it is important to understand the effect that antibiotics have on anaerobic cultures.

The beta-lactam antibiotic amoxicillin is one of the world's most widely prescribed antibiotic used to treat a wide variety of conditions including pneumonia, dental abscesses, and stomach ulcers (Public Health England, 2017; Klein *et al.*, 2019). However, whilst there has been some research into other antibiotics on anaerobic microbial communities (Massé *et al.*, 2000; Cetecioglu *et al.*, 2015; Aydin, 2016), there is very little research into the effects of amoxicillin. Additionally, whilst residual antibiotics entering a centralised wastewater treatment plant (WWTP) may be diluted by all the other influent streams within the catchment, the narrow catchment into a septic tank means that any contamination would enter in a shock load higher than would be seen in a WWTP.

24 This thesis aims to explore the effects of an acute shock exposure on amoxicillin on the methanogenic
25 activity, treatment efficacy, and microbial structure of anaerobic granules. The research was
26 conducted in three phases which explored (1) (Chapter 6) inhibition of methanogenic activity due to
27 acute amoxicillin exposure (2) (Chapter 7) the development of antimicrobial resistance and changes
28 in ecology due during acute exposure to amoxicillin; and (3) (Chapter 8) abiotic amoxicillin decay
29 in anaerobic culture medium.

30

31 **Thesis structure:**

32 **Chapter 3 – Methods and Materials**

33 This chapter contains methods used within the experimental chapters within this thesis as well as
34 method development for LC-UV and DNA extraction methods. As anaerobic culturing was used on
35 multiple experiments within this dissertation, to minimise repetition, culture conditions are
36 summarised in this chapter.

37 **Chapter 4 - Method Optimisation and Baseline Study - Specific Methanogenic Activity Assay**

38 A specific methanogenic assay (SMA) was conducted with the aim of establishing baseline activity
39 in terms of maximum rate of methane production, window of maximum activity, and time required
40 to exhaust the substrates provided. Additionally, the work aimed to optimise sample preparation
41 methods. The findings of this chapter were used to inform experimental design subsequently.

42 **Chapter 5 - Method Development for Repeat Batch Feeding of Anaerobic Granules**

43 This study used a repeat batch assay to assess whether anaerobic granules fed on glucose and a
44 complex broth could maintain activity over a two-week period without addition of trace nutrients.
45 An SMA was conducted at the beginning, middle and end of the study to measure changes in activity
46 with exposure to different substrate types. The work aimed to establish the health of the community
47 through measuring variation in methane volume over time, the rate of methane production, and
48 changes in the biomass in terms of grams of volatile suspended solids (gVSS) through time.

49 **Chapter 6 – Changes in Methanogenic Activity in Response to Acute Amoxicillin Exposure**

50 This chapter aimed to measure the inhibitory influence of amoxicillin on anaerobic systems
51 experiencing a shock exposure event. Centralised wastewater systems are exposed to chronic low
52 concentration exposure to amoxicillin and other pharmaceuticals within the influent. However, the
53 distribution of pharmaceuticals within small scale systems is likely to be of higher concentration and
54 irregularly timed. This study explored changes in methanogenic activity as a proxy for treatment
55 efficiency. As there was limited research into the inhibitory effects of amoxicillin in anaerobic
56 communities, it was initially necessary to determine how sensitive anaerobic communities are to
57 amoxicillin and at what exposure range inhibition of methanogenic activity was observed. A specific
58 SMA was used to track how amoxicillin influenced the activity of each sub population within the
59 anaerobic trophic cascade. This was conducted by feeding a known concentration amoxicillin to
60 anaerobic granules along with co-substrates associated with different trophic levels: acetate, ethanol,
61 propionate, butyrate, glucose, and a proteinaceous complex broth and the gaseous substrate $H_2/CO_2$.
62 As amoxicillin concentrations within influent is likely to vary highly depending on the influent type
63 and context, a range of nine amoxicillin concentrations were studied in two batches which ranged
64 from 0.5 - 5.0 mg/L and 10 – 100 mg/L. The activity was calculated through measuring the
65 cumulative methanogenic output. The influence of amoxicillin was assessed through comparing the
66 rate of methane production, total methane output, as well as length of the lag time between substrate
67 addition and methane generation. Methane volume was measured using pressure transducers and
68 quantified using a GC-FID gas analyser.

69 **Chapter 7 – Changes in Community Assembly and AMR Genes In Anaerobic Granules After**
70 **Acute Amoxicillin Exposure**

71 Chapter 4 established that a shock exposure of amoxicillin produced a measurable inhibition of
72 methanogenic activity within the anaerobic cultures used. This chapter aimed to explore the
73 development of beta-lactam resistance genes as well as changes to the anaerobic community in
74 response to shock exposure to amoxicillin. Anaerobic granules were exposed to four concentrations
75 of amoxicillin: 1.0 mg/L, 10 mg/L, 50 mg/L and a blank along with glucose as a co-substrate.

Cumulative pressure was measured over a 48-hour window during which time sub-samples of each replicate test condition were removed for analysis. Effluent was collected for sCOD quantification. Anaerobic sludge granules were flash frozen in liquid nitrogen for storage until DNA extraction. Anaerobic activity was measured via methanogenesis and effluent sCOD. Changes within the anaerobic community were measured using NGS profiling of 16S rRNA gene (V4 region) from whole community DNA by Earlham Institute (Norwich, England). The quantification of antimicrobial resistant genes and 16s rRNA within the sludge was measured using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland).

**Chapter 8 - The Influence of Anaerobic Culture Medium on The Structural Stability of Amoxicillin**

This chapter aimed to measure the decay rate of amoxicillin in the presence of different compounds within anaerobic media in order to determine the impact of biotic versus abiotic decay within anaerobic cultures. As the primary mechanism for beta-lactam resistance is the deactivation and decay of the beta-lactam ring, it is assumed that the decay and removal of beta-lactams within cultures are primarily a biological action. This study aimed to assess the extent to which the constituents of anaerobic culture media catalysed hydrolysis of the beta-lactam ring. The study exposed amoxicillin to each of the individual components of anaerobic medium and incubated them at 35°C as would be used during the culturing of anaerobic granules. Samples were taken at regular intervals at which point the amoxicillin was quantified using liquid chromatography with a UV detector. This data was then used to determine the contextual half-life of amoxicillin for each test conditions.

**Chapter 9 – Conclusions and Future Work**

The contributions made within this thesis are summarised and discussed as they related to the objectives of this work and wider understanding of beta-lactams within anaerobic communities. From these conclusions, future work is discussed in term of potential experiment for further understanding changes to the anaerobic community in response to beta-lactam exposure, confirmed beta-lactam resistance within anaerobic systems, and improvements to study design.

# Chapter 2
# Literature Review

## 2.1 Antibiotic Resistance in Water Treatment– An issue of global concern

Antibiotic resistance is an issue of increasing global concern (Torres *et al.*, 2021). Antibiotics are an invaluable lifesaving treatment to reduce mortality associated with bacterial infection, prevent the spread of disease, and make possible many surgical procedures regularly practiced today. As such, the use of antibiotics has increased year after year (Center for Disease Dynamics Economics & Policy, 2015). The increase in use of antibiotics however has been accompanied by an increase in microbial resistance to those drugs, resulting in an overall reduction in their efficacy. Furthermore, few new antibacterial drugs have been developed since the 1970s (Torjesen, 2013).

Estimations of the impact of antimicrobial resistance (AMR) vary. The Review on Antimicrobial Resistance states that in the period running up to 2016 as many as 700,000 people died each year due to complications from antimicrobial resistance; and that this number could rise to 10 million by 2050 (O'Neill, 2016) (**Figure 2-1**). There are an estimated 2.8 million antibiotic resistance infections each year in the United States which contribute to more than 35,000 deaths (Sriram *et al.*, 2021). In the European Union in 2009 over 25,000 deaths per year were attributed to AMR diseases (EMEA, 2009). A study by The Centre for Disease Dynamics, Economics & Policy (CDDEP) has suggested that the burden of antimicrobial resistance is greater in middle and lower income countries, likely due to variance in prescription policies as well as health and water infrastructure (Sriram *et al.*, 2021).

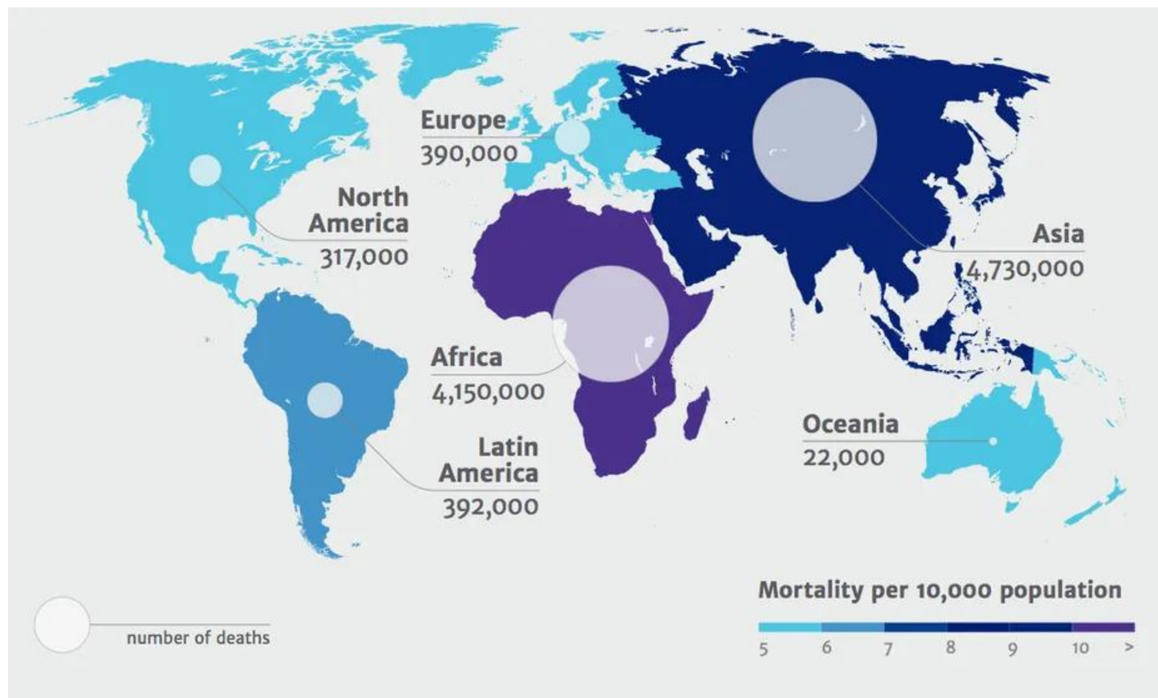



***Figure 2-1** - Predicted deaths attributed to AMR resistant diseases as of 2050.*

*Figure taken from (O'Neill, 2016).*

24 Whilst water infrastructure is recognised as key to limiting the spread of AMR, it is increasingly
25 recognised that wastewater treatment facilities can act as point sources for the proliferation and
26 spread of resistant microbes (Kümmerer, 2009a; Nguyen *et al.*, 2021). As such, the fate of antibiotics
27 in wastewater treatment and its spread is an area of active study (Singer *et al.*, 2016; Jong *et al.*,
28 2018; Hiller *et al.*, 2019; Ebomah and Okoh, 2020; Nguyen *et al.*, 2021). As the antibiotics that we
29 take leave our homes and hospitals in wastewater (**Figure 2-2**), they eventually enter our wastewater
30 treatment plants (WWTPs). Indeed, a wide variety of pharmaceutical compounds have been detected
31 at varying concentrations both in the influent and effluent of WWTPs (Matsuo *et al.*, 2011; Blair *et*
32 *al.*, 2013). Presently, the treatment processes at WWTPs are designed to breakdown and remove
33 digestible or settable organic materials and are not optimised for the removal of pharmaceuticals. As
34 such, these compounds may then eventually be discharged into the wider water environment.
35 Wastewater treatment is at its heart a biological process. Exposure of microbial communities (as
36 might be found in WWTPs) to sublethal concentrations of antibiotics has the potential to select for
37 antimicrobial resistance (Oberlé *et al.*, 2012; Singer *et al.*, 2016). Surveys of AMR within wastewater
38 treatment facilities regularly detect genes associated with resistance to antibiotics both within the

39 WWTP and in effluents (Amador *et al.*, 2015; Triggiano *et al.*, 2020; Wang *et al.*, 2020). These
40 effluents are usually discharged into local water ways and resistance genes are regularly detected
41 downstream (Singer *et al.*, 2016).

42 Whilst WWTPs are widespread, much of the world population do not have access to centralized
43 wastewater treatment and instead use decentralised treatment systems such as septic tanks and pit
44 latrines (Zhou *et al.*, 2018). And, while there is a significant body of research into the spread of AMR
45 in WWTPs, there is limited research into influence of antibiotics on the treatment efficiency or spread
46 of AMR in decentralised systems. As the technologies used in small scale and decentralised water
47 treatment systems are fundamentally different from large scale treatment facilities, understanding the
48 spread of AMR globally requires an increased understanding of AMR in decentralised wastewater
49 technologies.

50

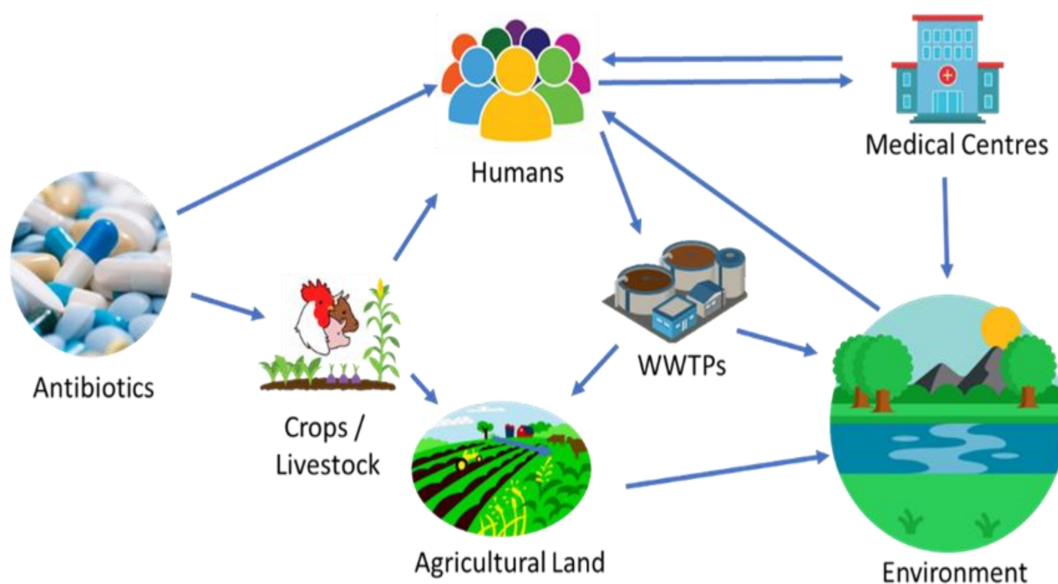


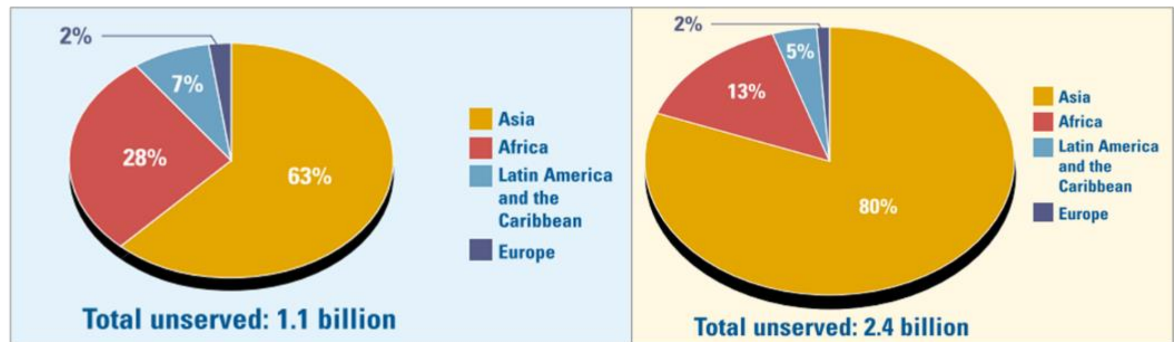

***Figure 2-2*** *- An example of how antibiotics move around our environment.*
*While humans use antibiotics medically, antibiotics are also applied to crops and livestock. This then runs off into the land and eventually into the water environment. The antibiotics we take ends up in our water treatment plants through sewage. As waste treatment effluent is usually discharged directly to the environment, any residual antibiotics within effluent will also enter the environment.*

51 ## 2.2 DECENTRALISED WASTEWATER TREATMENT

52 ### 2.2.1 The global sanitation crisis

53 Access to effective sanitation is essential for ensuring global human health. Indeed, improving global
54 access to effective sanitation has been a priority for the World Health Organisation (WHO) for the
55 last 30 years. Nevertheless, the latest report from the WHO on the state of the worlds sanitation found
56 that “over half of the world’s population, 4.2 billion people, use sanitation services that leave human
57 waste untreated, threatening human and environmental health” (UNICEF & WHO, 2020) despite its
58 aim to end this practice globally by 2030 (Harada and Strande, 2016) **(Figure 2-3)**. Despite an
59 increased reliance on centralised collection and treatment of wastewater within urban areas for the
60 last 100 years, it is currently estimated that 2.7 billion people worldwide rely on decentralised
61 sanitation technologies such as septic tanks and pit latrines (Harada and Strande, 2016). Due to their
62 versatility and low cost, an increase in the utilisation of decentralised or onsite waste treatment
63 technologies could be a feasible path forward in the effort towards meeting the WHO sustainable
64 development goals (SDGs). There are three primary drivers towards the decentralisation of waste
65 treatment systems in address of the SDGs: economic, technical, and environmental (Massoud,
66 Tarhini and Nasr, 2009). Decentralised systems are generally inexpensive to install and require less
67 maintenance than a large plant which by contrast requires large amounts of upfront funding as well
68 as ongoing maintenance and constant monitoring for continued operation (Sharma *et al.*, 2013).
69 Additionally, the transport of waste to a central facility is expensive and requires the construction of
70 pipes and pumps throughout the catchment area. These pipes are expensive to install and provide
71 opportunities for leaks and environmental contamination. Decentralisation can be a more cost-
72 effective treatment option as cities expand into low density suburban landscapes and regions with
73 limited local budgets and restricted access to funding. Finally, decentralised wastewater treatment
74 facilities have significantly lower carbon footprints than centralised systems as they do not have the
75 electricity and fuels costs required for transfer of sewage to and treatment at WWTPs (Arias *et al.*,
76 2020).

A. Distribution of global population not served with improved water supply in 2000




B. Global Sanitation Coverage 2017

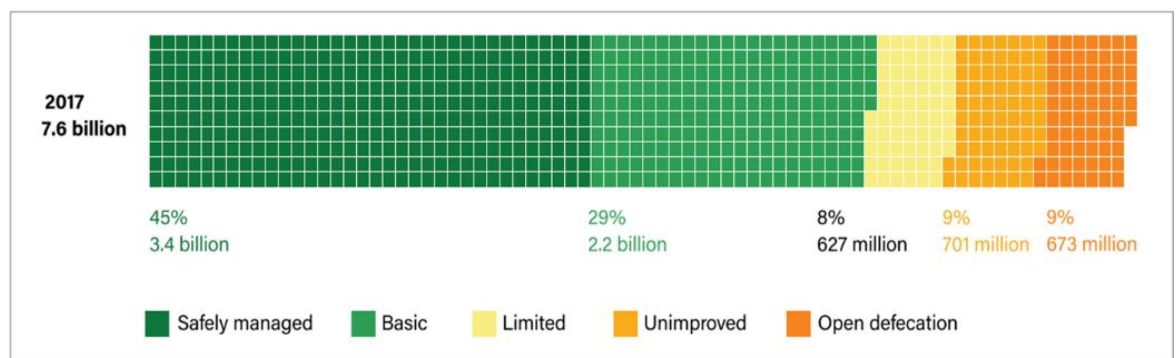



***Figure 2-3*** *- Distributions of Global Sanitation.*
*A Distribution of population not served with improved sanitation supply defined as "Use of pit latrines without a slab or platform, hanging latrines or bucket latrines" (WHO/Unicef 2000) B. Global sanitation coverage as of 2017. Each square represents a million people. (UNICEF 2020)*

Decentralised waste treatment systems are currently used throughout the world. In industrialized countries, decentralised waste treatment is commonly used in areas with low population density such as rural or suburban settings. While there are many decentralised technologies available, due to its simplicity and low maintenance, septic tanks are commonly utilised throughout the globe. For example, roughly 20% of households in the USA are served by septic tanks (Donovan *et al.*, 2013). It is estimated that there are 400,000 registered septic systems within England (DEFRA and EA, 2014). Although cities across Japan are primarily served by large centralised systems, in rural Japan, reliance on septic tanks can be as high as 65% (Mizuochi *et al.*, 2008). In developing regions or countries, decentralisation is a cost-effective option for treatment in areas where centralised treatment systems are otherwise not available. In Thailand, off grid facilities are the primary treatment used outside of cities (Withers *et al.*, 2014).

88 ### 2.2.2 Septic Tanks

89 Septic tanks are one of the oldest forms of modern sewage treatment. The first reported use of the
90 septic tank was by John Louis Mouras and Abbe Moigno in France in 1860 and consisted of a wooden
91 chamber that allowed for the separation of solids from effluent (Butler & Payne, 1995). This design
92 was later patented by Donald Cameron in 1895. A similar design, the Imhoff cone, which allowed
93 for increased separation of the solids and effluent was patented in 1907. These technologies were
94 designed to treat relatively small volumes of waste before discharge directly to the vadose zone.
95 Although there have been many technological advances in centralised wastewater treatment systems
96 at an urban scale, decentralised small-scale systems are remarkably similar in design and function to
97 the initial technologies developed in 19th century (Lofrano & Brown, 2010). Modern septic tanks
98 are available in many different forms and designs; however, their treatment processes are all
99 fundamentally the same.

100 Septic tanks operate as settling tanks in which settleable solids are gravimetrically removed from
101 suspension where they are slowly anaerobically digested (Diaz-Valbuena *et al.*, 2011). Wastewater
102 enters the tank via a T-shaped inlet pipe or baffle which minimises mixing as influent enters the tank

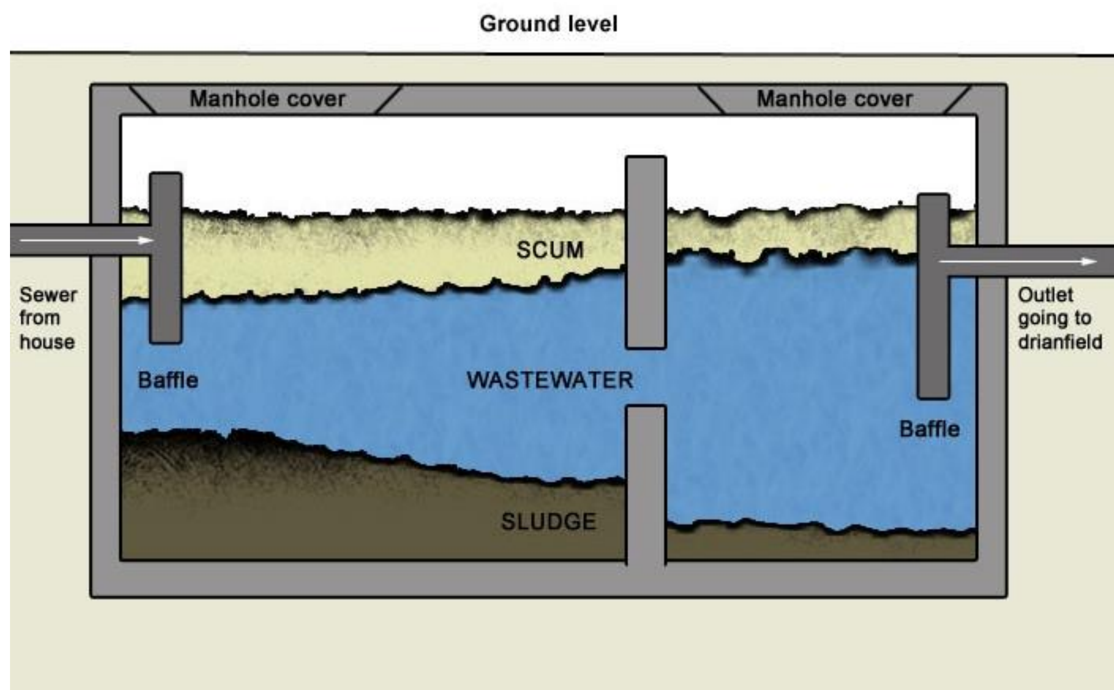



***Figure 2-4*** *- Diagram of a septic tank. Source www.owlshall.co.uk/*

103 (**Figure 2-4**). As wastewater moves through the chamber fats, oils, or any other buoyant materials
104 will collect on the surface where they are retained and form the scum layer. Settleable solids sink to
105 the bottom of the chamber where they form a layer of sludge, leaving behind a clarified effluent for
106 discharge. Anaerobic microbes slowly hydrolyse the organic matter from the influent. As the organic
107 matter is hydrolysed soluble metabolites, such as volatile fatty acids, dissolve into the clarified liquid
108 and are removed from the tank with the effluent. Generally, septic tanks only provide partial
109 degradation of the organic materials, and as a result requires additional treatment such as percolation
110 through the soils via underground pipes in a drain field (also called a soakaway) where the resident
111 soil microbiota will metabolise remaining organic materials. Nevertheless, methane and other
112 greenhouse gasses are emitted from septic tanks. The EPA estimates that septic system emissions
113 account for nearly 48.0 % of methane emissions from wastewater treatment, both centralised and
114 decentralised, in 2020 which make up 5.7 Tg of $CO_2$ equivalents (EPA, 2022).

115 As they have no moving parts, septic tanks do not require any additional power to operate, though
116 they do require regular maintenance. As sludge is digested, indigestible or inorganic materials build-
117 up in the tank and require removal, usually on an annual basis though frequency depends on the size
118 of the tank and the quality of the effluent. While septic tanks traditionally operate at ambient
119 temperatures, research into heated septic tanks has found that increasing the internal tank temperature
120 from 30°C to 40°C increased the methanogenic activity in the sludge layer of the septic tank. This
121 resulted in less total volatile solids formed, a decrease in the accumulation of settled sludge, and an
122 increase in the total methane produced (Pussayanavin *et al.*, 2015).

123 The composition of influent into septic tanks varies greatly throughout the world with difference in
124 application and usage. The main components of septic tank influents are comprised of black water
125 and grey water. Black water is primarily comprised of excreta and flush water, while grey water is
126 comprised of wastewater from washing, cleaning, and bathing. Brown water is comprised of a
127 combination of the two (Metcalf and Eddy, 2003; Karia and Christian, 2013). The ratio of black
128 water and greywater in the influent varies with region and application type, as well as throughout the
129 day (Patterson, 2003). For example, in industrialised countries utilising septic tanks, households will

130 often have appliances generating significant greywater discharge such as showers, washing
131 machines, and dishwaters. As a result, the influent into the septic tank will be generally less
132 concentrated with respect biochemical oxygen demand (BOD). By contrast, in areas with low or no
133 access to domestic water supply, where greywater is collected and reused, blackwater makes up a
134 much greater proportion of the influent. As a result, the influent of these systems is generally much
135 more highly concentrated with respect to BOD. In some applications, such as vacuum toilets, flush
136 water is not used, which will concentrate the influent even further. As the biological degradation of
137 organic matter within a septic tank is primarily by anaerobic digestion, to understand how antibiotics
138 will affect decentralised septic systems, it is important to understand anaerobic digestion and
139 anaerobic communities.

## 140 2.3 Anaerobic Digestion

141 Anaerobic digestion is a naturally occurring biological process in which organic matter is broken
142 down into simpler compounds in the absence of oxygen and is driven by a diverse community of
143 microorganisms. Anaerobic microbial communities can be found in marine and freshwater lake
144 sediments (Hinrichs *et al.*, 2000; Marchant *et al.*, 2017; Martinez-cruz *et al.*, 2018), fens and bogs
145 (Duddleston *et al.*, 2002; Steinberg and Regan, 2008), within digestive systems (Zhai *et al.*, 2020),
146 as well as within anthropogenic environments such as landfills (Tammemagi, 1999). Additionally
147 anaerobic digestion is used in industrial settings including for the removal of organics in wastewaters
148 (Karia and Christian, 2013) and food waste (Poggio *et al.*, 2016), methane production for biofuels
149 (Kiselev *et al.*, 2019), and the fermentation of cheese and alcohol (Ishtar Snoek and Yde Steensma,
150 2008; Button and Dutton, 2012).

151 ### 2.3.1 Anaerobic Digestion Process

152 Anaerobic digestion is undertaken by a diverse set of microbes via a syntrophic food chain whereby
153 the metabolites produced by some specialised members of the microbial community are the
154 substrates required by other members within the community (Gerardi, 2003). Anaerobic microbial
155 communities comprise a mix of bacteria and archaea. Although anaerobic digestion is only

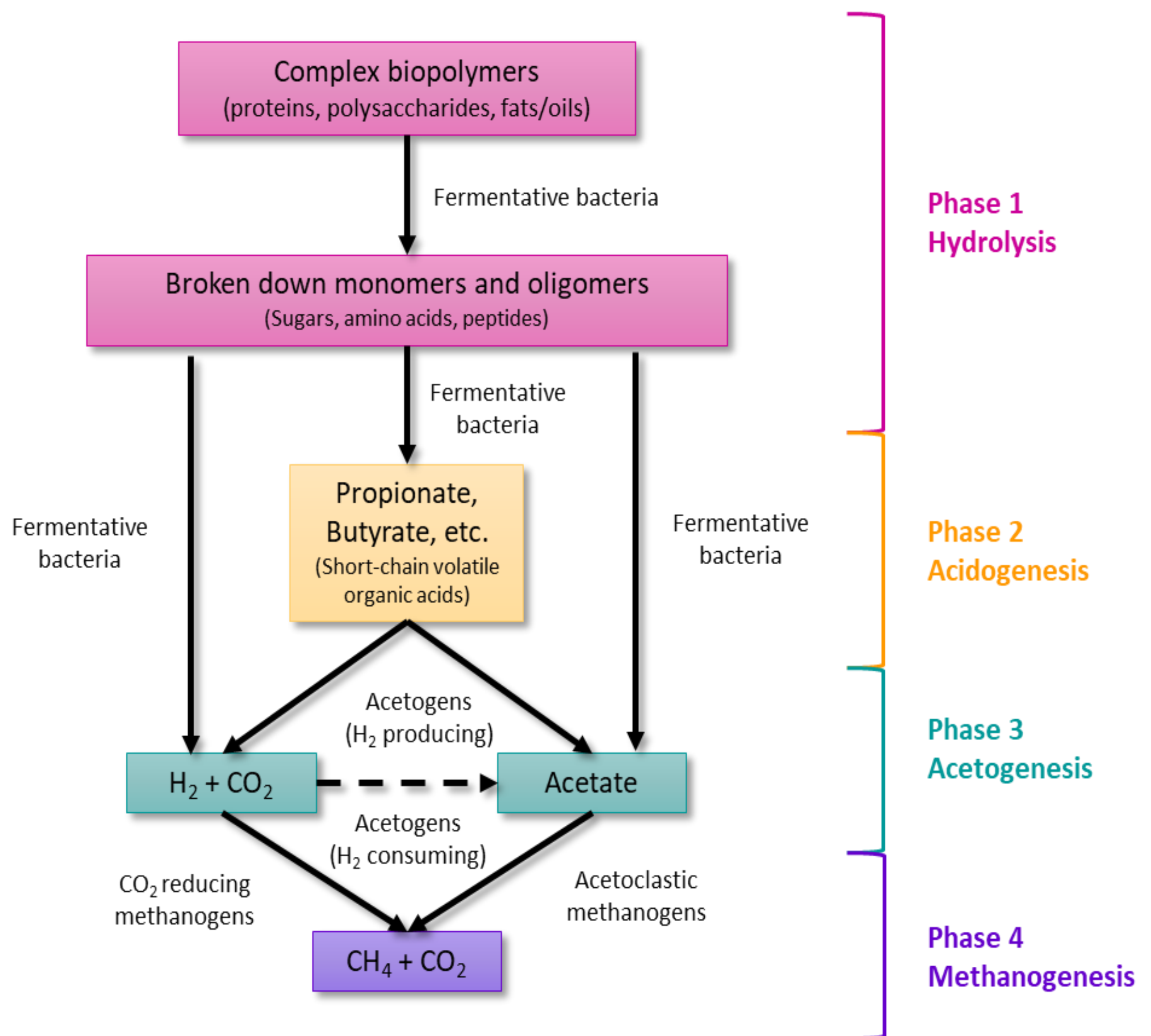



***Figure 2-5*** *- Schematic of four phases of biogas production.*
*Figure adapted from: https://www.e-education.psu.edu/egee439/node/727*

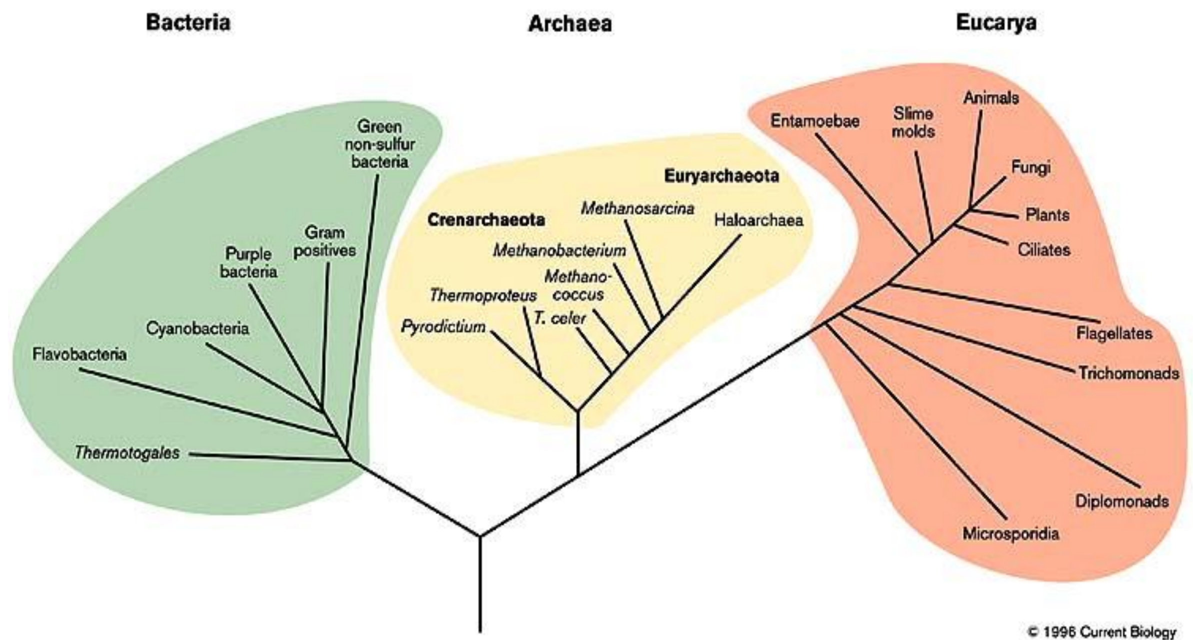



*Figure 2-6 - Phylogenetic tree of bacteria, archaea, eucarya (Woese, 1996).*

156 undertaken in the absence of oxygen, the microbes within anaerobic microbial communities have
157 varying levels of oxygen tolerance. Strict anaerobes cannot survive in the presence of oxygen.
158 Facultative anaerobes are capable of using oxygen as an electron acceptor when it is present and are
159 capable of growing in both aerobic and anaerobic conditions (Madigan, 2014). The anaerobic
160 digestion process is conducted sequentially over four stages (1) hydrolysis, (2) acidogenic
161 fermentation, (3) acetogenesis and (4) methanogenesis, in which each stage is conducted by different
162 subpopulations with the anaerobic microbial community (Sanders, 2001; Gerardi, 2003) (**Figure 2-**
163 **5**). While the first three stages are undertaken by bacteria, methanogenesis is undertaken exclusively
164 by archaea which sit in their own domain (Woese, 1996) **(Figure 2-6).**

165 The first stage of anaerobic digestion is hydrolysis in which complex insoluble compounds such as
166 proteins, lipids, and complex carbohydrates are broken down into smaller soluble substances. As
167 these large macromolecules cannot be transported across cell membranes, digestion occurs
168 extracellularly (**Figure 2-7**). Exoenzymes are excreted from the cell wall which hydrolyse the bonds
169 within the substrate and release the soluble monomers. In this way, proteins are broken down into
170 amino acids, carbohydrates release soluble sugars, and lipids break into fatty acids. In the treatment
171 of wastewater, as the wastewater influent usually contains high concentrations of complex solid

172 waste, the hydrolysis step is often rate limiting in that context (Bialek, Cysneiros and O'Flaherty,
173 2014). Hydrolysis is primarily undertaken by hydrolytic bacteria, facultative anaerobes, and strict
174 anaerobes which are each capable of generating enzymes that hydrolyse unique bonds within the
175 provided substrates. Complex substrates requiring hydrolysis often also contain essential materials
176 for the formation of biomass and other enzymes.

177 In the second phase of anaerobic digestion, acidogenic fermentation, the soluble products of
178 hydrolysis are brought inside the cell where they are further broken down to provide energy (ATP)
179 and biological building materials (Madigan, 2014) (**Figure 2-7**). The products of fermentative
180 digestion usually include volatile fatty acids, alcohols, lactic acid, carbon dioxide, hydrogen,
181 ammonia and hydrogen sulphide, as well as new bacterial cells. A large diversity of facultative and
182 strict anaerobes produces an equally diverse variety of compounds in many fermentative processes.
183 However, many of the microbes responsible for this process are the same as those responsible for
184 hydrolysis (de Lemos Chernicharo, 2015). Acidogenesis is exergonic in which the reaction is the
185 accompanied by the release of energy and therefore enables the rapid growth of fermentative bacteria
186 (Seghezzo *et al.*, 1998). As the products of acidogenesis are acidic, high levels of acidogenic activity
187 can lower the pH of the system and disrupt methanogenesis.

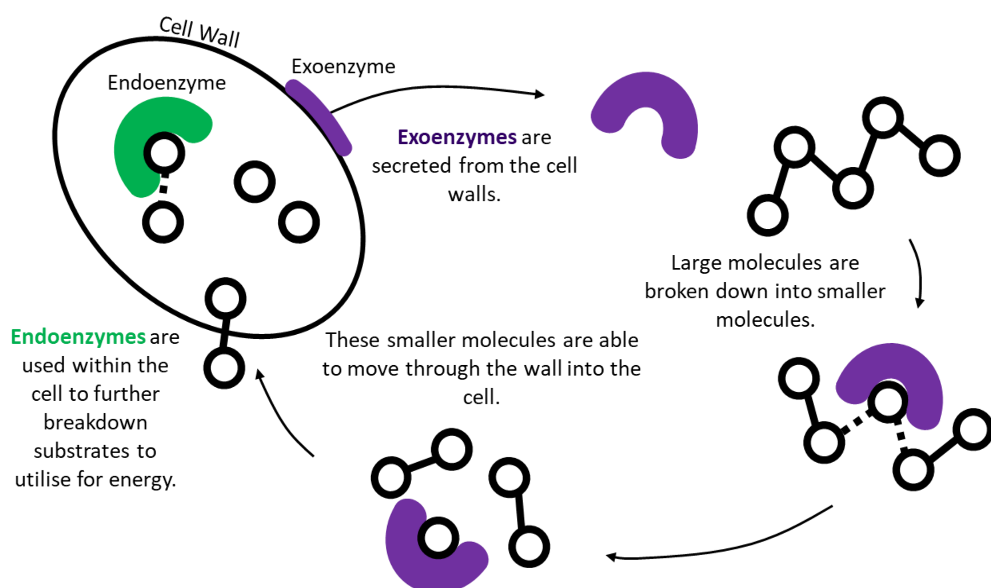



*Figure 2-7 – Diagram of exoenzymes and endoenzymes.*

188 Fermentation products are further oxidised by acetogenic bacteria into substrates appropriate for
189 methanogenesis. The primary products generated by acetogenesis are acetic acid, hydrogen and
190 carbon dioxide though the ratios of each depend on which products were produced during
191 fermentation as well as the composition of the microbial community. If not utilised in methane
192 formation this hydrogen can react with carbon dioxide and acetic acid to form other organic acids
193 lowering the pH of the aqueous medium in the process (de Lemos Chernicharo, 2015).

194 The final stage of the anaerobic digestion process is methanogenesis in which methane and $CO_2$ are
195 formed. The production of methane is primarily conducted by methanogenic archaea, a
196 phylogenetically diverse group of strict anaerobes, with some methanogenesis conducted by bacterial
197 species under rare conditions such as in phosphate starved ocean surface conditions (Thauer *et al.*,
198 2008; Carini *et al.*, 2014). Methane can only be produced using a limited number of substrates: acetic
199 acid, hydrogen/$CO_2$, formic acid, methanol, methylamines and carbon monoxide (Gerardi, 2003).
200 When molecular sulphur is present, methanogens will preferentially produce large amounts of $H_2S$
201 in addition to methane (Garcia, Patel and Ollivier, 2000). There are currently more than 150
202 identified species of methanogens though more are being discovered regularly. In general
203 methanogens can be grouped according to substrate use (Jabłoński, Rodowicz and Łukaszewicz,
204 2015). (1) Hydrogenotrophic methanogens oxidise $H_2$ and reduce $CO_2$ to form methane. The
205 hydrogenotrophic group also includes formatrophs which are capable of oxidising formate to form
206 methane. (2) Methylotrophic methanogens utilise methane compounds such as methanol,
207 methylamines, and dimethylsulfide as their primary substrates. (3) Acetotrophic (aceticlastic)
208 methanogens utilise acetate as their primary substrate (Garcia, Patel and Ollivier, 2000). While the
209 specific makeup of species varies greatly from culture to culture, the three primary groups found in
210 biogas producing anaerobic digesters are *Methanobacterium*, *Methanolinea* (hydrogenotrophic) and
211 *Methanosaeta* (acetoclastic) (Gerardi, 2003; Trego *et al.*, 2020).

212 ## 2.4 BETA LACTAMS

213 Beta lactams are some of the most widely used antibiotics in the world (Klein *et al.*, 2019)
214 constituting roughly 65% of the global market (Githinji *et al.*, 2011). In 2017 approximately 44.6%
215 of all antibiotics used in the UK were penicillin class (Public Health England, 2017). Beta lactams
216 are defined by the beta lactam ring at the centre of their molecular structure. They were among the
217 first antibiotics developed after Scottish researcher Alexander Fleming first discovered penicillin in
218 1928 (Bennett and Chung, 2001). Since then, hundreds of different types of beta-lactam derivatives
219 have been developed each with a central lactam ring. There are four classes of beta lactam:
220 penicillin's, cephalosporins, monobactam and carbapenems with a primary core structure containing
221 either a single lactam ring or a double ringed structure (**Figure 2-8**). Although the first beta lactams
222 were isolated from natural origin, as in the case of penicillin, novel beta lactams are synthesized
223 through the addition of side chains onto the four core structures resulting in semisynthetic compounds
224 with varying properties and potencies (Kong, Schneper and Mathee, 2010).

225

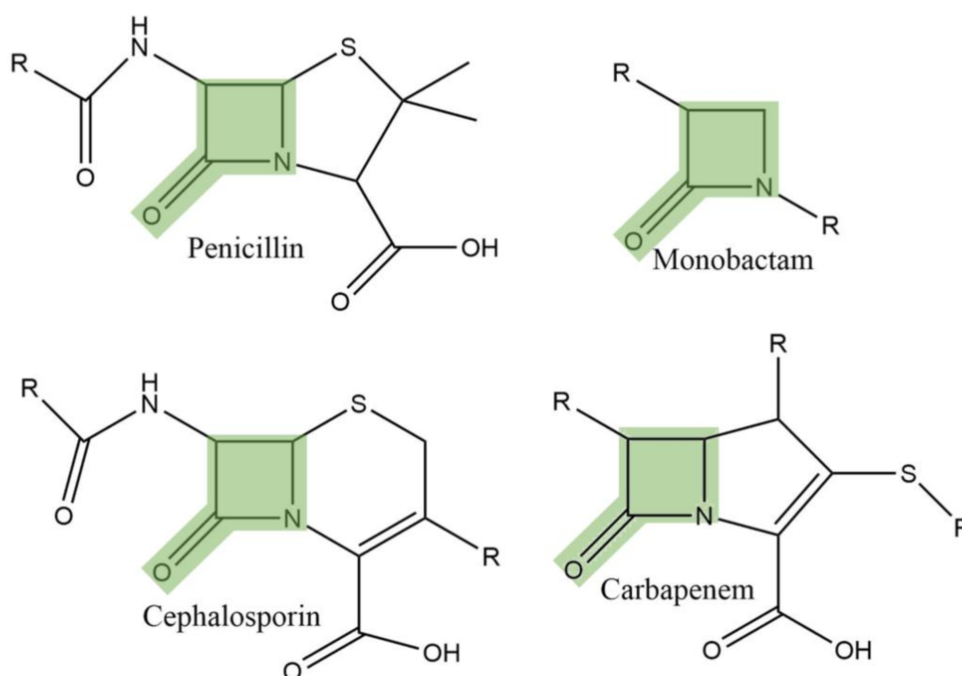



***Figure 2-8*** *- The four primary structures of beta-lactam antibiotics.*
*The central defining beta-lactam ring is highlighted in green.* (Wikimedia Commons, 2010)

226 The primarily mechanism of action for beta-lactam class antibiotics is through inhibition of bacterial
227 cell wall synthesis. Beta-lactams interfere with cell division and the maintenance of structures within
228 growing cells by inactivating penicillin binding proteins (PBP) therefore inhibiting the final
229 transpeptidation step of peptidoglycan synthesis (Wong *et al.*, 2021).

230 Peptidoglycan is a polymer composed of alternating units of the amide derivative N-
231 acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). To connect the chains of NAG and
232 NAM together and form the peptidoglycan layer, an enzyme called a transpeptidase removes the
233 ends of the amino acid chains and catalyses the crosslinking of a peptide bond between the amino
234 acid chains attached. One of the primary transpeptidase enzymes responsible for this action is PBP,
235 named for its affinity to binding with the beta lactam ring on penicillin class antibiotics (Madigan,
236 2014). As beta-lactam antibiotics deactivate the PBP the cell wall loses is structural integrity and
237 lyses (Wong *et al.*, 2021) **(Figure 2-9)**. There are a variety of different types of PBPs within each
238 cell and can differ between bacterial species. As the structure of the PBP can vary between different
239 species so too does the effectiveness of the various beta-lactam drugs (Kong, Schneper and Mathee,
240 2010).

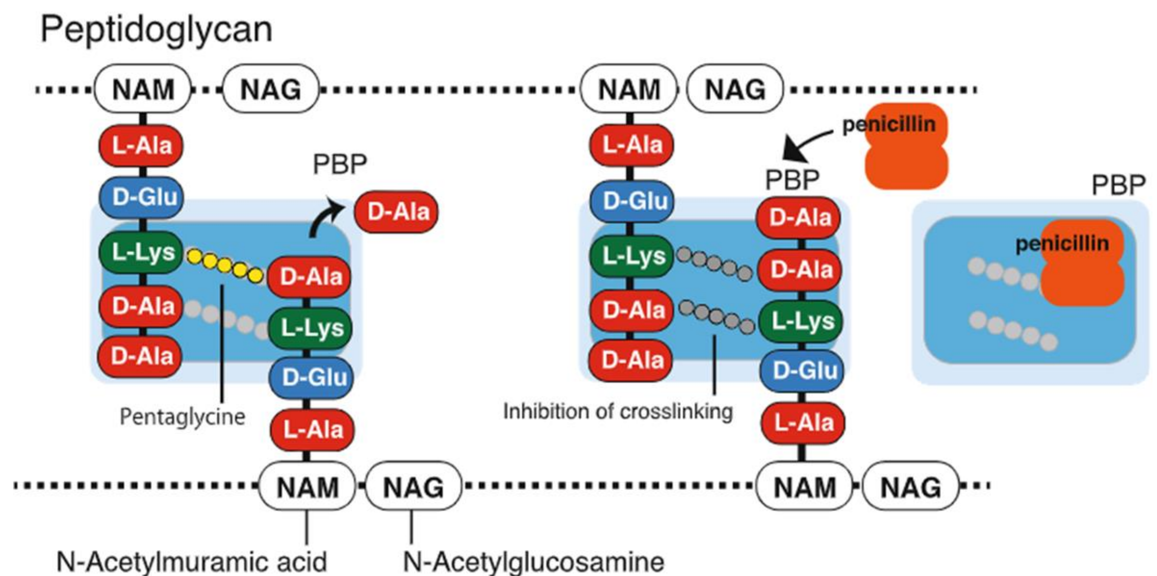



***Figure 2-9*** *– Diagram of peptidoglycan and the action of penicillin around PBP.*
*(Sawa, Kooguchi and Moriyama, 2020)*

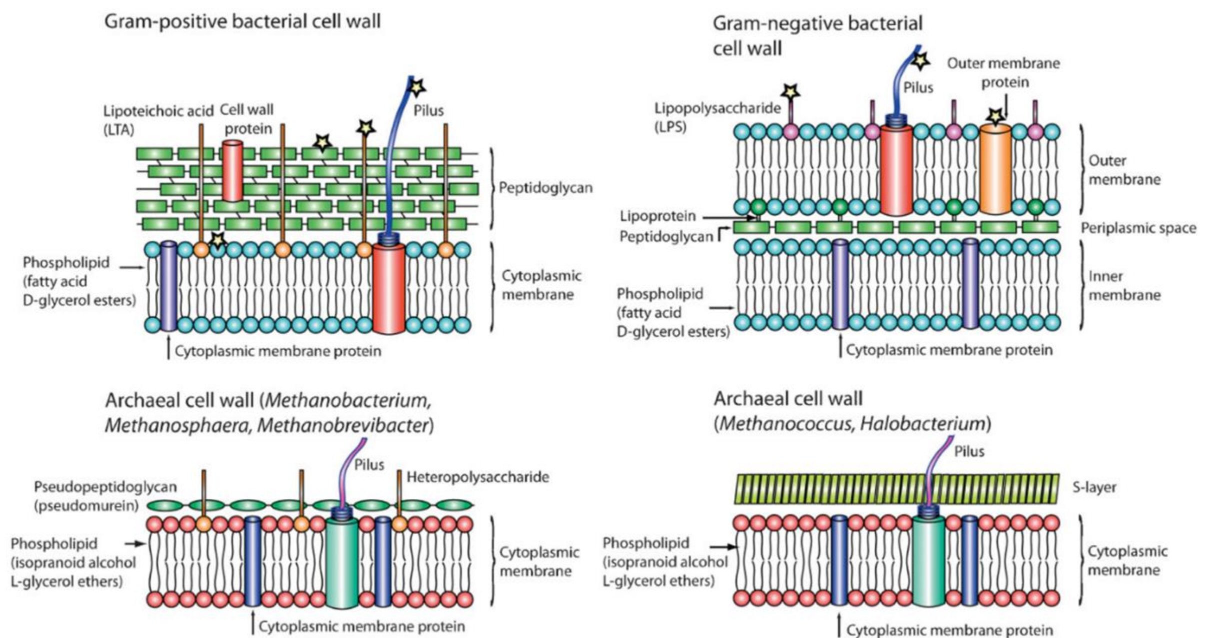



***Figure 2-10** – The cell wall of gram-positive bacteria, gram negative bacteria, and two types of archaea. Beta-lactam antibiotics act through synthesis inhibition and destruction of the peptidoglycan (green bar). As archaea do not contain peptidoglycan, they are naturally immune to beta-lactam antibiotics. (Gill and Brinkman, 2011)*

241 Beta-lactams affect both gram positive and gram-negative cells, although the effect is different for
242 each due to the differences in the structure of the cell walls (**Figure 2-10**). Gram positive cells are
243 comprised of a single phospholipid bilayer with a thick layer of peptidoglycan providing structure.
244 The cross linking in the peptidoglycan of gram-positive cells are held together through a peptide
245 inter-bridge in which the amino acids within the peptidoglycan are crosslinked at several points.
246 Gram negative cells, by contrast, have two phospholipid bilayers with a thin layer of peptidoglycan
247 in between. The crosslinking of the peptidoglycan in gram-negative cells is bonded with a single
248 peptide bond on the amino acid on the terminal D-alanine within the amino acid chain. The outer
249 layer of gram-negative cells is primarily comprised of phospholipid bilayer with a variety of
250 transmembrane proteins such as porins, channels, and pumps which facilitate the movement of
251 materials in and out of the cell. In between the inner phospholipid bilayer and the peptidoglycan of
252 the gram-negative cells is the periplasmic space (Madigan, 2014). This is comprised of a gel like
253 matrix known as periplasm which contains a store of enzymes, including those which confer
254 resistance for susceptible bacteria. Because the peptidoglycan on gram positive cells is more readily
255 exposed, it is more susceptible to the beta lactam antibiotics. Nevertheless, beta lactams are able to
256 enter into the periplasm of gram-negative cells where they are able to act upon the peptidoglycan

257 (Wong *et al.*, 2021). The beta lactam ring within all penicillin class antibiotics can irreversibly bind
258 to PBP. Binding inactivates the PBP protein by changing its shape and hence its ability to catalyse
259 the crosslinking between amino acids within peptidoglycan. This in turn prevents the generation and
260 repair of cell walls resulting in weak points. As weaknesses in the cell wall accumulate over time,
261 pressure within the cell causes the cell to rupture, killing the cell in the process (Wong et al., 2021).

262 Organisms of the archaeal domain, including those responsible for methanogenesis, are structurally
263 diverse and have a multitude of outer membrane structures which differ greatly to those observed in
264 bacteria. Unlike most bacteria, archaea do not contain murein, a type of peptidoglycan (Kandler and
265 König, 1998). In their place the structure of archaeal cell walls is obtained through a para-crystalline
266 structure commonly comprised of proteins or glycoproteins, though other structures have been found
267 as well. Because archaea do not contain peptidoglycan **(Figure 2-10)** they are not susceptible to beta-
268 lactam antibiotics (Hans and König, 1996; Khelaifia and Drancourt, 2012).

## 269 2.5 BETA LACTAM RESISTANCE

270 As beta-lactam antibiotics have been used extensively, there has been proliferation of antibiotic
271 resistance within bacterial populations. Three distinct mechanisms for resistance to the damage that
272 beta lactam rings cause are identified: alteration of the PBP binding site, removal though an efflux
273 pump, and degradation of the beta-lactam active site via beta-lactamase enzymes (Nikaido and Pagès,
274 2012; Bush, 2013; Zango *et al.*, 2019; Park, Sutherland and Rafii, 2020).

### 275 2.5.1 Penicillin Binding Proteins

276 The antibiotic properties of beta-lactams function by competitively bonding with the PBP activation
277 site and preventing the formation of cell walls (Madigan, 2014). However, changes to the structure
278 of PBP can prevent beta-lactam binding resulting in resistance to this class of antibiotics. For
279 example, one of the most studied examples of this mutation is the PBP2a which is carried on the
280 gene mecA which and resides on a large mobile genetic element and is believed to be transmitted
281 through horizontal transfer between staphylococcus species (King *et al.*, 2017). While the *mecA* gene
282 can be found in a wide variety of *Staphylococcus* species it is most widely associated with

283 Methicillin-resistant *Staphylococcus aureus* (MRSA) a pathogen of concern which has been
284 observed to be resistant to several widely used antibiotics including beta-lactams (Lakhundi and
285 Zhang, 2018).

### 286 2.5.2 Efflux Pumps

287 Efflux pumps form part of the xenobiotic removal system in which potential bacterial stressors are
288 removed from the cell through efflux transporters within the cell membrane (Madigan, 2014). Efflux
289 transporters exist as either single component pumps which transport the target from the cytosol into
290 the periplasm; or multicompetent pumps, which capture their targets from the inner membrane or
291 periplasm and transport them outside the cell. While single component pumps contain a single part,
292 multi component systems are typically comprised of a pump, an outer membrane channel protein
293 (OMP), and an accessory membrane fusion protein such as MFP (**Figure 2-11**). While the majority
294 of known efflux pumps reside in bacteria, they can also be found in eukaryotic as well as archaeal
295 organisms (Elbourne *et al.*, 2017). Major families of efflux transporters include RND, MFS (major

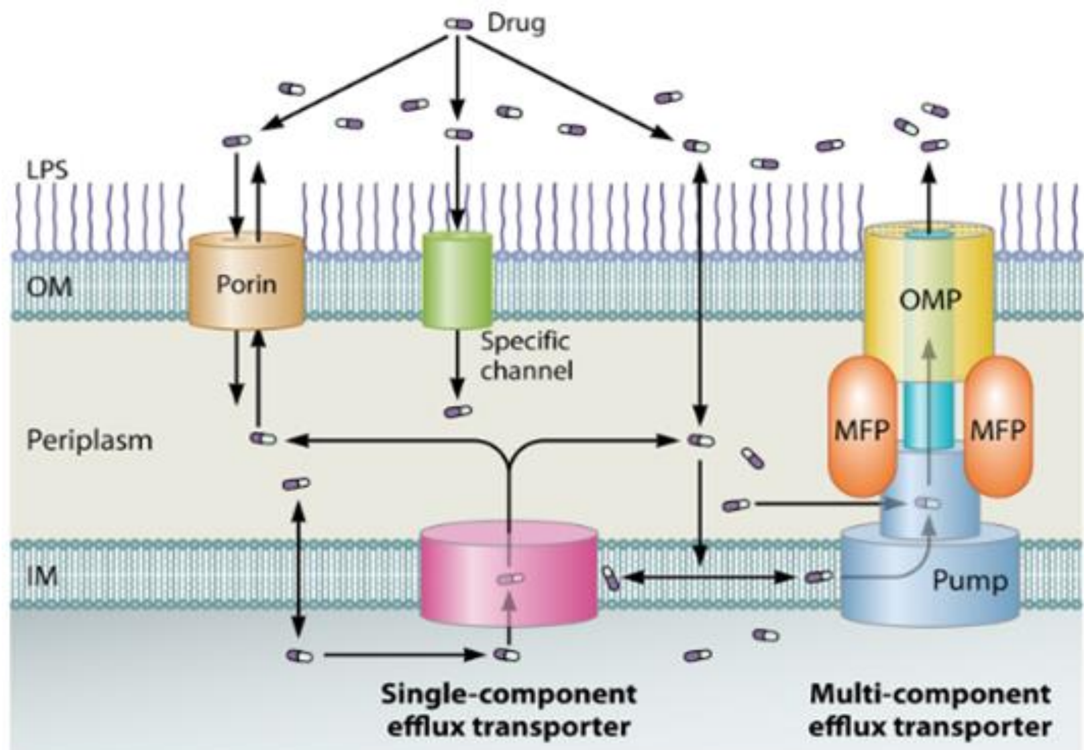



***Figure 2-11*** *– Diagram of single component efflux pumps and multi component efflux pumps.*
*(Li, Plésiat and Nikaido, 2015)*

296 facilitator superfamily), MATE (multidrug and toxic compound extrusion), SMR (small multidrug
297 resistance), and ABC (ATP-binding cassette) super- families or families (Poole, 2005).

298 Efflux pumps are nonspecific in what they remove and are therefore a part of multidrug resistance
299 (MDR) however, their role in beta-lactam resistance is highly complex (Lin and Scott, 2012; Li,
300 Plésiat and Nikaido, 2015). The influence of efflux pumps on beta-lactam resistance is actively being
301 investigated and appears to depend on drug properties (eg. hydrophobicity and ionic strength),
302 bacterial species, and beta-lactamase production (Li, Plésiat and Nikaido, 2015). Efflux mediated
303 beta-lactam resistance has been observed via chromosomally encoded multicomponent pumps of the
304 RND family AcrAB-TolCj for *H. influenzae* and MexAB-OprMk for *P. aeruginosa* and several
305 Gram-negative bacteria. Additionally, the ABC family LmrA pump has been observed to confer
306 modest beta-lactam resistance for *L. lactis* (Poole, 2005).

### 307 2.5.3 Beta-Lactamases

308 The primary mechanism for beta lactam resistance is mediated through the production of beta-lactam
309 enzymes which catalyse the hydrolysis of the amine bond within the beta-lactam ring inactivating
310 the beta-lactam ring in the process (Madigan, 2014). Both gram negative and gram-positive cells can
311 generate beta-lactamase, but as gram negative cells are capable of storing proteins in their
312 periplasmic space, they are able to react immediately when exposed to a beta-lactam antibiotic. As a
313 result, they can have a constitutive resistance as they are immediately able to respond to exposure.
314 Gram positive microbes, on the other hand, do not have this periplasmic space, and are not able to
315 store these proteins for later use. Therefore, gram positive microbes have inductive resistance and
316 only begin generating resistance proteins in response to exposure to the antibiotic (Madigan, 2014).

317 Beta lactamases are classified using two methods. The Ambler System classifies beta lactams based
318 on amino acid structure, whilst the Bush- Jacoby classification scheme classifies beta lactams based
319 on their function (Bush and George A. Jacoby, 2010) (**Table 2-1**). In the Ambler Classification
320 system, beta-lactamases are grouped into four classes by structure. Classes A, C, and D enzymes are
321 comprised of serine beta-lactamases (SBL) that hydrolyse the beta-lactam ring via a serine-bound
322 acyl intermediate within their active site. Class B are comprised of metallo-beta-lactamase enzymes

323 (MBL) which utilise zinc to directly hydroxide ion within the beta-lactam ring (Ambler, 1980). The
324 Bush – Jacoby Method classifies beta-lactamases into three groups depending on their function and
325 enzymatic susceptibility to inhibitors with each of the three groups approximately corresponding
326 with specific structural classes. Group 1 comprises enzymes targeting cephalosporins and
327 corresponds to class C beta-lactamases. The genes encoding for beta-lactamases in this group were
328 originally chromosomal (Prescott, 2013). Group 2 comprises all other beta-lactamases with a serine
329 active centre and correspond to the A and D structural classification. Group 3 comprises MBLs as
330 correspond with class B (Bush and George A Jacoby, 2010). As both classification systems convey
331 different information, they are commonly used in conjunction to describe the beta-lactamase
332 functions and activity (Sawa, Kooguchi and Moriyama, 2020) (**Table 2-1**).

***Table 2-1 -*** *Beta-lactamase classification systems adapted from Ambler (1980) and Bush and Jacoby (2010).*

| Ambler Molecular Class | Bush Jacoby group | Distinctive Substrate | Representative Enzyme | Enzyme Type |
|---|---|---|---|---|
| A | 2a | Penicillins | PC1 | Serine beta-lactamases |
| | 2b | Penicillins, early cephanosporins | TEM-1, TEM-2, SHV-1 | |
| | 2be | Extended - spectrum cephalosporins, monobactams | TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1 | |
| | 2br | Penicillin | TEM-30, SHV-10 | |
| | 2ber | Extended - spectrum cephalosporins, monobactams | TEM-50 | |
| | 2c | Carbenicillin | PSE-1, CARB-3 | |
| | 2ce | Carbenicillin, cefepime | RTG-4 | |
| | 2e | Extended - spectrum cephalosporins | CepA | |
| | 2f | Carbapenems | KPC-2, IMI-1, SME-1 | |
| B1 | 3a | Carbapenems | IMP-1, VIM-1,CcrA, IND-! | Metallo beta-lactamases |
| B2 | 3a | Carbapenams | L1, CAU-1, GOB-1, FEZ-1 | |
| B3 | 3b | Carbapenems | CphA, Sfh-1 | |
| C | 1 | Cephalosporins | AmpC, ACT-1, CMY-2, FOX-1, MIR-1 | Serine beta-lactamases |
| | 1e | Cephalosporins | GC1, CMY-37 | |
| D | 2d | Cloxacillin | OXA-1, OXA-10 | Serine beta-lactamases |
| | 2de | Extended - spectrum cephalosporins | OXA-11, OXA-15 | |
| | 2df | Carbapenams | OXA-23, OXA-48 | |

Class A beta lactamases are the most diverse group and is comprised of penicillinases, expanded spectrum beta- lactamases (ESBL)s, and carbapenemase encoding for several phyla such as Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria (Walther-Rasmussen and Høiby, 2007). Class A beta-lactamases were one of the first classes to be identified by Ambler in 1980, later classified as 2b by Bush - Jacoby. It was observed that *TEM* and *SHV* genes encode for enzymes capable of rapidly degrading penicillin and early carbapenems (Ambler, 1980). Since then, mutations to *TEM-3, TEM- 50* and *SHV-2* (Bush Jacoby Class 2be) have allowed for additional capabilities to degrade ESBLs along with *Cep A* (2e), *CTX-M, PER-1,* and *VIB-1 (2be).* Class A carbapenemases can be categorised into six separate groups comprised of *GES, KPC, SME*, *IMI/NMC-A* enzymes, while *SHV-38* and *SFC-1* each separately constitute a group. Class 2d and 2de enzymes are capable of degrading carbenicillin and are generally represented by *PSE, CARB,* and *RTG* genes (Naas *et al.*, 2017). At the time of writing there are 1701 enzymes associated with Class A beta-lactamases (Naas *et al.*, 2017).

Class C Beta-lactamases are primarily active against cephalosporins. Shortly after the initial Amber Classification scheme was proposed the cephalosporinase gene *AmpC* was sequenced and did not fit into either of the classes previously established (Jaurin and Grundstrom, 1981). The Bush-Jacoby systems breaks Ambler Class C into two subgroups. Group 1 contain exclusively cephalosporins and is represented by the *AmpC, CMY, ACT*, *FOX,* and *MIR* genes. Group 2 degrades cefradine and oxy-beta lactams and are represented by *GC1* and *CMY* gene families. Genes in this class are highly conserved and primarily occur in gram-negative bacteria where they are encoded into the chromosome (Zango *et al.*, 2019). At the time of writing there are 3578 enzymes associated with Class C beta-lactamases (Naas *et al.*, 2017).

Class D antibiotics were established by Huovinen et al in 1988 who identified the *PSE-2* serine, later named *OVA-10*, differed sufficiently in its structure from other SBLs to be designated its own class. (Huovinen, Huovinen and Jacoby, 1988). This class is comprised of oxacillinases (OXA) and are capable of degrading isoxazolyl β-lactams like methicillin and oxacillin (Medeiros, Cohenford and Jacoby, 1985). There are three Bush-Jacoby functional groups within Ambler class D. Group 2d are

360 comprised of cloxacillin- and oxacillin- hydrolysing genes *OXA-1* and *OXA-10*. In group 2de the
361 degradation substrate is extended to extended-spectrum cephalosporins (oxyimino-β-lactams), but
362 not carbapenems and contain genes *OXA-11* and *OXA-15* and are frequently encoded on plasmids
363 (Antunes and Fisher, 2014). Subgroup 2df contains carbapenem-hydrolyzing class D β-lactamases
364 (CHDLs). At the time of writing there are 1102 enzymes associated with Class D beta-lactamases
365 (Naas *et al.*, 2017)

366 Ambler Class B enzymes are comprised of carbapenem hydrolysing metallo-beta-lactamases (MBL)
367 in which the hydrolytic centres of each contain at least one active site zinc atom (Ambler, 1980).
368 Both Ambler and Bush Jacoby classification schemes subdivide MBLs into three subclasses based
369 on their functionality, sensitivity to inhibitory metal ion chelators, and number of zinc ligands within
370 their structure (Bush and George A Jacoby, 2010). However after the structural dissimilarities within
371 the *NDM-1* MBL within were discovered, it was suggested that a second B1 subclass be created to
372 accommodate these unique sequences (Yong *et al.*, 2009). Class B1 and B3 are both capable of
373 degrading most beta-lactams, though Class B2 primarily targets carbapenems. The primary genes
374 families with Class B are *IMP*, *VIM, SPM, GIM, NDM*, and *FIM*. Their activity can be inhibited by
375 the addition of the chelating agent EDTA. At the time of writing there are 549 enzymes associated
376 with Class B1, 23 associated with B2, and 225 associated with subclass B3 beta-lactamases (Naas *et*
377 *al.*, 2017).

### 378 2.5.4 Transfer of beta-lactam resistance

379 There are two methods for the transfer of resistance genes between bacteria: vertical gene transfer,
380 as happens when genes are passed from parent to daughter cells through replication, and horizontal
381 gene transfer when genes are transferred from one individual to another within the same generation.
382 The three main mechanisms for horizontal gene transfer are transformation, transduction and
383 conjugation. During transformation bacterium uptake RNA or DNA, often in the form of plasmids,
384 from within the surrounding environment. Transduction occurs when bacterial DNA is moved from
385 one bacterium to another via viruses or bacteriophages. Conjugation occurs when DNA is transferred
386 between bacteria directly via pili. Horizontal gene transfer plays a central role in the spread of

387 antimicrobial resistance as plasmids carry genes from one host to another. In this way genes
388 conferring resistance can spread throughout a bacterial community, even between species (Madigan,
389 2014).

### 390 2.5.5 Beta-lactam Resistance in Wastewater and Anaerobic Microbial Communities

391 The effluent from WWTPs is considered to be one of the most significant conduits for AMR
392 proliferation and the spread of beta-lactam resistant microbes into the environment (Kümmerer,
393 2009a; Ebomah and Okoh, 2020). Several studies have investigated the occurrence of antimicrobial
394 resistance genes (ARGs) in wastewater and the efficacy of wastewater treatment in reducing AMR
395 between the influent and effluent (Rizzo *et al.*, 2013; Biswal *et al.*, 2014; Bengtsson-Palme *et al.*,
396 2016). Although beta-lactams are one of the most widely prescribed and consumed antibiotics, they
397 are rarely detected within WWTP settings. As beta-lactams are highly susceptible to hydrolysis it is
398 generally believed that they are broken down within the sewers in transit to the treatment facilities
399 (Tran, Reinhard and Gin, 2018). Nevertheless, many studies reported beta-lactam resistance genes
400 in most if not all influent and effluent samples tested (Laht *et al.*, 2014; Rodriguez-Mozaz *et al.*,
401 2015; Neudorf *et al.*, 2017; Lin *et al.*, 2021). The dominant genes at each plant varied between studies
402 due to natural variances in treatment, ecology and design, as well as the scope of the project. However
403 the *blaTEM, blaVIM, blaCTX, blaSHV* and *blaOXA* are broadly considered dominant within the
404 wastewater biology (An *et al.*, 2018; Hiller *et al.*, 2019; Subirats *et al.*, 2019). A meta-study of AMR
405 reduction across different wastewater treatment technologies found that although beta-lactamase
406 associated genes were often most concentrated within activated sludge, they were typically lower in
407 the effluent than the influent (Hiller *et al.*, 2019).

408 Within WWTPs anaerobic digesters come in a wide variety of designs as well as both mesophilic
409 (25~45 °C) and thermophilic (50–60 °C) temperatures. Generally membrane based technologies are
410 the most efficient and removing ARGs from the effluent as they effectively separate the sludges from
411 the effluent where they remains within the reactor (Hiller *et al.*, 2019; Nguyen *et al.*, 2021). Do et
412 al., (2022) found that compared to storage or composting, anaerobic was more efficient at reducing
413 the microbial load, ARGs and MGEs in pig slurry. Additionally, anaerobic technologies have been

414 found to limit the spread of antimicrobial resistance compared to composting or conventional
415 activated sludge (Munir, Wong and Xagoraraki, 2011; Tong *et al.*, 2019). Temperature also plays a
416 key role in the reduction of ARGs within anaerobic digestion. Thermophilic digestion has been
417 shown to effectively remove 50–99% of tetracycline ARGs and class 1 integrons in lab scale and full
418 scale digesters (Ghosh, Ramsden and Lapara, 2009; Diehl and Lapara, 2010).

419 Methanogens, which are comprised exclusively of archaeal species, do not contain peptidoglycan in
420 their cell walls and as a result are naturally resistant to beta lactam antibiotics (Hans and König,
421 1996; Khelaifia and Drancourt, 2012). Nevertheless, as anaerobic microbial communities are
422 comprised of a combination of bacteria and archaea, anaerobic communities as a whole are
423 susceptible to, and develop resistance to, beta-lactams. Because anaerobic microbial communities
424 are highly syntrophic, instability within one sub population can lead to instability within the whole
425 system. As such, when exposed to high concentrations of beta-lactam antibiotics, anaerobic microbial
426 communities have been shown to decrease in methanogenic activity (Heidari, Fatemeh Nabavi,
427 Saffari Khouzani, Mehdi Amin, *et al.*, 2012).

428 While there is little research into the proliferation of resistance within anaerobic communities in a
429 wastewater context, many known beta-lactam resistant pathogens are known to grow under anaerobic
430 conditions and have been detected in wastewater (Zagui *et al.*, 2020). For example *Aeromonas sp*,
431 which are facultative anaerobes commonly found in WWTPs, has been shown to be a carrier for
432 beta-lactam resistant genes for all classes of the Ambler scale (Piotrowska *et al.*, 2017). Of the 12
433 antibiotic-resistant "priority pathogens" identified by the WHO as posing the greatest threat to human
434 health, seven are beta-lactam resistant strains and 11 are capable of growing under anaerobic
435 conditions (**Table 2-2**) (WHO, 2017). However, anaerobic treatment of sewage known to contain
436 beta-lactam resistant microbes has repeatedly been shown to reduce the relative abundance and total
437 counts of AMR within the effluent compared to influent (Resende *et al.*, 2014; Zhang *et al.*, 2016;
438 Kanger *et al.*, 2020). Zhang, Yang and Pruden (2015) found that biomatter within effluent from
439 mesophilic anaerobic digestion contained less than 10% the AMR genes counts relative to the
440 influent. However research by Ju (2016) found that while most ARGs were removed during

441 anaerobic digestion, the species that remained in the effluent (mainly *Collinsella aerofaciens,*
442 *Streptococcus salivarius* and *Gordonia bronchialis*) were of pathogenic importance (Ju *et al.*, 2016).

443 ***Table 2-2*** *- Antibiotic-Resistant "priority pathogens" adapted from the WHO guidelines from 2017. (WHO, 2017)*

| Species | Resistance | Oxygen Tolerance |
|---|---|---|
| **Priority 1: CRITICAL** | | |
| *Acinetobacter baumannii*, | carbapenem-resistant | aerobic |
| *Pseudomonas aeruginosa*, | carbapenem-resistant | obligate aerobe (although can grow anaerobically in the presence of nitrate) (Health protection agency, 2015) |
| *Enterobacteriaceae*, (including Salmonella, Escherichia coli, Klebsiella, and Shigella) | carbapenem-resistant, ESBL-producing | **facultative anaerobes,** |
| **Priority 2: HIGH** | | |
| *Enterococcus faecium*, | vancomycin-resistant | **facultative anaerobes** |
| *Staphylococcus aureus* | methicillin-resistant, vancomycin-intermediate and resistant | **facultative anaerobes** |
| *Helicobacter pylori*, | clarithromycin-resistant | **facultative anaerobes** |
| *Campylobacter* spp., | fluoroquinolone-resistant | **strict anaerobe** |
| *Salmonellae*, | fluoroquinolone-resistant | **facultative anaerobes** |
| *Neisseria gonorrhoeae* | cephalosporin-resistant, fluoroquinolone-resistant | obligate aerobic (although can grow anaerobically in the presence of nitrate) |
| **Priority 3: MEDIUM** | | |
| *Streptococcus pneumoniae*, | penicillin-non-susceptible | **facultative anaerobes** |
| *Haemophilus influenzae*, | ampicillin-resistant., | **facultative anaerobes** |
| *Shigella* spp | fluoroquinolone-resistant | **facultative anaerobes** |
| | | |

444

445 **2.5.6 Methods to Quantify AMR Resistance**

446 Early detection of antimicrobial resistance was developed from diagnostic culture techniques
447 developed for the assessment of drug suitability and disease identification (Madigan, 2014). Culture
448 methods aimed to measure the minimum concentration of antibiotic needed to prevent the growth of
449 the test culture. This was useful in a clinical setting in order to develop treatment, though was also
450 able to detect the presence of resistant pathogens (Shanmugakani *et al.*, 2020). However, there were
451 limitations to this method. Culture techniques were only suitable for microbes which were readily
452 culturable in plate and broth media. Additionally, only one strain could be detected at a time.

Furthermore the test would only detect phenotypic resistance and was not able to track any genes that were inactive or residing in non-culturable microbes (Shanmugakani *et al.*, 2020).

With the advancement of gene sequencing and polymerase chain reaction (PCR) technologies it became possible for the resistance genes themselves to be detected (Hiller *et al.*, 2019). As such environmental surveys began to be conducted on antibiotic restrained within wastewater and the environment. This allowed for a rapid identification of ARGs directly. There were two limitations to this technology. The equipment required was advanced and expensive. As the method was only capable of targeting a single gene target at a time, researchers needed to know ahead of time which genes they were aiming to find. Additionally, while it could detect the genes present, qPCR was not able to identify which species were resistant, only if resistance genes were present within the community.

The development of the high-throughput qPCR array in the late 2010s allowed for the simultaneous detection of hundreds of mobile genetic elements (MGEs) and ARGs (Looft *et al.*, 2012). Parallel analysis cut down on the time and the cost of running the tests (Waseem *et al.*, 2019). Furthermore, as thousands of primers have been developed, the large number of tests available made it possible to screen large gene sets, which is particularly useful for environmental and water samples (Stedtfeld *et al.*, 2018). However, as these systems run in parallel, all samples were run under the same PCR conditions, it was not possible to optimise conditions for the amplification of each primer. This resulted in variable sensitivity across the libraries (Waseem *et al.*, 2019). However, the technologies are still currently widely used with several commercial available tools available, of which the Takara (previously Wafergen) SmartChip instrument which can amplify up to 5184 qPCR assays per chip within 3–4 h allowing up to 384 primer sets can be analysed in parallel, is the most popular (Waseem *et al.*, 2019).

## 2.6 KNOWLEDGE GAPS

To fully understand how beta-lactams impact decentralised water treatment, it is first important to understand how they impact the treatment efficiency of anaerobic systems. However, there is limited

479 research into the effects of beta-lactams on anaerobic systems. When it is, the conditions of these
480 studies are varied and often focused on higher concentrations than would be found in water treatment
481 facilities and the results do not always agree. In general, it was found that the impact of beta lactams
482 on treatment efficiency are limited at low concentrations. For example, Sanz found a maximum
483 inhibition of 10 mg/L penicillin led to a partial inhibition of 25-45% methane production (Sanz,
484 Rodríguez and Amils, 1996). Anaerobic digesters running on pig slurry from animals with 16 mg/kg
485 of penicillin in their feed reported a 35% reduction in methane generation when compared to the
486 digesters running wastes from pigs fed a control feed (Massé *et al.*, 2000). Conversely, Zhang et al
487 found that anaerobic systems acclimated to amoxicillin could withstand concentrations up to 60mg/L
488 before displaying any inhibition of methane generation (Zhang *et al.*, 2015). A study by Su et al
489 (2019) did not report any inhibition of methane in anaerobic granules exposed to 100ppm amoxicillin
490 (Su *et al.*, 2019). As these studies vary in design and application, it is difficult to make broad
491 conclusions. **The aim of this thesis is to further explore how amoxicillin antibiotics influence**
492 **anaerobic microbial communities specifically in terms of activity, treatment efficiency, and**
493 **community ecology and resistome.**

# Chapter 3
# Materials and Methods

The aim of this chapter is twofold. Firstly, several of the studies within this thesis include the culturing of anaerobic granules, and, whilst some of the details of each experiment were unique, many of the methods remained consistent across all studies in which they appeared. Therefore, this chapter summarises those methods that are repeated. Secondly, method development was required for amoxicillin quantification using LC-UV as well as PCR. The optimisation and development of these methods are laid out in this chapter.

## 3.1 ANAEROBIC CULTURING

### 3.1.1 Source And Characteristics of Anaerobic Sludge

The sludge used in all trials was sampled on 6th July 2018 from a full-scale mesophilic Expanded Granular Sludge Bed (EGSB) bioreactor operated by the North British Distillery Company in Edinburgh, Scotland. The granules had historically been treating distillery waste. The sludge was transported back to the lab in two 30L jerry cans. The process engineers at the facility reported that the digesters had not been recently fed prior to sampling. The sludge was stored in the basement of the Rankine building on the University of Glasgow campus at room temperature and irregularly fed glucose with nutrient additives (Section 3.2.1) until use. Although the graneuls had previously been optimised to distillery waste as their primary substrate, with the alternative and intermittent feeding it is likely that they instead optimised for both glucose fermentation and / or dead cellular matter within the culture.

23 ### 3.1.2 Tracking Methanogenic Activity for Specific Methanogenic Assay (SMA) and
24 General Methanogenic Assay (GMA)

25 Methanogenic activity of the granular sludge was measured using an adapted version of the pressure
26 transducer technique in which the change in headspace pressure over time within sealed serum vials
27 containing sludge samples and a specific substrate (food) is used to estimate methane production
28 rates (Colleran et al., 1992; D. Coates, F. Coughlan and Colleran, 1996). The specific methanogenic
29 activity of each test culture was measured against simple substrates: acetate, ethanol, propionate, and
30 butyrate as described by (Colleran et al., 1992). Additionally, activity was measured using a general
31 methanogenic rate assay in which glucose and a complex broth were used as substrate (modified
32 from the OECD 224 (OECD, 2007)). During the experiment, a known mass of substrate was added
33 to a sealed bottle containing an anaerobic culture medium and granules. The change in pressure in
34 the headspace of the vials was intermittently monitored over time as the substrate was converted to
35 methane and carbon dioxide using a pressure transducer. The methane concentration in the headspace
36 gas was measured at the end of the experiment (**Figure 3-1**). The pressure changes and methane
37 concentration were then used to determine the methanogenic activity of the sludge against a specific
38 substrate. As each substrate used can only be broken down by a subset of the population, using an

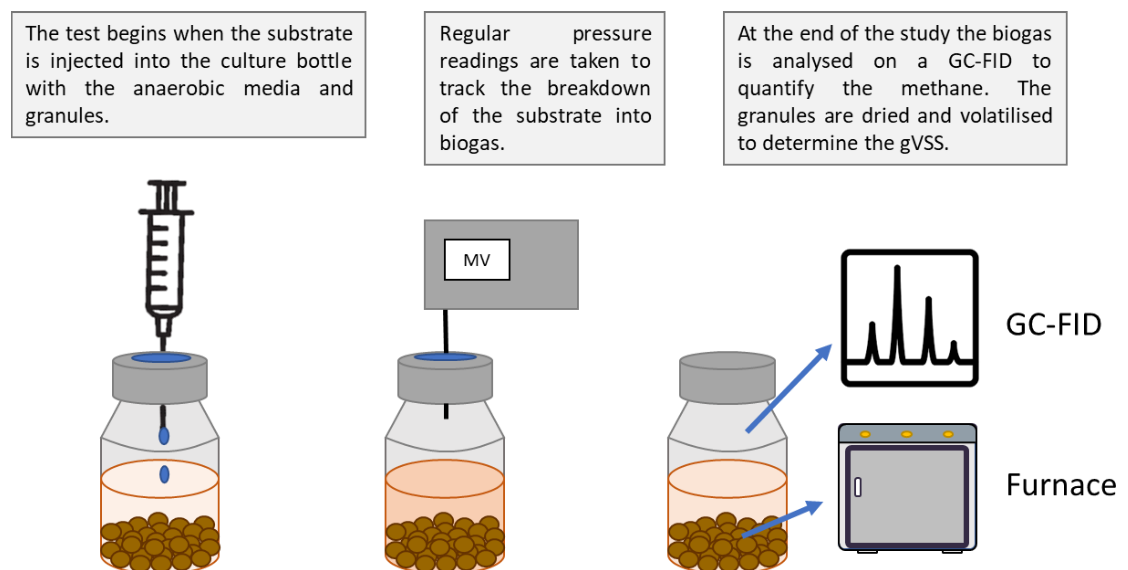



***Figure 3-1*** *- The specific methanogenic assay (SMA) tracks the changes in pressure over time. The pressure data and methane quantification data are used to estimate net methane production and to estimate the activity rate of the microbes within the culture when incubated with specific substrates.*

39 array of different substrates indicates the activity of each sub population associated with each
40 substrate type. While the design specifics for each experiment differed slightly, the preparation of
41 the bottle, granule acclimation, pressure readings, and analytical methods were all similar between
42 experiments. These methods are described below.

43 Sample bottles were prepared by soaking overnight in Virkon™ (Lanxess) solution followed by
44 triplicate rinsing with Milli-Q® Type 1 Ultrapure Water (Milli-Q) and air drying. Within an
45 anaerobic chamber, 0.5g of sludge granules were added to 30ml bottles (Sigma Aldrich, Catalogue
46 no. 33106) along with 9.5ml of anaerobic buffer to provide approximately 10 g/L VSS per assay.
47 The bottles were sealed using rubber bungs and aluminium crimp caps before removing from the
48 chamber (Sigma Aldrich Catalogue No. 508500). Once removed, the headspace gas in each bottle
49 was exchanged to 1 atmosphere (ATM) with a 20% $CO_2$ and 80% $N_2$ mixture by flushing for 30
50 seconds. Each test condition was prepared in triplicate. Once filled, sealed, and flushed, the culture
51 bottles containing granules and culture medium (a mixture of buffers, oxygen scavengers, indicators,
52 and metal nutrients) were acclimated at 37°C for 3 days prior to commencing the monitoring period.
53 To minimise the time to initial methane production, a method development study (Chapter 5) was
54 conducted. The study demonstrated that the addition of substrate during the acclimation period
55 reduced the length of the lag phase, reducing the experiment duration and resources for the research
56 team. Thus, each substrate was also added at the offset of the acclimation phase for each of the
57 experiments. The specific substrates used differed for each study. After the acclimation incubation,
58 each bottle was opened within the anaerobic chamber. The liquid medium within was removed using
59 a pipette and discarded. A mix of fresh anaerobic medium, buffer and nutrients were added to the
60 acclimated granules and amoxicillin added as appropriate to the specific test. Bottles were resealed
61 within the anaerobic chamber. The headspace gas was flushed for 30 seconds with a 20% $CO_2$ and
62 80% $N_2$ mixture to a final pressure of 1ATM to remove any methane or oxygen present. After the
63 head space gas was exchanged, the relevant liquid substrate was injected into each bottle through the
64 septa and placed into a 37°C shaking incubator with the time of feeding representing the
65 commencement of the experiment. The set of liquid substrates and amoxicillin concentrations used
66 were different for each experiment conducted and details are be described in the relevant chapter.

During each experiment, pressure readings were taken every 1 -4 hours using a Centrepoint pressure transducer until the pressure within each bottle began to plateau at which point the frequency of readings was reduced. At the end of each experiment the biogas within the headspace of each bottle was removed using a syringe needle through the septa. The methane content of the biogas was determined using a GC-FID biogas analyser (described in detail in section 3.3.1). The volatile suspended solids (VSS) of the biomass were measured as described in Section 3.3.2. The methanogenic activity was calculated as described in Section 3.3.3.

## 3.2 MATERIALS

### 3.2.1 Anaerobic Medium Components and Preparation

The anaerobic buffer was comprised of resazurin (0.4ml/L) as an anaerobic indicator and l-cysteine (0.56g/L) as an oxygen scavenger. The indicator and l-cysteine were dissolved in 1 L of Milli-Q water then pH adjusted using 8N NaOH until it reached 7.0 – 7.2 pH. This solution was then boiled until colourless, indicating that there is no dissolved oxygen in the solution, while the headspace was continually purged with nitrogen gas. Once the medium was completely colourless, the bottle was placed in an ice bath to cool. When the temperature of the solution reached 50°C, 3.05g of sodium bicarbonate was added to increase the alkalinity of the medium. The buffer was then stored under anaerobic conditions and used within 48 hours.

### 3.2.2 Trace Metals and Additives

Some of the studies contained additional nutrient additives (modified from Shelton & Tiedje, 1984) and buffer as detailed in Chapters 4, 5, 6, and 7 within the dissertation. The nutrient additives included a "Nutrient Mix", "Trace nutrient mix", and phosphate buffer solution. The additive mixes were prepared and stored separately to allow preparation at different stock concentration to make the dilutions easier to prepare and more accurate, and to reduce the likelihood of interaction between the chemical components in each solution. Bottles used to prepare stock solutions of each mix were soaked in a Virkon™ solution, rinsed three time with Milli-Q water and fully dried before use. The Nutrient Mix was prepared at 10x the final working concentration and contained 5.3 g/l of $NH_4Cl$;

93 0.75g/L of $CaCl_2$; and 1g/L Mg Cl * $6H_20$ in 100ml Milli-Q. The Trace nutrient mix was prepared
94 as a 500x mix of final concentration and was comprised of 20g/L of $FeCl_2$*$4H_2$; 0.5 g/l $MnCl_2$ *
95 $4H_2O$; 0.05g/l $H_3BO_3$; 0.05g/l $ZnCl_2$; 0.03 g/l $CaCl_2$; 0.01 g/l $NaMo_4$*$2H_2O$; 0.5 g/l $CaCl_2$ * $6H_2O$;
96 0.05 g/l $NiCl_2$ * $6H_20$; and 0.05 g/l $SeO_2$. The $FeCl_2$*$4H_2O$ was added to 10ml of 25% (7.7M) HCl
97 before dilution and mixture with the trace nutrient stock solution. A phosphate buffer was added to
98 the anaerobic medium to maintain 7.0pH within the bottles. The phosphate buffer was mixed at 10x
99 the final concentration and contained: 2.7g/L $H_2KPO_4$, and 3.5g/L of $K_2HPO_4$. Both nutrient mixes
100 and the buffer were autoclaved after preparation and stored in the refrigerator at 4°C for the length
101 of the experiment. The nutrient composition was adapted from Shelton & Tiedje, (1984).

102 ## 3.3 ANALYTICAL METHODS AND CALCULATIONS

103 ### 3.3.1 Quantifying Methane on the GC-FID

104 To quantify the methane content of the biogas in the headspace of sample bottles, a 2.0 ml sample of
105 headspace gas was removed from each bottle using a standard 20ml syringe and a luer lock. As the
106 upper limit of detection for the equipment was 5.0% methane, each gas sample taken from the vial
107 was diluted in the syringe 1:10 with $N_2$ to 20ml total volume before analysis. Methane content in the
108 biogas was quantified using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a
109 GS-CarbonPlot capillary column and a flame ionization detector. Instrument parameters were: inlet
110 temperature - 250 °C; detector temperature - 250 °C; $H_2$ flow, 30 mL/min; air flow - 400 mL/min,
111 nitrogen as a carrier gas at a constant pressure of 15 psi. The oven temperature was 150 °C for 10
112 min.

113 ### 3.3.2 Calculating VSS

114 The volatile solids content of the biomass was measured in accordance with Standards Methods 2540
115 B and 2540 E (Association., Association. and Federation., 1998). Volatile suspended solids (VSS)
116 are a measure of the volatile material within the sample. This material is assumed to be organic and
117 is used as a proxy for biomass. To determine the VSS in each sample vial at the end of each test, the
118 total contents of the culture bottle were filtered through 0.45um Whatman® membrane glass fibre

119 filter papers using a vacuum pump and filter apparatus. The filters and sample were dried in a 100°C
120 oven for 48 hours then the combined mass of each filter and sample was determined by weighing.
121 The filters and samples were then transferred to a furnace and volatilised at 500°C for 4 hours to
122 remove any volatile solids and the combined mass of the filter and ash determined by weighing. The
123 mass of each filter was recorded before measurement and filtration to enable calculation of the dried
124 sample mass and ash mass.

125 The mass (g) of the volatile solids (VSS) was calculated:

*VSS(g) = (Final Mass dried sample(g) – Mass Filter (g)) – (Mass Sample Ash (g)– Mass Filter(g))* ***Equation 1***

126

127 ### 3.3.3 Calibrating Pressure Transducer

128 Measuring changes in microbial activity using the specific methanogenic assay requires tracking
129 pressure changes in the headspace of culture bottles. This is achieved with the use of a pressure
130 transducer and is reported in millivolts (mv). Thus, to enable reporting of pressure in units ATM, a
131 calibration curve was obtained by injecting known volumes of gas into a container of a known
132 volume and measuring the corresponding pressures. This was done using 30ml serum vials, as were
133 used in the SMA test, with 10ml of water added to each bottles to simulate the 10 ml of medium and
134 granules present during the studies leaving 27ml of headspace. Five bottles were stoppered and
135 sealed. The stoppers were pierced with a needle and left to equilibrate for 5 seconds, after which an
136 initial reading was taken. After the initial readings were taken, 2ml of air was injected into each of
137 the bottle and a pressure reading was taken. This was repeated until a total of 30 ml was injected into
138 each of the bottles with corresponding pressure readings taken for each step.

139 The gas volume added (mL) was then plotted against the pressure readings (mv) to produce the
140 calibration curve (**Figure 3-2**). A best fit regression line was calculated for the collected data to
141 produce a set of constants which could be used to calculate gas volume (ml) from experimentally
142 measured pressure readings (mv) **Equation 2** (n=80, slope = 4.0797, intercept = 1.6624, $R^2$ =
143 0.9984).

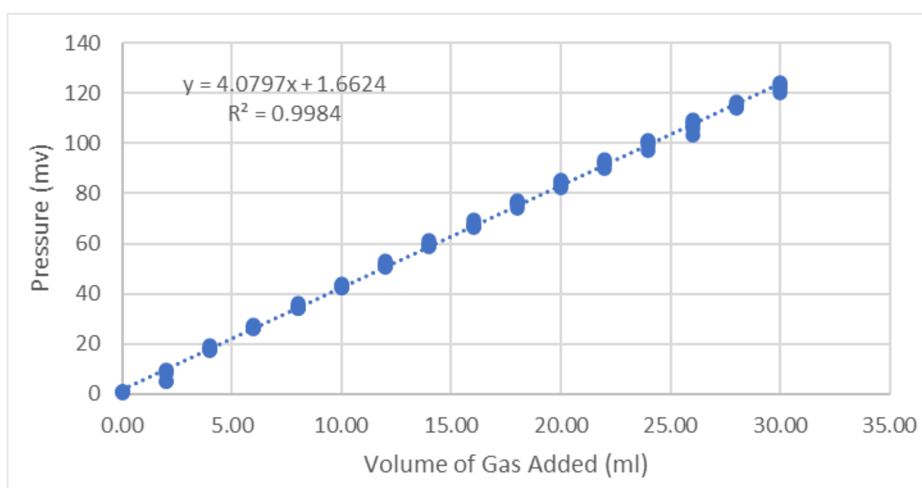



***Figure 3-2** – Calibration curve for gas volume.*

*The curve compared the known volume of gasses added to the bottle and the corresponding pressure readings they produced. A best fit line was produced using experimental*

144

$$Volume\ (ml) = \frac{Pressure\ (mv) - 1.6624}{4.0797} \qquad \textit{Equation 2}$$

145

146 ### 3.3.4 Calculating Methane Volume

147 The pressure within culture bottles was measured (as mV) using a pressure transducer. The recorded

148 values were converted into total biogas volume (ml) using the conversion calculation in **Equation 3**.

$$Total\ Biogas\ Volume\ (ml) = \frac{Pressure\ (mv) - 1.6624}{4.0797} \qquad \textit{Equation 3}$$

149

150 The methane volume produced in each test vial was then calculated using the pressure data combined

151 with the methane concentrations as measured by the GC-FID as is described in **Equation 4**.

$$Total\ Methane\ (ml) = Total\ Biogas\ Volume\ (ml) * Methane\ Percent\ (\%) \qquad \textit{Equation 4}$$

152

153 ### 3.3.5 Rate of methane production

154 The methanogenic activity rate was calculated for each test by determining the slope of the best fit
155 line through the steepest straight-line section of each plot of methane produced over time. The
156 number of points used varied for each test condition, though no fewer than 3 points were used in this
157 calculation and details are discussed in each experimental chapter.

158 A best fit slope was calculated (using Excel software) for each of the consecutive points using mls
159 of methane per gVSS as the response variable (y) and time in hours at the treatment variable (x). The
160 best fit slope with the greatest value was taken as the maximum rate value. This value was then
161 divided by 24 to convert the figures to the units ml of $CH_4$ / gVSS *day.

162 ### 3.3.6 Lag Time

163 As microbial populations adjust to their new environment there is often a delay between substrate
164 addition and activity, known as the lag phase. The addition of amoxicillin within the culture medium
165 was also found to influence the length of lag between substrate addition and biogas production
166 (Chapter 6). To capture the influence of the amoxicillin on lag time, it was necessary to identify the
167 first point of biogas production. For this study, length of the lag phase was defined as the time
168 between substrate addition and first point used to calculate the maximum rate of methane production
169 as found in (Section 3.3.5).

170 ### 3.3.7 Calculating Percent Inhibition

171 To determine the inhibitory effect of amoxicillin on the potential treatment capacity of the microbial
172 communities, the percent inhibition of activity was calculated. Percent inhibition calculations were
173 modified from the calculations described in section 37 The OECD: Determination of the inhibition
174 of the activity of anaerobic bacteria: reduction of gas production from anaerobically digesting
175 (sewage) sludge (OECD, 2007). Although the OECD guidelines focus on total biogas produced, this
176 study has expanded this calculation to include relative inhibition of total biogas produced (mL), total
177 methane produced (mL), and maximum rate of methane production (ml of $CH_4$ / gVSS *day). Total
178 biogas and total methane production (reported as mean of triplicate tests) was defined as that which
179 had been produced in a given test condition 66 hours after the addition of substrate. The maximum

rate of methane compares the values as calculated in section 3.3.5. Each condition compares the relevant value for the test condition (with amoxicillin) to the control condition (without amoxicillin) (**Equation 5**).

$$Percent\ inhibition = \frac{Pc}{\overline{Pt}} * 100$$

*Equation 5*

$Pc$ – *The mean value within the control condition.*

$\overline{Pt}$ – *The mean of the values for the test condtions*

## 3.4 MOLECULAR MICROBIOLOGY METHODS

DNA was extracted using the Fast DNA spin kit for soil (MP Biomedical) and Fast Prep -24 instrument. The kit was primarily used as directed with additional optimisation for the sample mass of homogenised granule and length of bead beating for cell lysis (as detailed in **Table 3-1** and **3-2**) to maximise DNA yield. Method optimisation was conducted using the same batch of granules from which the experimental samples were taken. DNA concentrations were determined using the Qubit 2.0 Fluorometer and Qubit dsBR (Broad Range) Assay kit (Invitrogen).

***Table 3-1*** *- Testing granule mass (g) used in the DNA extraction kits which would result in the highest concentration of DNA recovery (ng/ul).*

| Granule Mass (g) | DNA Concentration (ng/ul) | Conclusion |
|---|---|---|
| 0.15 | 164 | Use 0.3g sample in the future. |
| 0.25 | 236 | |
| 0.35 | 225 | |

200 *Table 3-2 Testing the lysis time for the DNA extraction kits for the highest concentration of DNA recovery (ng/ul).*

| Lysis Time | DNA Concentration (ng/ul) | Conclusion |
|---|---|---|
| 20 sec | 185 | Run the lysis procedure for 40 seconds, as recommended by the kit instructions. |
| 30 sec | 195 | |
| 40 sec | 323 | |

201
202
203
204
205

206 The quality of the extracted DNA during optimisation was inspected for shearing on a 1% agarose
207 gel with a SYBR® Safe DNA Gel Stain (Invitrogen Life Technologies) with visualisation again a
208 1kb Plus DNA ladder (Invitrogen Life Technologies) and run at 90V for 40 minutes. It was found
209 that, although some shearing was observed, the quality of the primary DNA was adequate for use in
210 the downstream processes: PCR for next generation sequencing and qPCR for AMR quantification.

## 211 3.5 Amoxicillin Quantification my LC-UV Method Development

### 212 3.5.1 LC-UV Instrumentation and Operating Conditions

213 The amoxicillin was analysed by a Shimadzu LC20AT liquid chromatograph (Kyoto, Japan)
214 equipped with an SIL 20AHT Autosampler and a DGU-20 A Degassing unit. Chromatographic
215 separation was achieved using a Purospher® STAR RP-18 end capped column (5µm, 150mm x 4.6
216 mm). Detection was conducted using an SPD-10 UV Detector. The system was controlled by
217 LabSolutions (Agilent Technologies).

218 The column was conditioned by flushing a mixture of 1/9 Methanol and Acetate Buffer (0.01M, pH
219 5.0) through the column for 30 minutes. At the end of each set of runs the column was flushed with
220 4/1 Methanol and MilliQ (Millipore) water for one hour before storage to prevent the crystallisation
221 of salts on the column.

222 The optimal operating conditions for the 25 min method were: injection volume 100µl, flow rate 0.9
223 ml/min, mobile phase A: acetate buffer (0.01M, pH 5.0), mobile phase B: HPLC grade methanol.
224 The elution gradient: 0 - 4 min 10% B, 4 - 18 min 10 - 30% B, 18 - 20 min 30% B, 20 - 25 min 10%.

225 All samples were analysed using pH and conductivity. Conductivity readings were taken using an
226 Orion 013605MD conductivity cell. pH readings were taken using an Orion 8156BNUWP Ross Ultra
227 combination pH probe. Reads were measured using the Orion 5 Start meter.

228 ### 3.5.2 Reagents and Chemicals

229 Amoxicillin was supplied by Sigma Aldrich. Methanol was HPLC grade and supplied by Sigma
230 Aldrich. Solutions were prepared with Milli-Q® Type 1 Ultrapure Water.

231 Acetate buffer used in mobile phase was comprised of: Sodium Acetate 0.55g, Acetic Acid 0.12g,
232 MilliQ water 1000ml. The buffer was pH adjusted to pH 5.0 by adding NaOH (1M) as needed. All
233 bottles were washed with methanol and MilliQ water before use during mixing. The mixed buffer
234 was filtered through a 0.45µm filter (type) before use.

235 ### 3.5.3 Preparation of Standard Stock Solutions

236 Stock solutions were prepared by weighing 0.2g of Amoxicillin powder into 200ml of MilliQ water
237 to create a 1000 ppm stock. This bottle was covered with foil and stored at 3°C for the next several
238 weeks to be used as a base for other stock dilutions. A 10ml sample of this solution was filtered
239 through a 0.45um Whatman® membrane glass fibre filter and diluted with MilliQ to create a 100
240 ppm stock solution which was used as the base for all other dilutions.

241 ***Table 3-3 -*** *Stock solutions used to optimise LC-UV method.*

| Final Stock Volume (mg/L) | Concentration used for dilution (mg/L) | Volume of stock used (ml) | Milli Q Added (ml) | Total Volume (ml) |
|---|---|---|---|---|
| 10 | 100 | 1.0 | 9.0 | 10 |
| 1 | 10 | 1.0 | 9.0 | 10 |
| 0.5 | 1 | 5.0 | 5.0 | 10 |
| 0.4 | 1 | 4.0 | 6.0 | 10 |
| 0.3 | 1 | 3.0 | 7.0 | 10 |
| 0.2 | 1 | 2.0 | 8.0 | 10 |
| 0.1 | 1 | 1.0 | 9.0 | 10 |

242

243 ### 3.5.4 Repeatability

244 Repeatability measures the performance of a method on repeated samples within a single day, or
245 intra-day variation of the peak area, retention times, and migration times. Each of the five

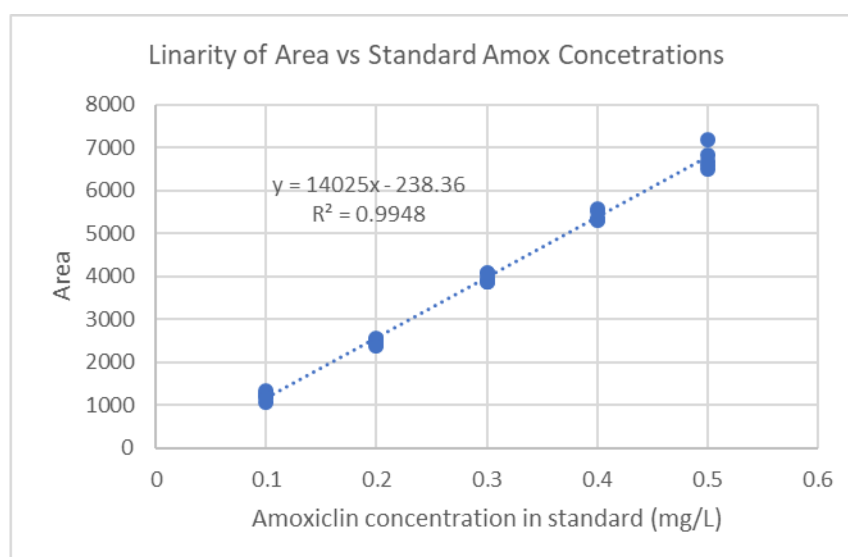
246 concentrations were analysed five times, three running from lowest to highest, and two running from
247 highest to lowest, with two blank samples of pure MilliQ water run between each set of standards.

248 The average values for the retention time for the 25min method (n=25) was 4.4088 min with an RSD
249 of 0.95%. The RSD values for the peak areas of each of the five concentrations (n=5) ranged from
250 1.97 to 7.44%. Over the five sets, the mean set run migrated from 4.42 min (set 3) to 4.42 (set 5) in
251 which set order and mean retention time for each set are not statistically significant ($r^2 = 0.306$). This
252 indicates that the repeatability of this method is acceptable.

### 253 3.5.5 Linearity

254 Linearity is determined to include a series of at least five different concentrations within the target
255 range (FDA, 1995; US FDA, 1996). Linearity for the 25 min method was checked from a range of 0.1
256 – 0.5 which included the concentrations (0.0, 0.1, 0.2, 0.3, 0.4, 0.5). Linearity was measured for
257 peak area for each of the standards resulting in a slope of 14025 and an intercept of -238.36 with an
258 $r^2$ value of 0.995.

259




260

261 *Figure 3-3 – Linearity of the area for standard amoxicillin concentrations.*

262

263 ### 3.5.6 Limit of Detection

264 The limit of detection and quantification was calculated using the signal to noise ratio method as
265 explained in *Guideline for Industry: Text on Validation of Analytical Procedures* (FDA, 1995; US
266 FDA, 1996). The baseline noise and signal to noise ratio (S/N) for each sample was calculated by
267 the LabSolutions (Agilent Technologies) software. Per these guidelines, the lower limit of detection
268 (LOD) was defined as a S/N ≥ 2 or a sample that causes a peak twice the height of the background
269 noise. The lower limit of quantification (LOQ) was defined as S/N ≥ 10. The S/N values for each
270 standard were fitted to a regression line. These values were used to calculate the LOD and LOQ for
271 each method. The 25 min method generated a S/N regression with a slope of 61.994, an intercept -
272 4.6478 of, and an $R^2$ value of 0.9369. These values resulted in a calculated LOD of 0.123 and an
273 LOQ of 0.236.

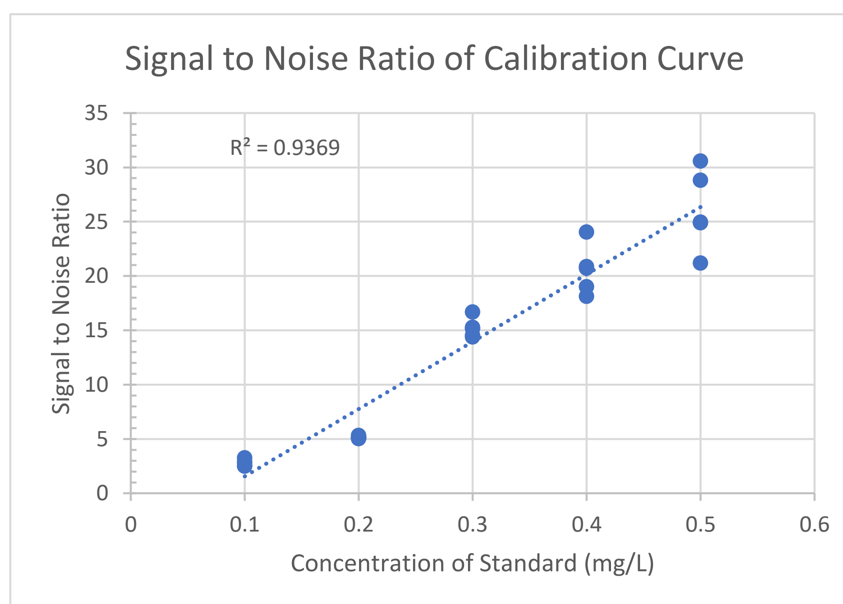



***Figure 3-4*** *- Signal to Noise Ratio of Calibration Curve*

274

# Chapter 4
# Method Optimisation and Baseline Study - Specific Methanogenic Assay

## 4.1 INTRODUCTION

The specific methanogenic assay (SMA) was utilised to study the effect of amoxicillin on methane production and to infer the relative impact of amoxicillin on different trophic groups in anaerobic sludge granules. The SMA was developed by D. Coates, F. Coughlan, & Colleran, (1996) and Shelton & Tiedje, (1984) and tracks the change in pressure in sealed bottles containing anaerobic medium and a known feed type to infer the rate of methane produced by the anaerobic culture. Like larger ecosystems, the mixed microbial communities found in anaerobic sludge granules contain microbes mediating a trophic cascade in which different members of the population are capable of metabolising organic matter of differing complexities which is sequentially broken down and eventually converted into predominantly methane or carbon dioxide by methanogens (Stams, 1994; Gerardi, 2003; Pol *et al.*, 2004). Within the system, a wide variety of complex polymers are hydrolysed and fermented by an equally diverse set of microbes into a relatively few simple compounds most notably acetate, ethanol, propionate, and butyrate. Each of these compounds are metabolised and transformed by a subpopulation of specialist organisms. The SMA aims to test the activity of these specific specialised subclasses within the microbial community. When the test

bottles are fed specific substrates of differing complexities corresponding to a specific level of the anaerobic trophic cascade, the rate of methane produced can be used to infer the relative activity of the members of that trophic level. An imbalance in microbial activity at any point in the methanogenic food chain in natural or engineered environments can result in the build-up of a single food source which can in turn change the environmental growth conditions, such as pH or ammonia concentration, which can be toxic to the system setting off a chain reaction further reducing activity. Thus, the methanogenic activity rates can be used to infer the activity of the community as a whole and its capacity to degrade certain substrates, as well as overall system stability.

Whilst the monitoring and measurement methods associated with the SMA are well established, the duration of the SMA and methods for sample preparation vary with sample type and substrates used. **Therefore, to inform subsequent experimental methodology and design, a preliminary experiment was conducted to**

**(i) establish a baseline of activity in terms of maximum rate of methane production, window of maximum activity, and time required to exhaust the substrates provided; and**

**(ii) to optimise the sample preparation method.**

## 4.2 METHODS

### 4.2.1 Preliminary Study Design Overview

An array of culture bottles were prepared and monitored in which each bottle contained a single substrate. Four substrates were used: acetate, ethanol, propionate, and butyrate (**Table 4-1**). Each culture bottle contained anaerobic granules, anaerobic medium, and one substrate (or water in the case of the no substrate controls subsequently called 'blanks'). Each condition was prepared in triplicate. Activity was measured through changes in headspace pressure (mv) using a pressure transducer. The pressure (mv) data was subsequently converted into total biogas and total methane volumes (ml). The bottles were monitored intermittently for 152 hours after feeding (6 days).

49 *Table 4-1 - Study Parameters for Anaerobic Cultures*

| Parameter | Design Value or Condition |
|---|---|
| Temperatures | 37°C |
| Feed Type | Acetate (3M), Ethanol (3M), Propionate (3M), Butyrate (1.5M) |
| Replicates | 3 |
| Bottles Used | 18 |
| pH | 7.0 |
| Bottle Volume | 30ml |
| Granule Mass | 0.5 g |
| Total Volume of Liquid Medium (Anaerobic Medium + granules) | 10ml |
| Length of study | 152 hours (6 days) |

50

### 51 4.2.2 Investigation of sample preparation: Drain v Pipette

52 Optimum SMA preparation requires a consistent food to biomass ratio in each test vial to ensure that
53 results are comparable between assay replicates and conditions. This is achieved through consistent
54 culture inoculation with equal masses (gVSS/L) of sludge granules within each culture bottle. When
55 weighing sludge granules however, liquid medium may be transferred to the weighing boat along
56 with granules which could impact consistency of measurement. Thus, here, two different methods
57 for removing the water from the sludge granules before weighing them for use in SMA assays were
58 tested. The "drain method" removed the water by pouring a portion of granules over a sieve such that
59 the water would flow away from the granules, which would then be transferred to a weigh boat using
60 a spatula. This is the standard method used within the lab. In an alternative method, the "pipette
61 method", the water was removed using a pipette then the granules transferred to the weigh boat using
62 a spatula. Both methods were conducted inside an anaerobic chamber to preserve anaerobic
63 conditions during the test. Granules were re-submerged in liquid in test vials within 5 minutes of
64 weighing to ensure granules did not dry out prior to testing (**Figure 4-1**).

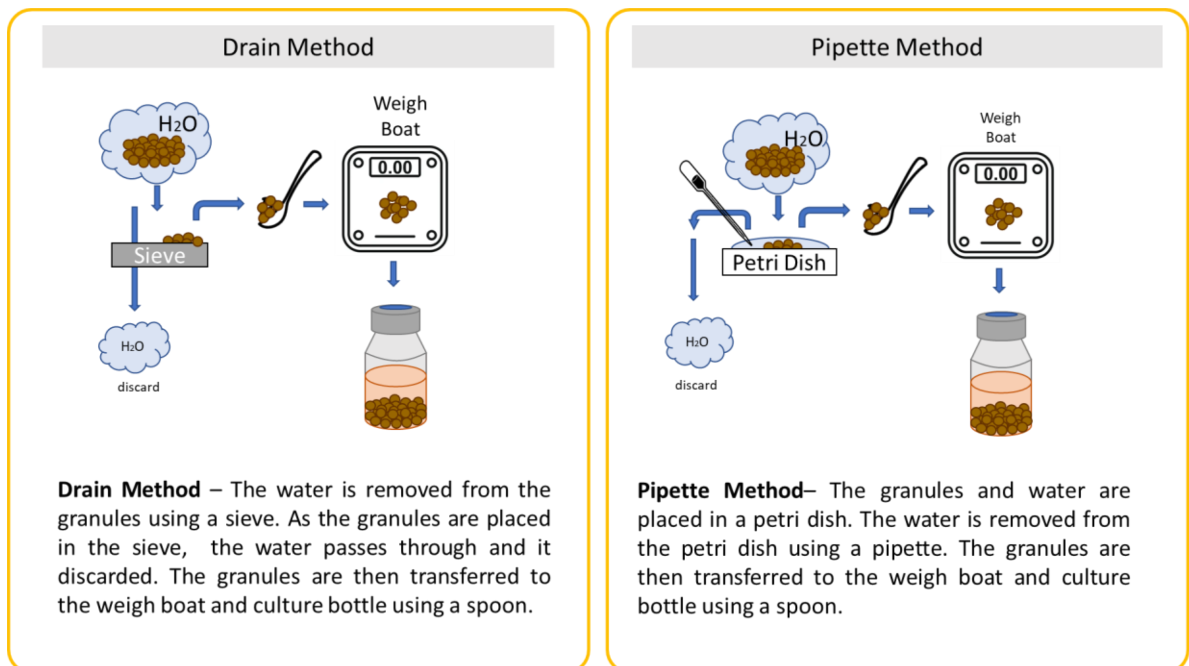



***Figure 4-1*** *- Drain versus pipette method summary.*
*The drain vs the pipette method for removing the water from the granules before weighing and transporting into the culture bottles.*

65

66 Each method posed relative pros and cons. Visual inspection determined that the pipette method
67 preserved a wider range of granule sizes than the drain method as smaller granules were removed
68 when the water drained out of the sieve. This is important because emerging evidence suggests that
69 granules of different size, putatively at different points in the life cycle of the granule (Trego *et al.*,
70 2020), are both physiologically and ecologically different. It is possible that some of the biomass lost
71 through the sieve are flocs rather than active granules. Nevertheless, retaining a diversity of granule
72 sizes should better preserve the microbial community composition to reflect that within the larger
73 reactor from which the granules were sampled. Furthermore, as the pipette method ensured retention
74 of more small granules, the granules sample would have comparatively more surface area which
75 could therefore result in higher rate of methane production. Whilst the pipette method was effective
76 at preserving granule size heterogeneity, removing the water from the granules reliably and
77 completely, using a pipette was challenging and inconsistencies in water removal could potentially
78 lead to variation in the water content and therefore the volatile suspended solids mass (gVSS) within

each bottle. As the water was removed uniformly in the drain method there should be less variation of gVSS.

To test the influence of the different sample preparation methods, the baseline study was prepared (as described in Table 4-1) using the drain method and an additional set of three acetate fed bottles were prepared using the pipette method. Other than the difference in granule handling, the two experiment sets were the same. The preparation of all bottles and reagents was in accordance with the methods described in Section 3.1 of the Materials and Methods chapter and the analytical methods, including methane quantification, gVSS quantification and maximum rate of methane calculations, were as described in Methods Section 3.3.

### 4.2.3 Statistics

The effectiveness of the Drain and Pipette methods were analysed by comparing the mean rate of methane production in samples prepared by each method, and, by comparing the mean amount of gVSS in samples prepared by each method. It is noted that one of the bottles prepared using the drain method leaked (loss of gas) during monitoring and was therefore not included in analysis of data. Thus, the conditions were compared using the Welch two sample t-test which allows for a t-test comparison with uneven sample sizes and small counts as low as 2 (de Winter, 2013).

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Methane Production over time

The pressure readings and methane quantification measurements were used to estimate the methane production volume over time for each test condition (**Figure 4-2**). All bottles showed an immediate increase in pressure due to differences in temperature between the bench where they were sealed and the incubator (where the pressure in the fed bottles increased with no lag or delay in gas production). Reported methane production volumes were blank normalise, that is the mean volume of methane produced in the blank (unfed) bottles was subtracted from each of the test conditions to calculate the methane yield attributable to the substrate tested. For all feed types except for propionate, measurable

104 biogas production ceased after between 16 to 32 hours. Bottles fed propionate did not appear to
105 generate any further biogas after 100 hours of incubation and monitoring.

106 Thus, when designing future studies, measurements within the first 48 hours should be considered
107 the most time sensitive with regular readings taken at intervals of between 1 and 4 hours in order to
108 capture a minimum of four observations during the period of the greatest rate of gas production.
109 Furthermore, it can be assumed that in the absence of an inhibitor, that simple VFAs such as acetate,
110 ethanol, and butyrate will have been consumed within 48 hours of the substrate addition whereas
111 more complex substrates such as propionate may require longer observation periods to reach
112 complete substrate conversion.

### 113 4.3.2 Granule handling- Drain v Pipette

114 The mean specific methanogenic activity rate using acetate as substrate was determined for samples
115 prepared by two distinct methods: drained and pipette methods. The average activity rates for the
116 pipette and drain bottles were 235.4 (mg CH4 /gVSS *day) and 258.0 (mg CH4 /gVSS *day)
117 respectively. Although the mean of the drain method was slightly higher than the pipette method,

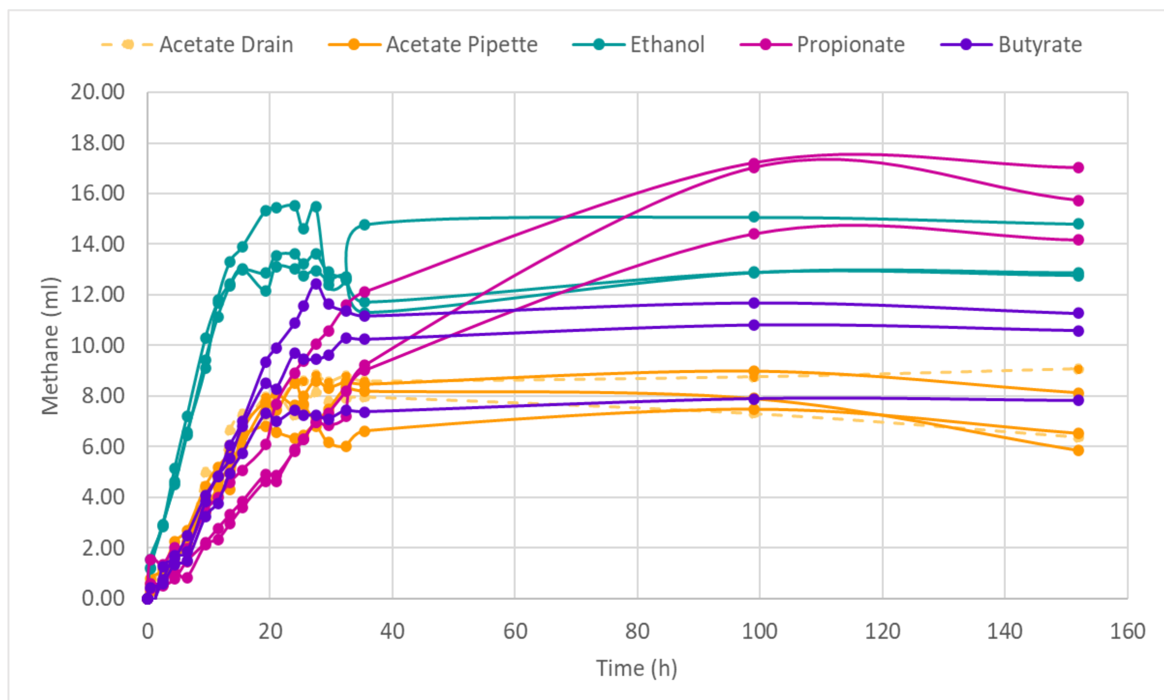



***Figure 4-2*** *- The substrates provided were converted into methane.*
*The accumulation of methane over time can be used to calculate the rate of methanogenic activity. The length of the experiment was determined by the time required for all the provided substrate to be converted to methane after which the rate of methanogenesis would level off*

118 indicating that the acetate was converted to methane at a slightly faster rate, there was no statistical
119 difference between the rates of methane production of the two methods was observed (p-value =
120 0.80) (**Figure 4-3**). Thus, as the drain method was less time consuming, this method was adopted for
121 use in all future experiments. As there were no statistical difference in the drain and pipette methods
122 the two methods will be treated as a single set of acetate fed bottles for the rest of this analysis.

123

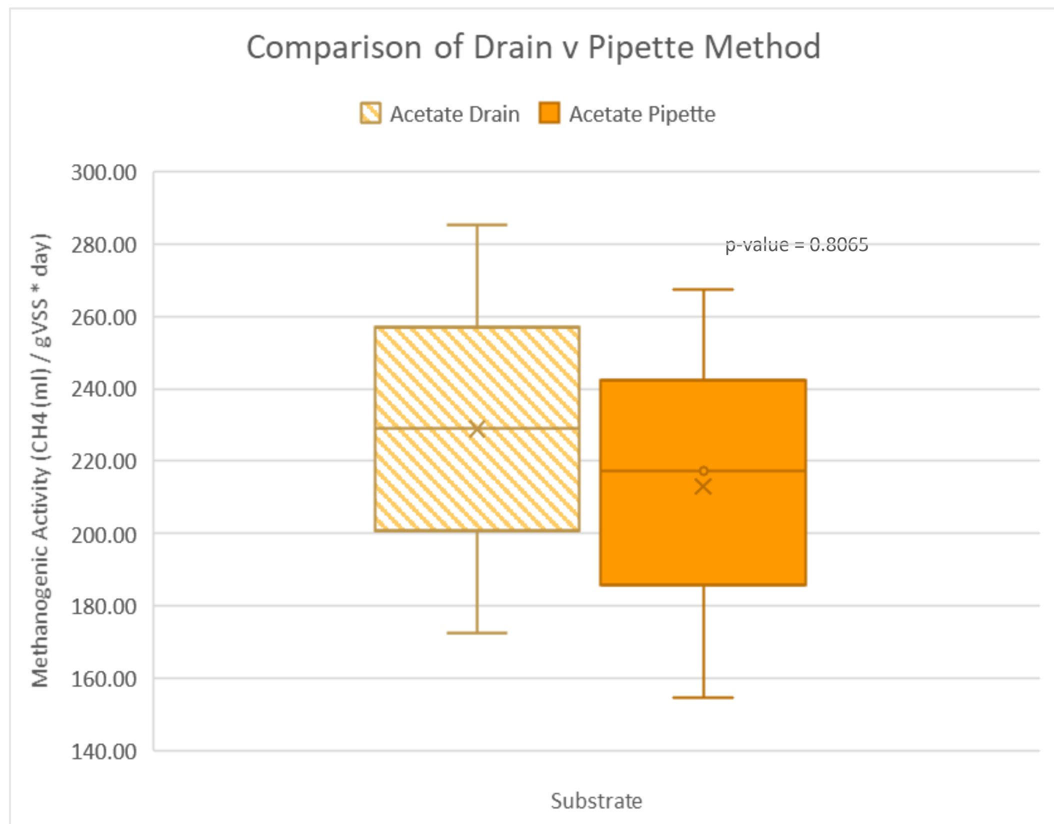



***Figure 4-3*** *– Comparison of drain and pipette method.*
*A comparison of the methanogenic activity of the granule handling using the pipette versus drain method. Each condition was fed acetate. Although the rates for the drain method were slightly higher, no statistical difference was found between them. The x represents the mean value, the centre line represents the median value. Each of the boxes represent the lower (Q1) and upper (Q3) quartile.*

124

### 4.3.3 Methanogenesis and Study Length

The specific methanogenic activity rate of the sludge granules was determined for each of the substrates tested (acetate, ethanol, propionate and butyrate). The bottles fed with ethanol had the greatest rate of methanogenic activity rate of 274.1 (SD = 47.7 RSD = 0.2) ml CH4/ gVSS × day (**Figure 4-4**). The bottles fed with acetate, ethanol, and butyrate had similar rate of gas production with mean rates of 219.4 (SD = 57.2, RSD = 0.3), 184.7 (SD = 15.3 RSD = 0.1), and 0.44 (SD = 0.02, RSD = 0.056) ml CH4/ gVSS × day respectively. The rate of methane production in bottles fed with propionate were lower with a mean of 124.1 (SD = 15.1, RSD = 0.1). The mean rate of methane production in unfed 'blank' bottles was 0.11 ml $CH_4$ / gVSS × day (data not shown). Despite the lack of added substrate, unfed blank cultures generally produce small amounts of methane, likely due to the use of dead cellular materials or extracellular polymeric substances (EPS) as a substrate. A one-way analysis of variance model with a Tukey's post hoc assessing rate of methanogenic activity in terms of feed type found that only the propionate and ethanol activity rates were statistically different ($p < 0.05$). The higher rate of methanogenesis in for the ethanol fed samples is possibly due to the cultures history treating distillery wastes.

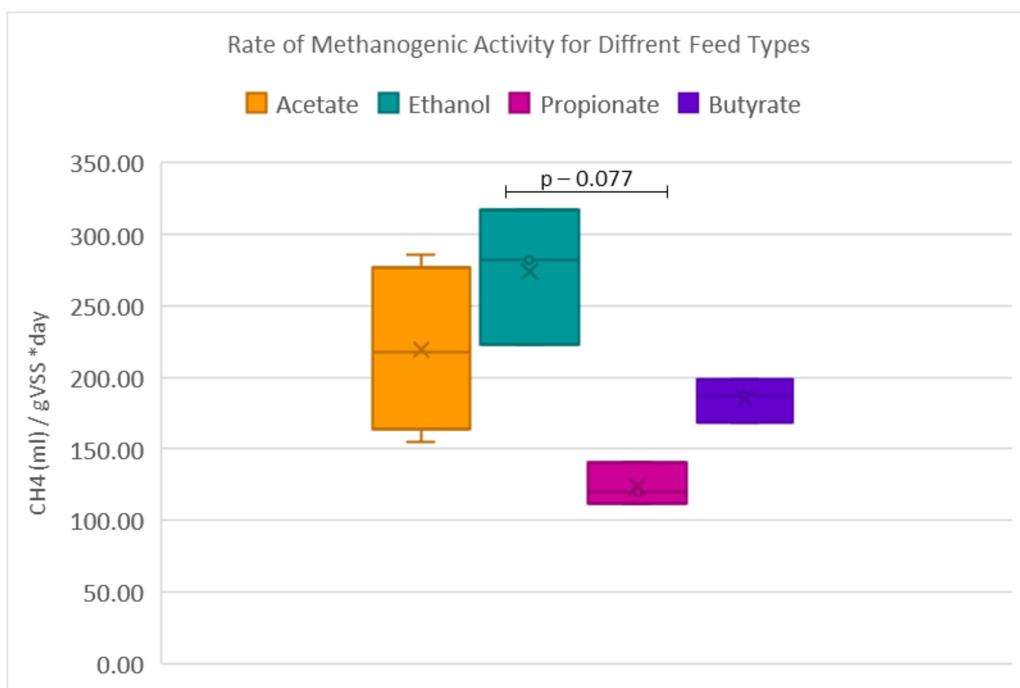



***Figure 4-4*** *– Rate of Methanogenic activity for different feed types.*
*The rate of methanogenic activity for each of the provided substrates indicated the activity of the sub communities required to convert the substrate to methane.*

## 4.4 SUMMARY

Based on this work the following conclusions were made and used to inform future experimental design.

- There was no significant difference in activity observed using the drain and pipette methods for sample preparation. As the drain method was less time consuming and more practical to do while in the anaerobic chamber, this method was chosen for all future experiments.
- To capture the maximum activity rate in assays without inhibitors, the first 20 hours require regular observation at 1–4 hour intervals.
- In assays without addition of inhibitors, it can be assumed that simple VFAs such as acetate, ethanol, and butyrate will have consumed all the available feed within 48 hours after initial reading. More complex substrates such as propionate may require longer periods of to reach complete substrate conversion. As such, the minimum window of time for observation should allow for at least 60 hours for an SMA.

# Chapter 5
# Method Development for Repeat Batch Feeding of Anaerobic Granules

## 5.1 INTRODUCTION [1]

In a traditional SMA, anaerobic cultures are fed once. After the provided substrates are fully consumed the study ends (Angelidaki et al., 2009; Shelton & Tiedje, 1984). Thus the SMA is useful for determining 'as sampled' methanogenic activity, or, for comparing short term effects of inhibitory compounds on activity (Cetecioglu *et al.*, 2012). Capturing chronic effects of inhibitory compounds on adaptation of a microbial community by contrast requires longer term studies. Additionally, longer term studies of inhibition necessitate the maintenance (or approximation) of steady state conditions which are maintained through the cycling of anaerobic medium and the regular addition of organic substrates (Gerardi, 2003; McHugh *et al.*, 2003). This is often achieved using bioreactors. Although these systems are usually automated or semi-automated, they are also large and require specialised and complex equipment to run, such as pumps, heaters, agitators, fume cupboards, and gas collection rigs. The complexity and physical footprint, in addition to their associated costs, reduces the potential to deploy such rigs in a study underpinned by a large number of test conditions or biological

[1] *Note: This section of the thesis reports the development of a repeat batch culturing assay for anaerobic granules adapted from the SMA method. The method was developed to enable longer term repeat batch culture via manual removal and replacement of anaerobic media over several weeks incubation. Although the repeat batch method was not used experimentally due to the Covid 19 lockdowns and loss of laboratory access, the results of this work, which demonstrated the value of nutrient additives and feeding during acclimation, were adopted in the design of other work reported within this thesis. Thus, the development study is reported in detail here.*

replicates. Anaerobic culture bottles, such as those used in the SMA, require little physical space, and limited specialised equipment beyond an anaerobic chamber in which to fill the bottles, a pressure transducer to measure pressure changes, and a shaking incubator to heat and mix the cultures. As the physical space required for this method is much smaller, it is possible to run more replicates and conditions within a single study. However, as each bottle requires manual handling to refresh medium and feed, manual repeat batch feeding has the potential to be labour intensive.

Whilst enabling observation of a large number of different test conditions, the traditional SMA assay does not provide trace elements for growth as may be required in a longer-term study. If an anaerobic culture is not provided with the substances required for maintenance and growth, the cells will begin to die, resulting in a reduction in activity and biomass. The time required for starvation and activity suppression differs between cultures and with the substrate makeup. Nutrients that may be essential to support the growth of many microbes, if added to cultures in such a way that they build up within a system, can however become toxic and inhibitory to growth and methanogenesis (Thanh *et al.*, 2016). Metals such as copper, nickel, zinc, cadmium, and chromium, all of which are essential at low concentrations, have been shown to inhibit acetogenesis and methanogenesis when concentrations are sufficiently high (Mudhoo and Kumar, 2013). Therefore, to conduct a repeat batch assay, understanding the requirement for and impact of nutrient dosing is essential.

**This study used a repeat batch assay to assess whether anaerobic granules fed on glucose and a complex broth could maintain activity over a two-week period without addition of trace nutrients**. Additionally, the effect of a nutrient additive mix on the activity of anaerobic granules fed on the same substrates was investigated to determine if the presence of the additives would increase activity and overall health or build up and become toxic. The 'health' of the community was assessed by monitoring:

(1) Variation in the volume of methane produced over time, whereby it was assumed that steady methane production indicated a healthy culture while variable or diminished methanogenesis indicates an unstable culture
(2) Methanogenic activity rate was measured three times during the study.

(3) Biomass in terms of gVSS – biomass was measured at the beginning and end of the study to determine if the culture was stable or had declined and lost biomass as a result.

## 5.2 Methods and Materials

### 5.2.1 Study Design - Repeat Batch Method

This study measured the methanogenic activity of a granular sludge using simple (glucose) and complex feeds (complex broth) with and without added essential nutrients over 20 days using a repeat batch culturing method (**Figure 5-1**). Anaerobic granules were placed in culture bottles with anaerobic medium, substrates, and medium and incubated at 35°C. Every 48 hours, 24ml of the medium was removed and replaced with fresh medium and the appropriate substrate/ nutrient combination which resulted in a 2.5day hydraulic retention time (HRT). The conductivity of the removed effluent was assessed to infer nutrient uptake. Immediately before effluent replacement, the pressure in the headspace of the bottles was measured and the biogas was sampled methane quantification. Pressure readings were taken once every 24 hours to measure net biogas accumulation. At three points during the study (day 2, day 10, and day 18) a general methanogenic assay (GMA) was conducted in which biogas was measured every 2-6 hours over a 48-hour period, similar to the readings as would be taken during an SMA, and the data was used to assess the differences in the overall methanogenic activity rate of the granules for each test condition. The primary difference between an SMA and a GMA is that an SMA uses an array of substrates with

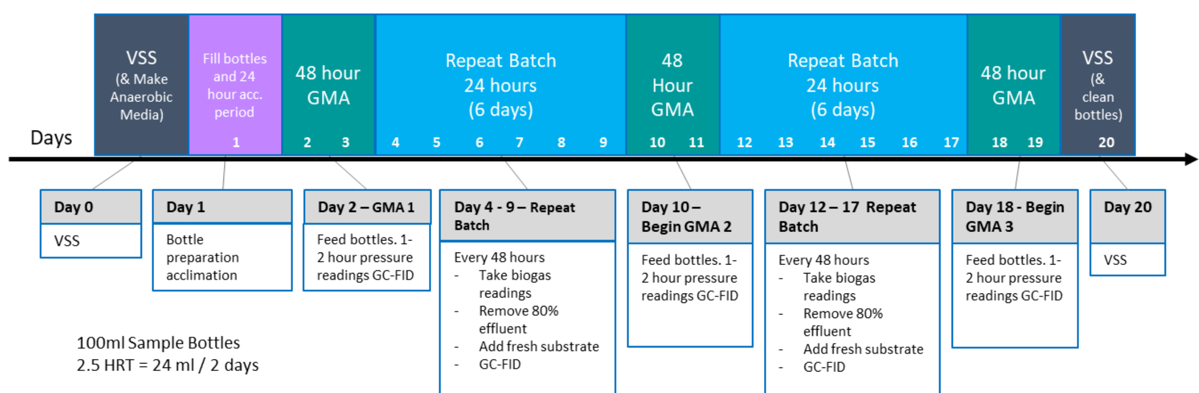



***Figure 5-1*** *- Summary of the study design and the monitoring for each phase.*

65 different complexity to target individual trophic levels, while the GMA used more complex
66 substrates and tracks methane for the community as a whole. The gVSS of the granules was measured
67 at the beginning and end of the study to enable estimation of the change in biomass within each bottle
68 during the study period. Six test conditions were tracked: three substrate types (blank, glucose,
69 complex broth) and two nutrient conditions (with and without nutrient additives). All test conditions
70 were conducted in triplicate for a total of 18 test bottles.

### 71 5.2.2 Culturing

#### 72 *5.2.2.1 Bottle Preparation and monitoring*

73 The bottles used were 100ml in volume. All samples were prepared in triplicate including six blanks
74 (three with nutrient and three without) into to which no substrate was added. All bottles were filled
75 under anaerobic conditions within an anaerobic chamber. Each bottle contained 1.5g of sludge (10
76 g/L of VSS) to which 28.5ml of anaerobic medium was added. After inoculation, the headspace in
77 the bottles was exchanged for 90 seconds with a 20% $CO_2$ and 80% $N_2$ mixture to 1 ATM to ensure
78 an anaerobic environment within each bottle. Samples were then acclimated without food for three
79 days within a 37°C incubator. The activity of cultures were monitored according to the protocol by
80 Colleran et al (1992) as described previously (Section 3.3).

#### 81 *5.2.2.2 Repeat feeding*

82 After acclimation, the bottles were returned to the anaerobic chamber and the liquor was completely
83 removed from each of the bottles using a pipette, leaving the granules behind. Then, 27.5ml fresh
84 anaerobic medium was added to the acclimated granules. Bottles were sealed within the anaerobic
85 chamber. The headspace was exchanged for 30 seconds with a 20% $CO_2$ and 80% $N_2$ mixture to 1
86 ATM. After the head space gas was exchanged, the substrate and nutrients were injected into each
87 bottle through the septa. The bottles were then incubated for 48 hours (with the monitoring period
88 adopted from results of Methods Section 3). Pressure readings were taken using a Centrepoint
89 pressure transducer at 24 and 48 hours after the addition of the substrate. After the 48-hour pressure
90 reading biogas was sampled from the headspace for methane quantification. This process was

91 repeated every 48 hours for the following 18 days (**Figure 5-1**). The removed effluent was placed in
92 sterile test tubes to await processing.

93 ***5.2.2.3 Methanogenic Activity Measurements***

94 Pressure readings were taken approximately every 1-2 hours until gas production began to level off,
95 after which readings were taken every 4-6 hours. These assays were conducted at three points in the
96 study (day 2, day 9 and day 18) to determine the rate of methanogenic activity for each of the test
97 conditions. After approximately 48 hours biogas and effluent samples were taken for analysis and
98 the medium was replaced at described above. The final pressure measurements were read at 44 hours,
99 46 hours, and 40 hours from substrate addition for the first second and third assays respectively. As
100 the presence of added nutrients might have an effect on the blank conditions, these were treated as
101 test conditions in their own right rather than being used to blank normalise the data of the fed test
102 conditions. Therefore, in order to compare the rate of the methane in the fed and unfed conditions,
103 the reported rates were not blank normalised as is done in other studies. The method used to calculate
104 the rate of methanogenic activity was as described in Methods chapter Section 3.3.

105 **5.2.3 Reagents and media**

106 In contrast to the VFAs used in a typical SMA, the GMA used two different substrates as feed for
107 the microbes, one complex (beef broth, yeast extract, and glucose) and one simple (glucose). These
108 were selected because utilisation of the complex broth would require activity from all levels in the
109 methanogenic food chain whilst utilisation of glucose, a soluble sugar, would not require hydrolytic
110 activity. The complex broth (modified from the OECD 224 (OECD, 2007)) contained a mix of 5g of
111 beef broth, 5g of yeast extract, 5g of D-glucose in 100ml of DI water. The 1.0 M glucose solution
112 was comprised of 18.16g of glucose in 100ml of DI water. As the complex broth has a shelf life of
113 24 hours, it was portioned into 2ml vials and frozen at -20c immediately after being made. Vials were
114 thawed immediately before use. The glucose solution was stored in the refrigerator at 4°C for the
115 length of the experiment. All mixes were autoclaved before storage to prevent contamination. The
116 nutrient mixes, buffers, and anaerobic medium were prepared as described in section 3.2.2 and the
117 final solutions prepared by mixing as described in **Table 5-1.**

118 ***Table 5-1-*** *Substrate additives.*
119 *There were six feeding conditions for this study with two test conditions: feed type (blank, glucose, and complex broth)*
120 *and nutrient (with and without). The nutrient mix included several solutions which were stored separately and had to be*
121 *mixed on the day. The volumes of substrate used where normalised such that each contain 8mg TOC/L in each bottle*
122

| Test condition | Complex Broth (ml) | Glucose-1M (ml) | Phosphate Buffer (ml) | Nutrient mix (ml) | Trace Nutrient mix (ml) | Mili-Q (ml) | Anaerobic medium (ml) | Total Volume added (ml) |
|---|---|---|---|---|---|---|---|---|
| Blank + No Trace Nutrient | 0 | 0 | 0 | 0 | 0 | 15.00 | 15 | 30 |
| Blank + Trace Nutrient | 0 | 0 | 1.5 | 1.5 | 0.15 | 11.85 | 15 | 30 |
| Glucose + No Trace Nutrient | 0 | 0.3 | 0 | 0 | 0 | 14.70 | 15 | 30 |
| Glucose + Trace Nutrient | 0 | 0.3 | 1.5 | 1.5 | 0.15 | 11.55 | 15 | 30 |
| Broth + No Trace Nutrient | 0.36 | 0 | 0 | 0 | 0 | 14.64 | 15 | 30 |
| Broth + Trace Nutrient | 0.36 | 0 | 1.5 | 1.5 | 0.15 | 11.49 | 15 | 30 |

123

124 ### 5.2.4 Analytical methods and Calculations

125 #### *5.2.4.1 Methane calculations*

126 Methane volume and rate was calculated as described in Method Chapter Section 3.3.

127 #### *5.2.4.2 gVSS*

128 Similarly to the SMA assay, volatile solids measurement was used to imply the biological content of
129 the samples. The gVSS of the cultures was measured at the beginning and end of the study as a means
130 of tracking changes in total biomass in response to the provided feed types. A decrease in VSS over
131 time was assumed to indicate that the given substrate did not contain the necessary constituents for
132 maintenance and cell growth. The gVSS methods and calculations were as described in Section 3.3.

133 ### 5.2.5 Statistics

134 All statistics were calculated using R version 4.1.2 and MS Excel. To measure variation in the volume
135 and relative proportion of the methane produced, the standard deviation (SD) and relative standard
136 deviation (RSD) was calculated for the methane volume (ml) for each of the feed types. Deviations
137 were computed including all time points and all replicates during the study for each test condition.
138 A Welch's 2 sample t-test was used to determine the influence of nutrient additives on the rate of
139 methanogenic activity for each feed conditions and p-values reported. An ANOVA was used to

140 determine the influence of feed composition on difference in gVSS between the beginning and end
141 of the study and 'X'-values reported.

## 142 5.3 Results and Discussion

### 143 5.3.1 Variation in Methane Volume

144 The volume of methane during the repeat batch method was consistent for most of the test conditions
145 (**Figure 5-2**). The glucose with supplemented nutrient, complex broth with, and complex broth
146 without nutrient produced mean of 281 ± 42 (RSD = 15.05%), 252 ±42 (RSD 16.68%), and 240 ±41
147 (RSD 17.26%) ml $CH_4$/gVSS respectively. The glucose fed cultures without supplemented nutrients
148 produced less overall methane with an average of 155 ± 68.2 ml CH4 /gVSS and production was
149 relatively more variable (RSD 44.07%) (**Figure 5-3**). Similarly, both complex broth conditions as
150 well as the glucose with added nutrient conditions showed an increase in net methane production
151 throughout the study, while the glucose without nutrient additives decreased in overall methane
152 output during the study. The variation in methane volume in the glucose without nutrient additives
153 is possibly due to deterioration of the community as the lack of essential nutrients prevented efficient
154 protein and enzyme production.

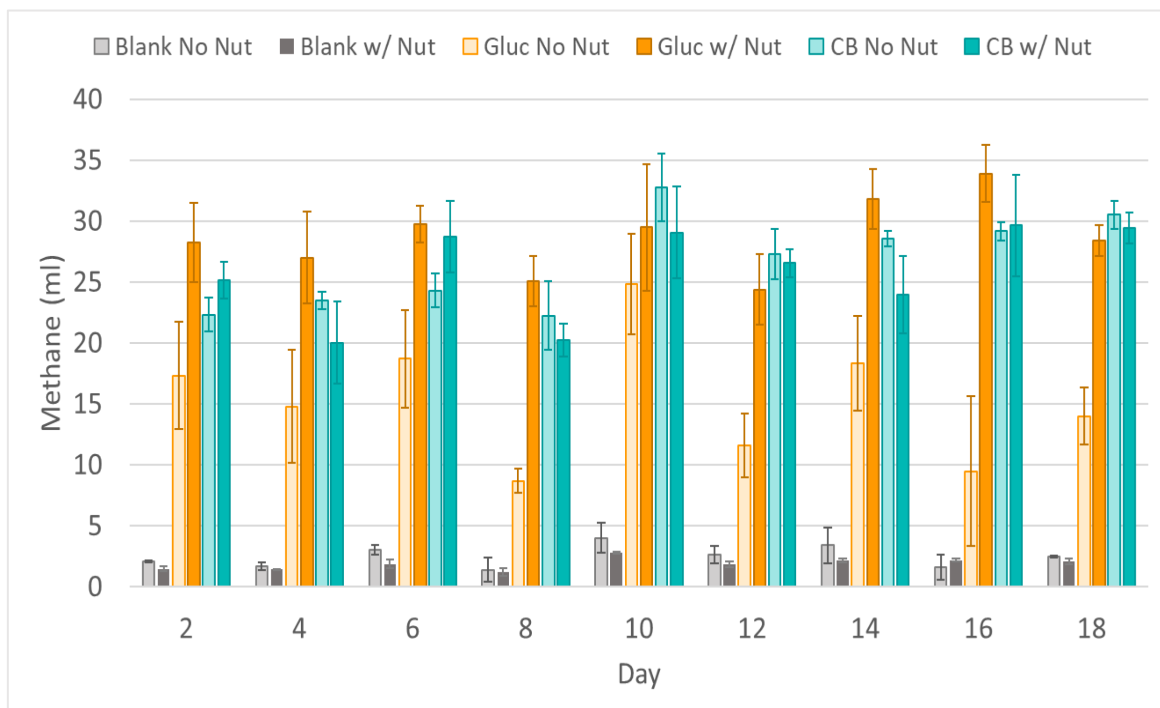



***Figure 5-2*** *- The mean volume of total methane at each changeover throughout the study. The error bars represent one standard deviation from the mean.*

155

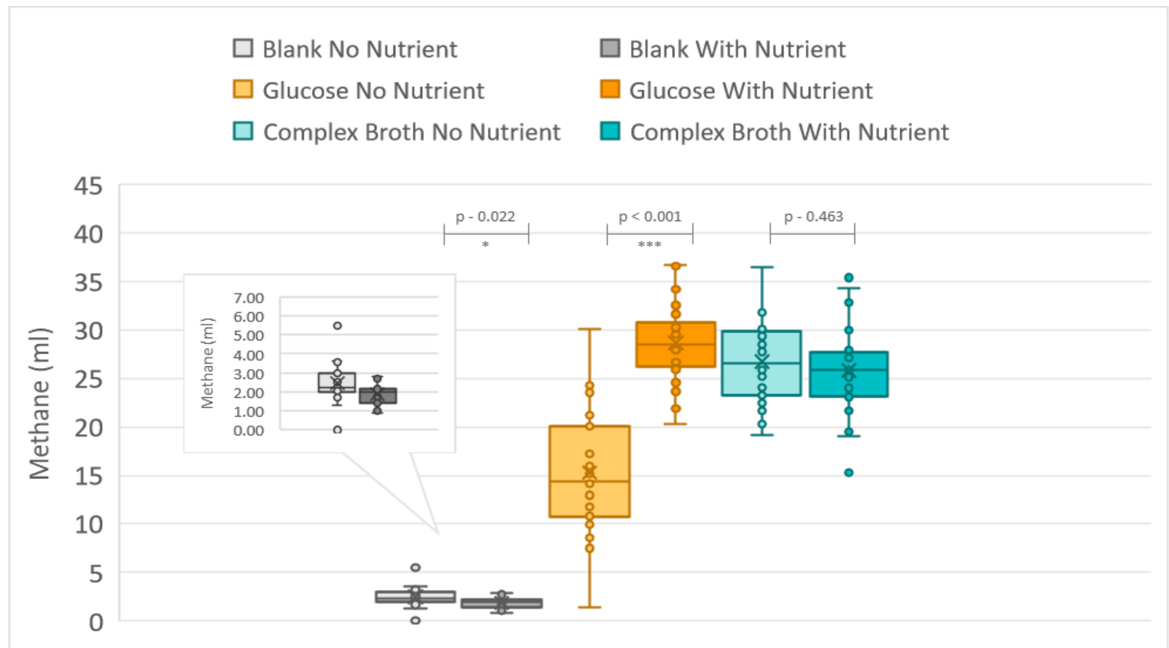



*Figure 5-3 - A comparison of the total methane generation at each time point measured during the study. Box plot represent 1 quartile of variation with an x representing the mean value. The effect of nutrient for each feed type was determined using a t-test ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).*

156

157 ### 5.3.2 Methanogenic Activity

158 The GMA was measured at the beginning (Day 2), middle (Day 9), and end (Day 18) of the study
159 (**Figure 5-4**). Biogas production was nearly complete by 35 hours for all test conditions except those
160 that were fed with glucose and no nutrient, which did not reach stationary phase during the 48-hour
161 observation period (**Figure 5-4a**). A lag phase was observed for all the test conditions for the initial
162 GMA. Complex broth fed cultures, both with and without nutrient addition, began biogas production
163 between 5 to 7 hours into the experiment. The cultures with glucose and nutrient additives began
164 producing methane around 17 hours while the bottles without glucose did not begin producing
165 methane until after 20 hours into the study. The lag period was not observed in later GMA tests,
166 rather, for the remainder of the study biogas production was observed immediately after addition of
167 additional substrate for all substrates (**Figure 5-4 b & c**). The initial lag observed was due to the time
168 required for the available nutrient to be taken up and utilised by the microbial communities. However,
169 the lack of a lag time observed in the later study is likely due to some residual substrates remaining
170 within the granules.

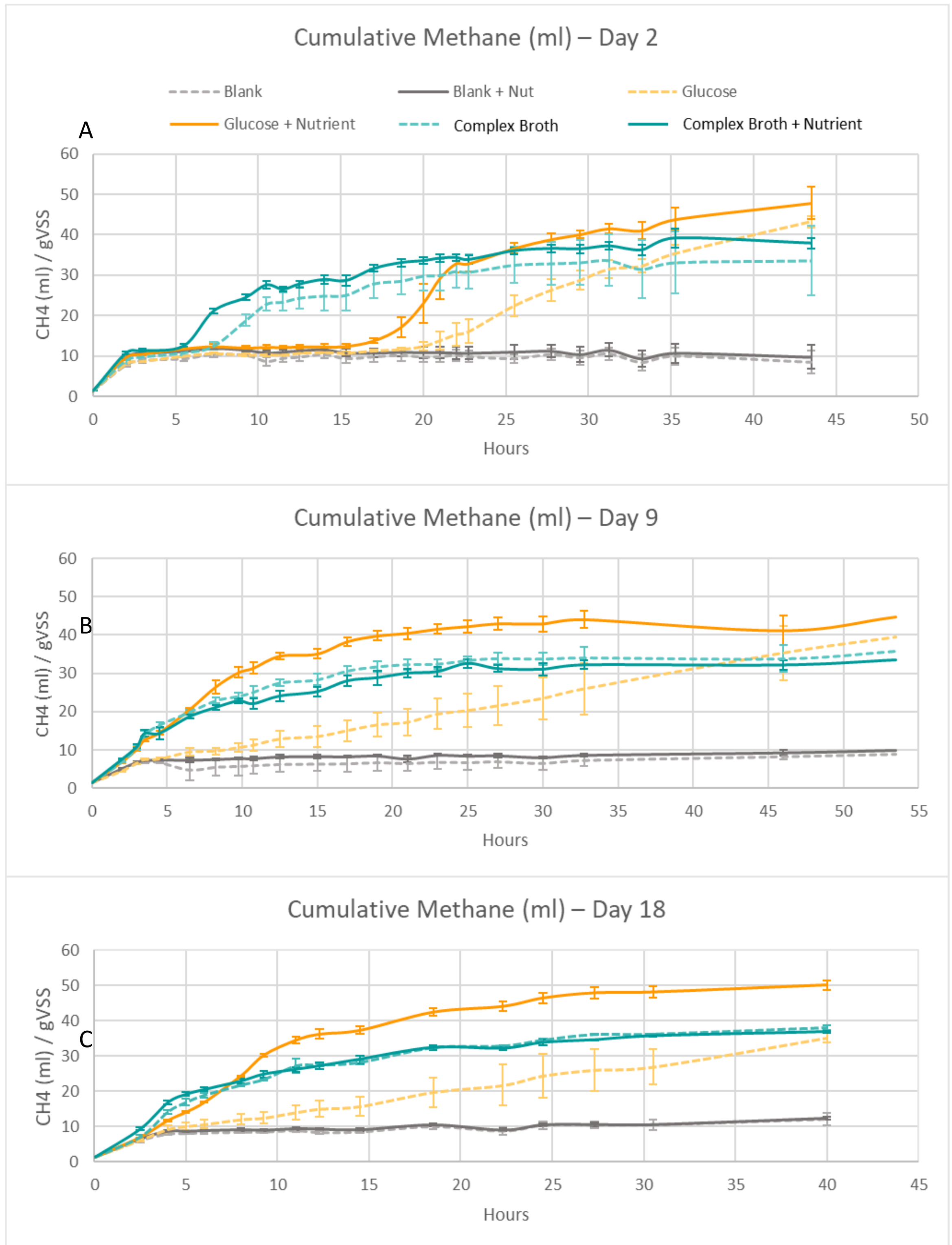



***Figure 5-4*** *- Cumulative methane produced for each of the GMA assays conducted - day 1(a) day 9 (b) and day 20(c). Each line represents the mean value for each time point. The error bars represent one standard deviation from the mean.*

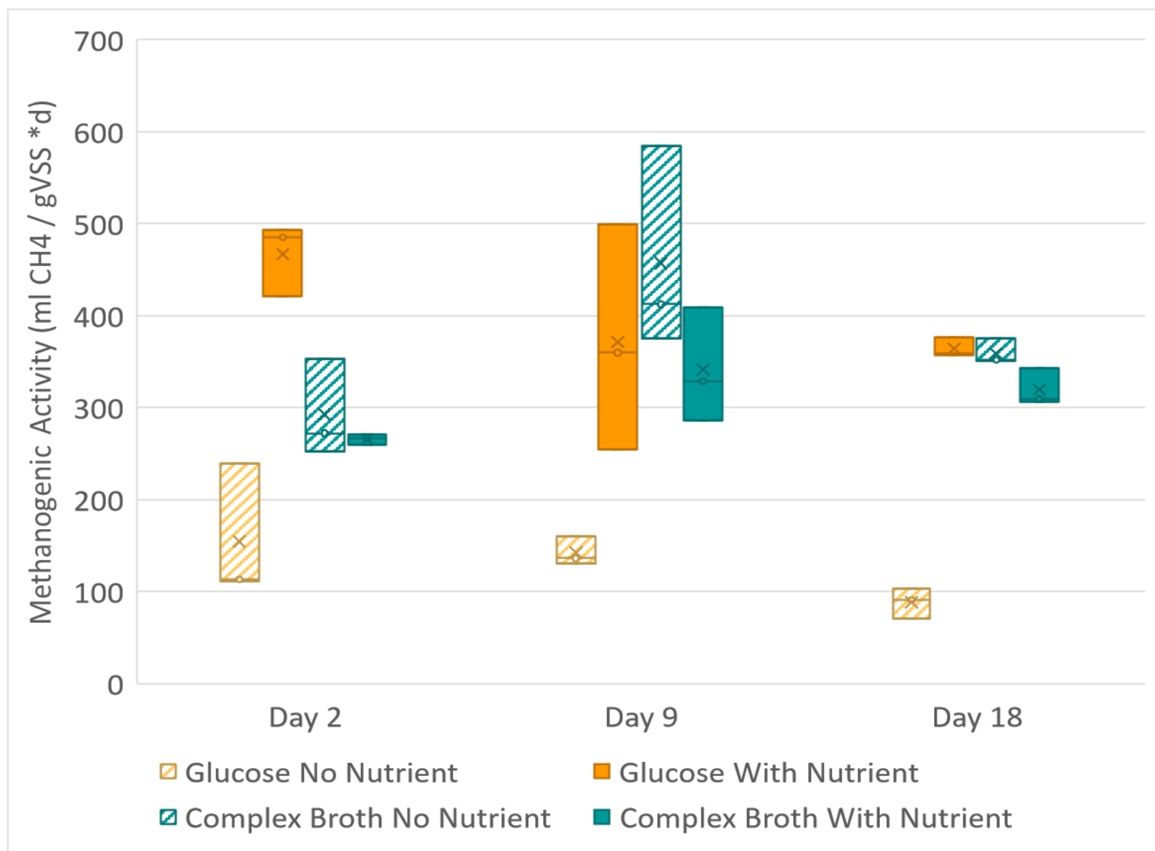



***Figure 5-5*** *- A comparison of the rate of methanogenic activity for each of the GMAs conducted at Day1, Day 9 and day 20 of the experiment.*

The influence of nutrient additives on methanogenic activity (**Figure 5-5**) varied significantly (p – values <0.001 – 0.08) between each substrate type used. Glucose with nutrient additives had the highest activity with a mean rate of 312.2, 228.96, and 275.79 ml ch4 / gVSS*day for the first, second and third GMAs respectively. The rate of methanogenesis declined throughout the study for cultures fed glucose, regardless of nutrient supplementation. By contrast, methanogenic activity for bottles fed with complex broth both with and without nutrients increased between the first and second GMA, though decreased slightly for the third GMA although not significantly (p>0.05) (**Table 5-2**). The higher rate of methanogenesis for the glucose fed bottles with added nutrients is likely related to the cultures previous exposure to glucose leading to a community adapted to that as its primary substrate. The slower rathe of methane production for the complex broth suggested that the community was not adapted to utilise this substrate as efficiently. As the community adapted through the experiment, the rate of methane production for the cultures fed the complex broth increased.

***Table 5-2*** *- The influence of nutrient additives on methanogenic activity for each of the different substrate types. Nutrients increased the methanogenic activity rate produced for glucose substrates such that the difference was strongly significant for the first and final GMA (p<0.001). The nutrient decreased the activity rate for broth fed bottles, though this effect was not found to be statistically significant. (P>0.5)*

| | | **Mean Methanogenic Activity (Ml CH4 / gVSS *day)** | | **Difference in Mean from Nutrient addition** | **t** | **df** | **p-value** |
|---|---|---|---|---|---|---|---|
| | | **With Nutrient** | **Without Nutrient** | | | | |
| **Glucose** | GMA 1 (Day 2) | 466.5 ±73.2 | 154.3 ±39.2 | 312.20 | 6.5 | 3.06 | <0.001 |
| | GMA 2 (Day 9) | 371.0 ±15.7 | 142.1 ±122.5 | 228.96 | 3.2 | 2.07 | 0.08 |
| | GMA 3 (Day 18) | 364.2 ±16.3 | 88.4 ±10.4 | 275.79 | 24.7 | 3.40 | <0.001 |
| **Complex Broth** | GMA 1 (Day 2) | 265.5 ±53.5 | 292.4 ±5.7 | -26.91 | -0.9 | 2.46 | 0.4756 |
| | GMA 2 (Day 9) | 341.1 ±111.5 | 457.2 ±62.4 | -116.06 | -1.6 | 3.14 | 0.298 |
| | GMA 3 (Day 18) | 319.3 ±13.6 | 359.2 ±20.1 | -39.93 | -2.9 | 3.51 | 0.05396 |

### 5.3.3 gVSS Before and After the Study

The volatile solids content of the granular sludge was measured at the beginning and the end of the study to determine whether the substrate and nutrient conditions provided were sufficient to sustain biomass (**Figure 5-6**). An ANOVA determined that the gVSS at the beginning and the end of the study was associated with the feed type (p-value <0.001) but not to the addition of nutrient (p-value - 0.874). While the unfed bottles had 0.24g less gVSS on average by the end of the experiment, the glucose and nutrient fed bottles had 0.006g and 0.00027g less gVSS on average respectively. T-tests comparing the gVSS in the seed community at the beginning of the study with the gVSS at the end of the study found that both the blank conditions were statistically different from the seed community (with nutrient p = 0.003, without nutrient p < 0.01) while all the glucose and complex broth conditions were not statically different from the seed (p > 0.10). These results suggest that in the absence of additional feed sources, the microbes in the unfed bottles utilised other biomatter in the bottles as feed and for biogas production. As the complex broth contained proteins, lipids, and carbohydrate all the macromolecules required for cellular construction were present. As such, the biomass was maintained throughout the study for those conditions.

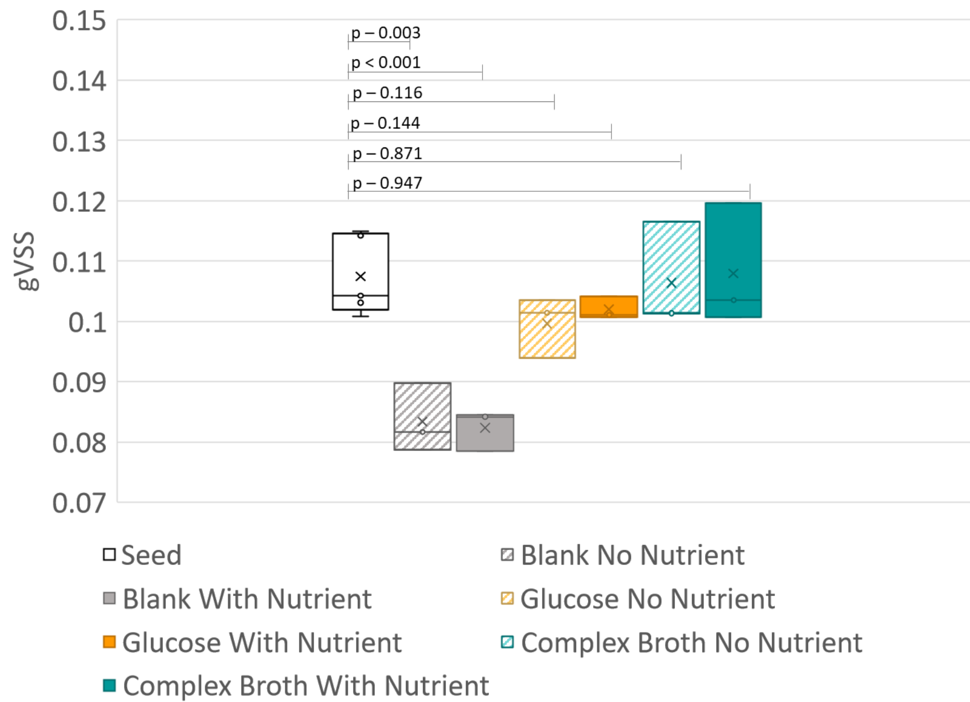



***Figure 5-6*** *- The gVSS for each test bottle. Differences between gVSS for each feed condition. The box plots represent the first quartile, and the x represents the mean value. The p-values are from t-test comparison from the seed condition.*

## 5.4 CONCLUSIONS

The effect of nutrients on methanogenic activity and VSS content of the biomass depended on the substrate complexity. Nutrient additives had no influence on the volume, rate, or variability of methane generation for cultures fed a complex substrate. Furthermore, no statistical difference in biomass (VSS) was detected between the beginning and end of the study for both feed conditions. Conversely the rate, volume, and stability of methane produced in cultures fed the simple substrate glucose differed greatly with and without the addition of nutrient. While the addition of nutrient additives resulted in methanogenic activities and volumes similar to those fed a more complex substrate, the biomass at the end of the study had decreased for both of the glucose fed conditions.

In summary:

- Nutrient additives will be used for all future analyses as their presence has an immediate positive effect on the rate, volume, and stability of methane generation, even in short studies.

217 • Substrate will be added at the offset of the acclimation phase as this eliminates the lag phase.
218 This will shorten the length of the overall observation widow and increase the likelihood of
219 observing the period of maximum methanogenic activity.
220 • The maintenance of community biomass requires the addition of complex substrates for long
221 term studies. Glucose and nutrient additives on their own are not sufficient to maintain
222 community biomass.

# Chapter 6
# How Acute Amoxicillin Exposure Effects Methanogenesis within Trophic Cascade of Mixed Anaerobic Communities

## 6.1 INTRODUCTION

Beta-lactam antibiotics, such as penicillin and amoxicillin, are amongst the most widely used antibiotics throughout the world (Van Boeckel *et al.*, 2014). They are used in a variety of industries and applications including human health, veterinary care, and agriculture (Patel *et al.*, 2019). Globally, beta-lactams make up roughly 65% of the antibiotics market, though usage varies regionally (Githinji *et al.*, 2011). As with all pharmaceuticals, some portion of ingested antibiotics will pass through our bodies largely unchanged and enter our sewers and eventually wastewater treatment facilities (Tran, Reinhard and Gin, 2018). These facilities, which are designed to remove organic matter from the influent, are not optimised for pharmaceutical removal (Patel *et al.*, 2019). As a result, many pharmaceuticals pass through wastewater treatment plants and are discharged into the receiving water environment with the treated effluent (Kümmerer, 2009a, 2009b; Githinji *et al.*, 2011; Surridge and Watson, 2012; Fekadu *et al.*, 2019; Peña-Guzmán *et al.*, 2019).

However, in much of the world conventional centralised wastewater treatment plants such as those monitored in the studies reported above are not viable due to technical, economic, or environmental

constraints. Rather, small or decentralised wastewater treatment technologies are used, of which septic tanks are the most common (EPA, 2002; Sharma *et al.*, 2013). Unlike centralised systems, influents into the tank are not diluted by external waters such as storm water or industrial effluents. Thus, septic tanks often contain a higher relative proportion of blackwater than influents to centralised water treatment facilities (Conn *et al.*, 2006). Resultantly, the concentration of organics within septic tanks as well as pharmaceutical pollutants can be one or two orders of magnitude more concentrated (Kujawa-Roeleveld and Zeeman, 2006; Luostarinen *et al.*, 2007; Conn, Lowe, *et al.*, 2010; Strande *et al.*, 2018).

Due to the low detection rates of beta lactams in centralised western wastewater treatment facilities, they are often cited as low risk pollutants (Kümmerer, 2009a). However, given the differences in concentration between the influent to septic tanks and centralised water treatment facilities coupled with the low rates of monitoring for septic systems, it is possible that the reports of beta-lactams in water treatment systems may underestimate true global exposures. High concentrations of undigested antibiotics in influent have the potential to harm treatment systems, the wider environment, and human health in several ways. First, if concentrations are high enough to inhibit biological treatment, then the quality of effluent exiting into the environment could become compromised (Amin *et al.*, 2006; Cetecioglu and Orhon, 2018) and could become a risk to human health. Secondly, undigested antibiotics and potentially antibiotic resistant microbes could leave the systems in the effluent to then enter the environment and waterways and become vectors for disease and impact natural microbial diversity in receiving environments (Kümmerer, 2009a). Due to this lack of research into amoxicillin in the water environment, in 2018 amoxicillin was added to the latest update to the Water Framework Directives emerging pollutant watch list (Loos *et al.*, 2018). Given the global reliance on septic tank technologies, to understand the impact of beta lactams on water treatment, it is important to understand the effect that amoxicillin would have in septic tanks specifically and anaerobic systems generally.

Research into the effects of beta-lactams on anaerobic systems specifically is rarely studied. Unlike bacteria, methanogenic archaea, the primary microbes responsible for the production of methane, are

50 naturally resistant to beta-lactam antibiotics due to their lack of peptidoglycan in their cell walls
51 (Kandler and König, 1998; Khelaifia and Drancourt, 2012). However, anaerobic communities are
52 comprised of a wide variety of microbes (Trego *et al.*, 2020), with a potential for varying degrees of
53 susceptibility to beta-lactam antibiotics. When research into the impacts of beta-lactam antibiotics
54 on anaerobic communities is conducted, the conditions of these studies are varied and often focused
55 on concentrations higher than we might expect in wastewater environments, and the results do not
56 always agree. In general, it was found that the impact of beta lactams on treatment efficiency are
57 limited at low concentrations. For example, Sanz found that the addition of 10 mg/L penicillin
58 inhibited total methane production by 25-45% (Sanz, Rodríguez and Amils, 1996) when VFAs were
59 used as substrate. Anaerobic digesters running on pig slurry from animals with 16 mg/kg in of
60 penicillin in their feed reported a 35% reduction in methane generation when compared to the
61 digesters running wastes from pigs fed a control feed (Massé *et al.*, 2000). Zhang et al found that
62 anaerobic systems acclimated to amoxicillin could withstand concentrations up to 60mg/L before
63 displaying any inhibition of nitrogen removal (Zhang *et al.*, 2015). A study by Su et al (2019) did
64 not report any inhibition of methane production in anaerobic granules exposed to 100mg/L
65 amoxicillin (Su *et al.*, 2019).

66 **The aim of this study is to assess the impact of short-term exposure to amoxicillin on the**
67 **methanogenic activity of different trophic groups within a mixed anaerobic microbial**
68 **community**. The range of amoxicillin concentrations used in this study aimed to include those that
69 might conservatively be found in small septic tanks in western countries (0.5 – 2.5mg/L) through to
70 higher concentrations more potentially found in areas of less regulated distribution (5 – 25mg/L) and
71 extreme concentrations (50-100mg/L). The study asks if there is a relationship between the specific
72 methanogenic activity of each trophic group with respect the rate and volume of methane/biogas
73 production and the amoxicillin concentration in the influent.

74

75

76

77 ## 6.2 METHODS

78 ### 6.2.1 Experimental Design

79 The experimental design was based on the specific methanogenic activity (SMA) (Colleran *et al.*,
80 1992; D. Coates, F. Coughlan and Colleran, 1996) assay, in which the rate of change of pressure
81 within sealed bottles containing the test microbial community and a range of substrates, is used to
82 assess the activity of the microbes within the culture capable of breaking down those substrates
83 (**Figure 6-1**). Both the rate of production and volume of methane produced were determined. To
84 explore differences in susceptibility to amoxicillin inhibition within specific groups in the trophic
85 cascade, substrates of varying complexity ($H_2/CO_2$, acetate, ethanol, propionate, butyrate, glucose,
86 and a protein rich complex broth) were used as substrate. The experimental set up consisted of 135
87 sealed bottles, each containing anaerobic sludge granules and anaerobic medium comprised of
88 buffers, oxygen scavengers, and an oxygen indicator as described in Sections 3.2.1 and 3.2.2.

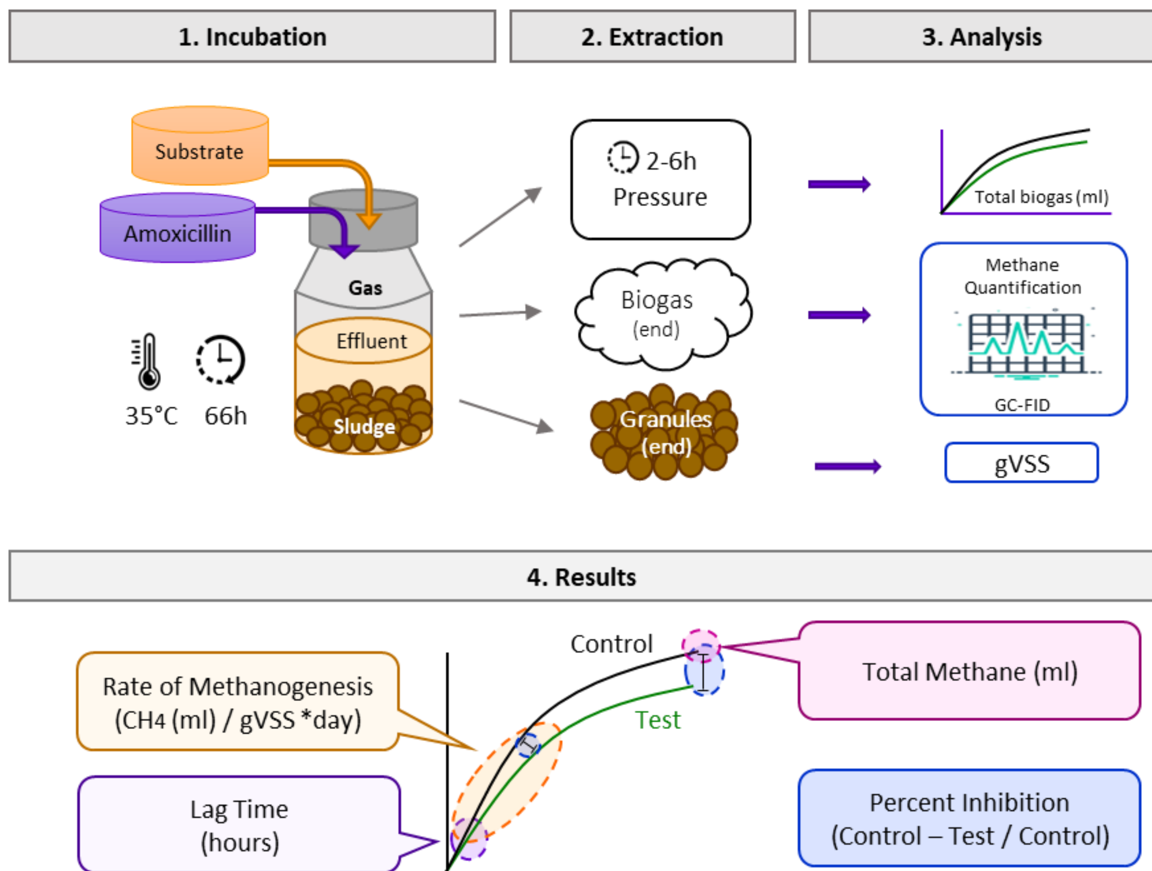



*Figure 6-1 - Experimental design.*

The effect of amoxicillin on exposure was assessed through changes in 1) rate of methanogenic activity, 2) lag time of methanogenic activity, 3) total volume of methane produced. Each condition was compared against a control.

### 6.2.2 Reagents and Media

Seven substrates of varying complexity were used, targeting each level of the anaerobic trophic cascade. Activity against each substrate was assayed at each antibiotic concentration (from 0-100mg/L) and all combinations were assayed in triplicate (**Figure 6-2**). Six liquid substrates were used: acetate (3M), ethanol (3M), propionate (3M), butyrate (1.5M), glucose (1M), and a complex broth solution at (150g/L). A mix of 20% $H_2$ and 80% $CO_2$ was used as a gaseous substrate. The VFAs (acetate, propionate, ethanol, and butyrate) were first neutralised using 8.0M NaOH to a pH of 7.0 then diluted with MiliQ water to the target concentration and then placed into vials and sealed. The 1M glucose was prepared by mixing 18.0g of glucose granules with 100ml MiliQ water. The complex broth was prepared by mixing glucose (10g), yeast extract (10g), and beef broth (10g) into 200ml MiliQ water. After preparation, the complex broth was stored at -20°c until use.

To understand the inhibitory effects of amoxicillin on methanogenesis within an anaerobic community two feed types were used as primary substrates: acetic acid as a liquid substrate and a mix of carbon dioxide and gaseous hydrogen as a gaseous substrate. Methane is formed by three

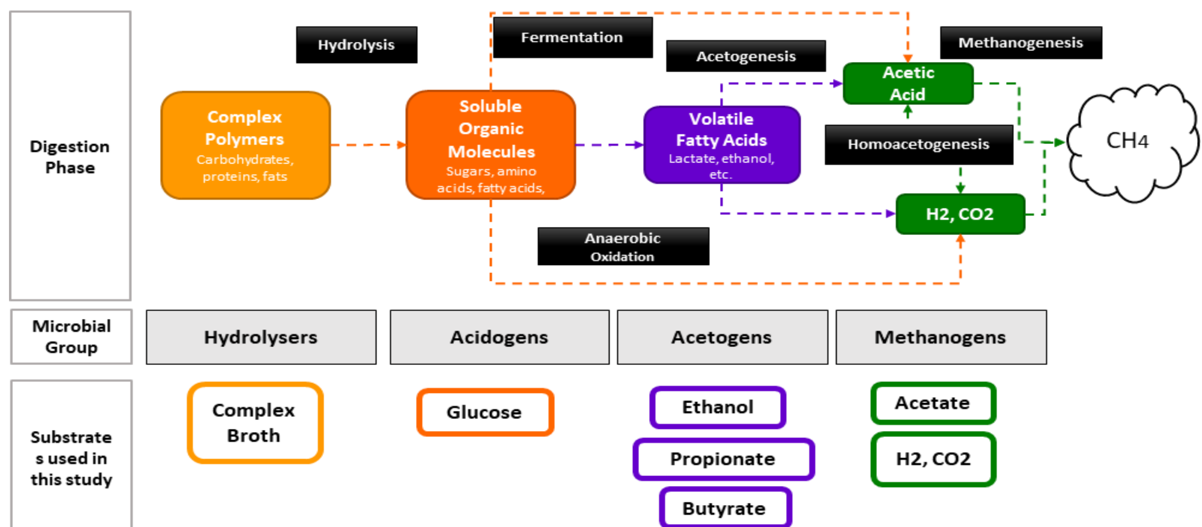



*Figure 6-2 – The anaerobic trophic cascade and the associated substrates used in this study.*

different groups of bacteria: (1) hydrogenotrophic methanogens which directly convert $CO_2$ and $H_2$ into methane, (2) acetoclastic methanogens which use acetate, and (3) methylotrophic methanogens which degrade containing one carbon such as formate and methanol. Acetoclastic methanogenesis is the most common form of methane production and it is estimated that two thirds of all biogenic methane in the atmosphere is from the degradation of acetate (Gerardi, 2003; Fournier and Gogarten, 2008). When macromolecules are consumed during anaerobic digestion $H_2$ and $CO_2$ are commonly generated as by-products (Vanwonterghem *et al.*, 2016). As methane production is the final stage in anaerobic digestion it is important to understand the influence of amoxicillin on methanogens.

To understand the inhibitory effects of amoxicillin on acidogenesis within an anaerobic community glucose was used as the primary substrate. The second state of anaerobic digestion is acidogenesis in which fermentative bacteria utilise simple sugars for energy, generating volatile fatty acids in the process. The fermentation of glucose can take a variety of different pathways which are conducted by a variety of bacterial types, though the most common products are propionate, butyrate, ethanol, acetate, formate, and $CO_2$ (Gerardi, 2003).

To understand the inhibitory effects of amoxicillin on hydrolysis within an anaerobic community, a mix of proteins and carbohydrates (complex broth) was used as the primary substrate. Unlike other processes of anaerobic digestion, in which the substrates are directly consumed by the microbes, hydrolysis is undertaken by exoenzymes excreted from the microbe and takes places outside the cell. As a result, the microbe does not directly gain energy from this step. However, the products of hydrolysis – simple sugars, amino acids, and fatty acids – are consumed, often, by the same bacterial types which produced the hydrolysing enzymes (Gerardi, 2003). The amino acids produced by the hydrolysis of proteins vary greatly in size and structure. As a result the products of the amino acid fermentation are equally diverse and nearly half of the products are gaseous and would be available for conversion to methane without requiring acetate formation as part of the pathway (Breure, Mooijman and van Andel, 1986).

To understand the inhibitory effects of amoxicillin on acetogenic bacteria within an anaerobic community ethanol, propionate, and butyrate were used as primary substrates. Fermentation is

133 undertaken by a wide variety of bacteria which syntrophically degrade soluble sugars and amino
134 acids into acetate and other by-products such as $CO_2$ and $H_2$ (Stams, 1994). While all three substrates
135 in this study are commonly utilised in acetate production, the over production or build-up of
136 propionate in anaerobic systems can suppress methane formation and is sometimes used as an
137 indicator of system instability (Ren *et al.*, 2007). Therefore, it is important to understand how acute
138 exposure to amoxicillin might influence methane production and substrate utilisation of VFAs.

139 The base anaerobic medium was prepared as described in Methods Section 3.2.1 of the methods
140 chapter to which antibiotic and substrate were added, the amount of which depended on the phase of
141 the experiment. All conditions contained nutrient additives which were prepared as described in
142 section 3.2.2 (Trace Metals and Additives) of the methods section. The make-up of each of these
143 solutions and the proportion of each in the final mixture is outlined in **Table 6-1** below.

144 ***Table 6-1*** *- Components within each bottle.*

| Compound | Unit | Concentration of Stock | Phase | |
|---|---|---|---|---|
| | | | *Acclimatization* | *Test* |
| Granule | (g) | | 0.5 | 0.5 |
| Feed Substrate | (ml) | *Various* | 0.1 | 0.1 |
| Antibiotic | (ml) | *10 x Final Concentration* | 0.0 | 1 |
| Phosphate Buffer | (ml) | *20x* | 0.5 | 0.5 |
| Nutrient mix | (ml) | *20x* | 0.5 | 0.5 |
| T. Nutrient mix | (ml) | *200x* | 0.05 | 0.05 |
| Anaerobic medium | (ml) | *1x* | 8.35 | 7.35 |
| *Total Volume added* | *(ml)* | | *10* | *10* |

145

146 ### 6.2.3 Conducting a Specific Methanogenic Activity Test

147 #### *6.2.3.1 Bottle Preparation and Granule Acclimation*

148 Bottles were prepared in accordance with methods section 1.1. Two bottle sizes were used: 30ml for
149 liquid substrates and 60ml bottles for gaseous substrate. All samples were prepared in triplicate
150 including three blanks for each bottle size to which no substrate was added. A method development
151 study (See Methods section 3.1) indicated that providing the microbes with some feed during the
152 acclimatisation phase would reduce the lag phase. This was advantageous as doing so increases the

likelihood of documenting the active phase of methane production. To commence the acclimation phase (bottles fed and incubated with the specific substrate but in the absence of antibiotic), 0.1 ml of substrate was injected into the bottles through the septa before transfer to an incubator. The headspace gas in bottles prepared for gaseous substrates were pressurised for 60 sec at 1.4 psi of CO2 $H_2$ before transfer to an incubator. The acclimation incubation was at 37°C for 5 days.

#### *6.2.3.2 Running the SMA*

After the acclimation incubation, bottles were opened in the anaerobic chamber and the liquid medium within each bottle was removed using a syringe. A mix of fresh anaerobic medium, buffer, and nutrient were added to the acclimated granules. These were then sealed within the anaerobic chamber. The headspace in the bottles was exchanged for 90 seconds with a 20% $CO_2$ and 80% $N_2$ mixture to 1 ATM to ensure an anaerobic environment within each bottle. At this point the SMA was conducted as described in Methods Section 3.1.2. The appropriate amount of substrate was inject into the bottles through the septum at the beginning of each experiment. The study was conducted over 66 hours.

### 6.2.4 Analytical Methods

#### *6.2.4.1 Quantifying Methane*

Methane in the headspace of bottles was quantified using a GC-FID (as described in Section 3.2 of the Methods chapter). The methane content of the biogas in each bottle was measured at the end of the study after the last pressure reading was taken, and before opening the bottles to remove the sludge granules and effluent. Due to an instrumentation error, methane quantification was not measured for the gaseous substrates using GC-FID. However, assuming that the pressure differential during the experiment is the result of gaseous substrate utilisation as described in D. Coates *et. al.* (1996).

#### *6.2.4.2 gVSS*

The gVSS methods and calculations were as described in Section 3.3.

178 ***6.2.4.3 Converting bottle pressure into volume of methane***

179 To calculate the rate and total gasses produced, the pressures measured for each bottle must be
180 converted to volume (ml) of total biogas. This is achieved through calibrating the bottle calibration
181 and is descried in detail in the Methods Section 3.3.3.

182 ***6.2.4.4 Rate of methane production***

183 Specific methanogenic activity rate is reported as ml of $CH_4$ / gVSS / day. The SMA rate was
184 calculated for each test by determining the slope of the best fit line through the steepest straight-line
185 section of each plot of methane produced over time. The number of points used varied for each test
186 condition, though no fewer than 4 points were used in this calculation. More details about the exact
187 calculations used can be found in the methods section 3.3.5.

188 ***6.2.4.1 Defining Lag Time***

189 Lag time was calculated as defined in section 3.3.6 of the Methods

190 ***6.2.4.2 Total Methane Volume***

191 The total methane values were taken from the total methane calculated for at 66 hours. The values
192 are reported in volume (ml).

193 ***6.2.4.3 Results Statistics***

194 To determine if the two runs could be treated as a single run, the rate of methanogenesis and total
195 methane produced in the control conditions for each of the groups was assessed using a Student's T-
196 Test. To determine the relationship between increasing amoxicillin concentration on methanogenesis
197 and the total volume of methane an aov was conducted. Each batch was analysed separately set (Run
198 1: 0.5 – 5.0 mg/L amoxicillin; Run 2: 10 – 100 mg/L) using amoxicillin. All statistical analyses were
199 performed using R statistical package and RStudio 4.1. Additionally, the percent inhibition values
200 were calculated in comparison to controls.

201 ## 6.3 RESULTS

202 This study was designed to explore the short-term effects of amoxicillin exposure on the activity of
203 anaerobic microbial communities. This was observed through changes in the specific methanogenic
204 activity rates and the volume of methane produced in response to specific substrates and amoxicillin
205 exposure.

206 ### 6.3.1 Measuring the Batch Effect for Each Run

207 Due to the number of bottles, the study was split into two runs. Run 1 contained all substrates with
208 amoxicillin concentrations 0.5, 1.0, 2.5, and 5,0mg/L. Run 2 contained all substrates with amoxicillin
209 concentrations 10, 25, 50, and 100mg/L. The difference between the blank adjusted specific
210 methanogenic activity rate observed for each substrate without the addition of amoxicillin (i.e. the
211 controls) between runs was assessed using a T-Test for each individual substrate type.

212 No statistical difference between the methanogenic activity was found for ($p > 0.05$) any condition
213 between the two runs except for propionate. The rate of methanogenic activity in the propionate fed
214 bottles in the first run was significantly higher than in the second run ($p = 0.002$) (**Figure 6-3**).
215 However, this is likely due to the differences in the rate of methane production in the first run
216 compared to the second run. Whilst there was also variation in methanogenic activity between the
217 replicates in the glucose and $H_2/CO_2$ fed bottles for each run, no significant difference was observed
218 between the rates determined. The Student's t-test determined no significant difference ($p>0.05$) in
219 the volume of methane produced in the controls for each substrate tested between Run 1 and Run 2.
220 (**Figure 6-4**).

221 The batch effect on the activity is likely caused by the time between the test runs and possible
222 differences in feeding. While seed community had regularly been fed glucose for a week before the
223 analysis began, by the time the second run was conducted the seed community would have had an
224 additional 4 weeks of glucose. The results from Chapters 5 and 7 of this thesis suggest that that would
225 have been enough time to drive changes in the community make up. Nevertheless, the analysis of the
226 control conditions for the two runs indicates that although there were some differences in the rate
227 and volume of methane generated for each of the different feed types, the runs were similar enough

228 to justify further analysis of amoxicillin inhibition on these cultures. The relative changes will be
229 reported as a change from the batch control for the respective run. Additionally, linear models
230 assessing the effects of amoxicillin will be run separately for each batch.

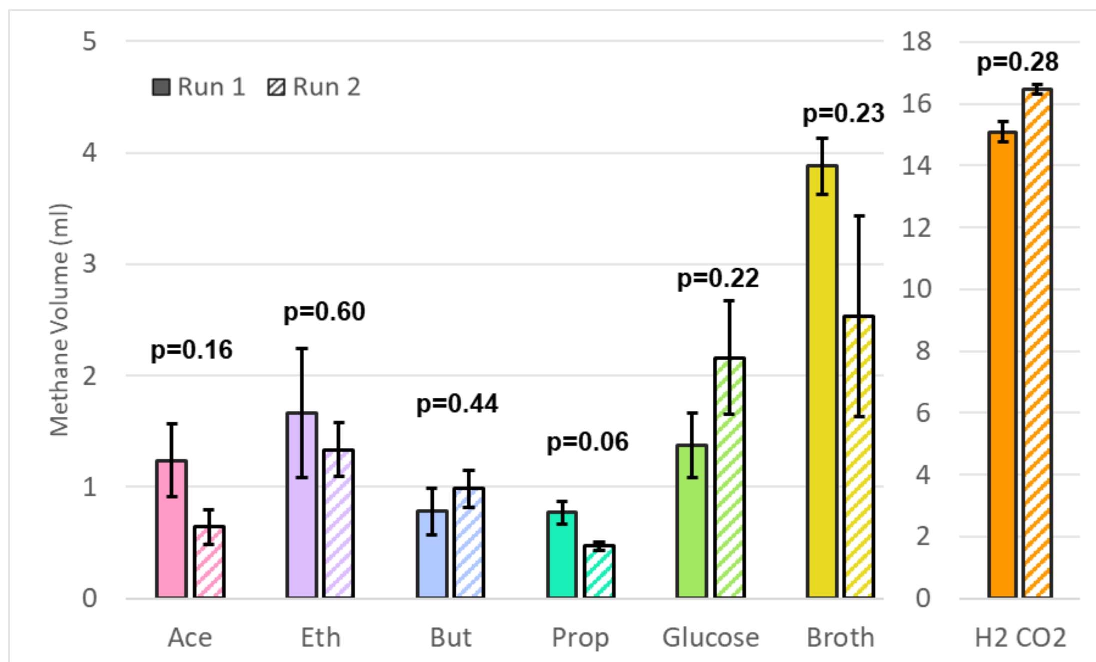



***Figure 6-3-*** *The total volume of the methane produced (ml).*
*The bar represents the mean value. The error bars represent one standard deviation from the mean. The solid bars represent the first run while the striped bars represent the second run. None of the conditions were statistically different from eachother. Both the left and right axis represent methane volume on different scales.*

231

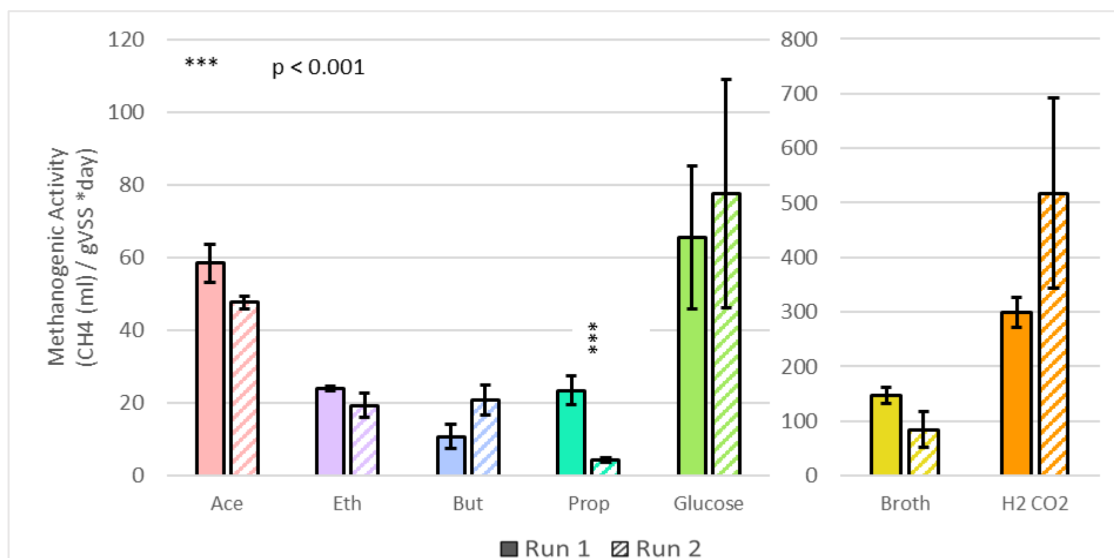



***Figure 6-4*** *- The rate of methanogenic activity (CH4 (ml) / gVSS * day).*
*The bar represents the mean value. The error bars represent one standard deviation from the mean. The solid bars represent the first run while the striped bars represent the second run. Only the propionate values were statistically distinct from eachother. Both the left and right axis represent methanogenic activity (CH4 (ml)/gVSS *day) on different scales*

232 ### 6.3.2 Amoxicillin and Rate of Methanogenic Activity

233 This study aimed to assess how acute exposure to amoxicillin would influence the activity of different
234 subpopulations of an anaerobic community. To accomplish this, specific methanogenic activity tests
235 (SMA) were run for a suite of 7 feed types (complex broth, glucose, butyrate, propionate, ethanol,
236 acetate, and $H_2/CO_2$) with and without exposure to amoxicillin. This was conducted over two runs.
237 The first run tested the amoxicillin concentrations 0.5, 1.0, 2.5, and 5.0 mg/L. The second run tested
238 amoxicillin concentrations 10, 25, 50, and 100 mg/L. The effect of amoxicillin on the community
239 was measured against the methanogenic activity, lag in activity, and total volume of methane
240 produced. Plots of the cumulative methane produced can be found in **Figure 6-5** plotted by feed type
241 with curves showing the response in the presence of each concentration of amoxicillin addition
242 alongside the response of the controls (bottles with feed but without amoxicillin).

243 #### *6.3.2.1 Methanogens – Acetate and $H_2/CO_2$*

244 The addition of amoxicillin had an inhibitory effect on acetolactic methanogenesis (**Figure 6-6**). The
245 rate of methanogenesis in acetate fed bottles was lower for all amoxicillin treatment conditions. The
246 greatest difference in rate of methane production was for the 25mg/L condition which had an average
247 of 32% of the rate of methanogenesis compared to the control. The 100mg/L amoxicillin conditions
248 produced methane at 52% the rate of the control. However, not all of these differences were found
249 to be statistically significant. Only the 1mg/L, 2.5mg/L, and 5mg/L were found to be statistically
250 lower than that of their respective control ($p<0.05$) (Appendix C).

251 Although the presence of amoxicillin results in consistent inhibition in the rate of methane produced
252 for both runs, there was no statistically singificant relationship between the addition of amoxicillin
253 and the rate of hydrogenotrophic methanogenesis (**Figure 6-6**). The greatest difference appears to be
254 a result of differences between each run in which all conditions have a slower methanogenic activity
255 in run 1 than in run 2. When these values are assessed in terms of relative percentage of the control,
256 no trend is observed for amoxicillin conditions of 5mg/L and below (**Figure 6-6B**). Values of
257 10mg/L through 100mg/L of amoxicillin in the substrate produce a gradual reduction in the rate of
258 methanogenesis is observed. The 50mg/L and 100mg/L have a mean of 56% and 57% of the control

259 rate respectively. None of the observed differences are statistically different from the control
260 ($p>0.05$) (Appendix C).

261 ***6.3.2.2 Acetogens - Ethanol, Propionate, and Butyrate***

262 There was no clear change in the methanogenic activity of ethanol fed bottles with the addition of
263 amoxicillin. The production of methane in the ethanol fed bottles varied greatly during both in terms
264 of cumulative methane production (**Figure 6-6A**) as well as the maximum rate values calculated
265 (**Figure 6-6B**). Test conditions containing 2.5, 5.0, 50, and 100mg/L of amoxicillin all produced
266 methane at an increased rate compared to the control while bottles containing 0.5, 1, 10, and 25 mg/L
267 of amoxicillin each produced methane at a slower rate compared to the control. None of these
268 differences were found to be statistically significant (Appendix C).

269 When fed butyrate as its primary substrate, the presence of 50mg/L of amoxicillin and below
270 appeared to produce an increase in the rate of maximum methanogenic activity **(Figure 6-6)**. For
271 these test conditions, the methanogenic activity was between 134-198% of the control. However,
272 inhibition appeared to be observed in the 100mg/L exposure condition produced methane at 56% of
273 the control. However, the correlation between amoxicillin concentrations and methanogenic rate was
274 not found to be statistically significant for the first run ($p >0.05$) though was significant for the second
275 run ($p < 0.05$) (Appendix C).

276 There are strong batch effects for the propionate fed control cultures in which the control conditions
277 significantly differed from eachother (Section 6.3.1). Therefore, it is difficult to compare the rate
278 between runs (**Figure 6-6**). The rate of methanogenesis was inhibited for all conditions compared to
279 the control within Run 1. In the second run, the rate of methanogenesis was relatively supressed with
280 the exception of 10 mg/L of amoxicillin which had a mean relative increate in the rate of
281 methanogenesis of 124% (**Figure 6-6B**). The correlation between amoxicillin concentrations and
282 methanogenic rate was not found to be statistically significant for either of the runs ($p >0.05$)
283 (Appendix C).

284

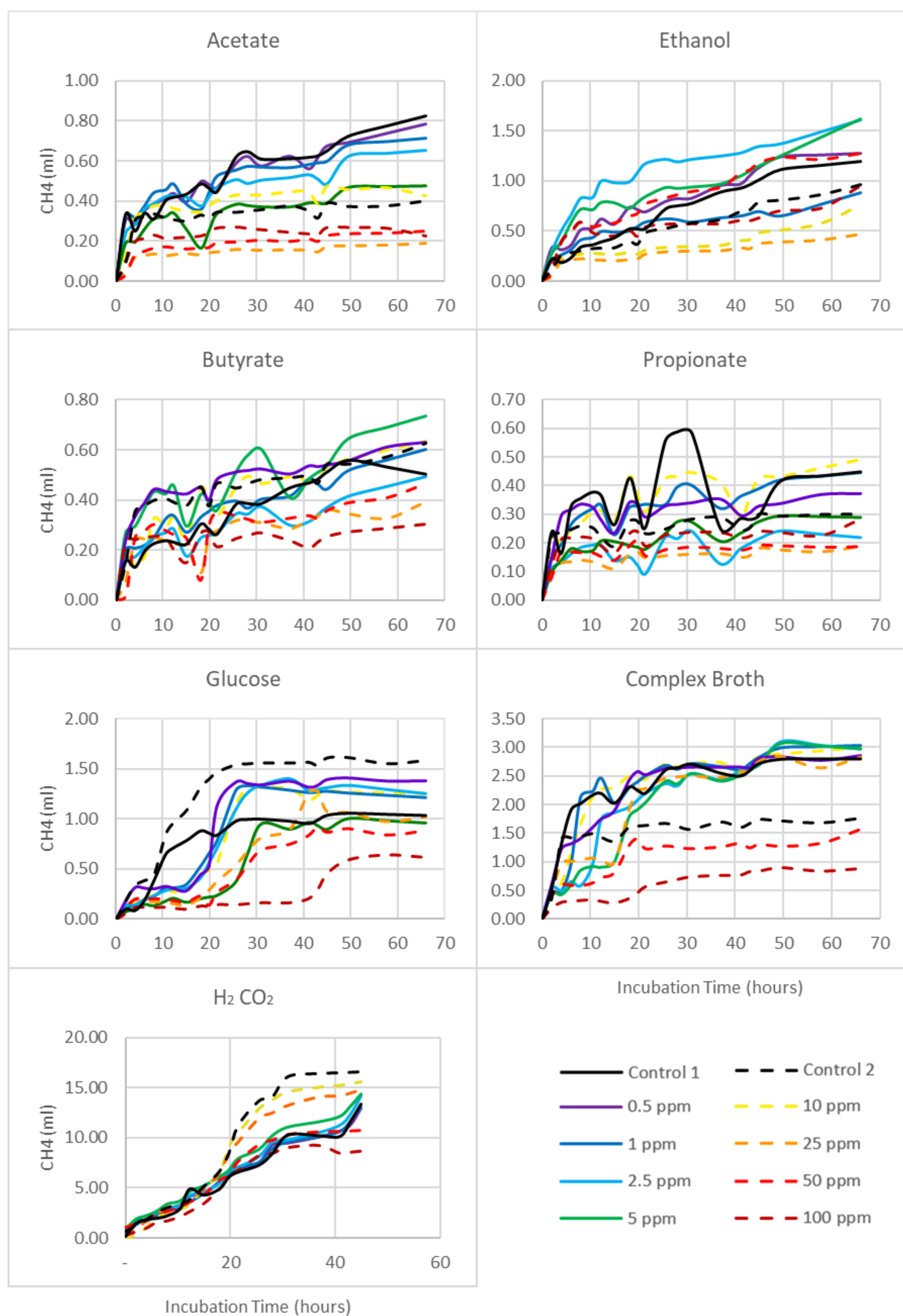



*Figure 6-5 - Cumulative methane produced for each SMA test run and report total methane produced (ml). Each colour represents the mean total methane for that time point. Control 1 was run with amoxicillin conditions 0.5ppm, 1ppm, 2.5ppm, 5ppm conditions. Control 2 was run with amoxicillin conditions 10ppm, 25ppm, 50pp, and 100ppm.*

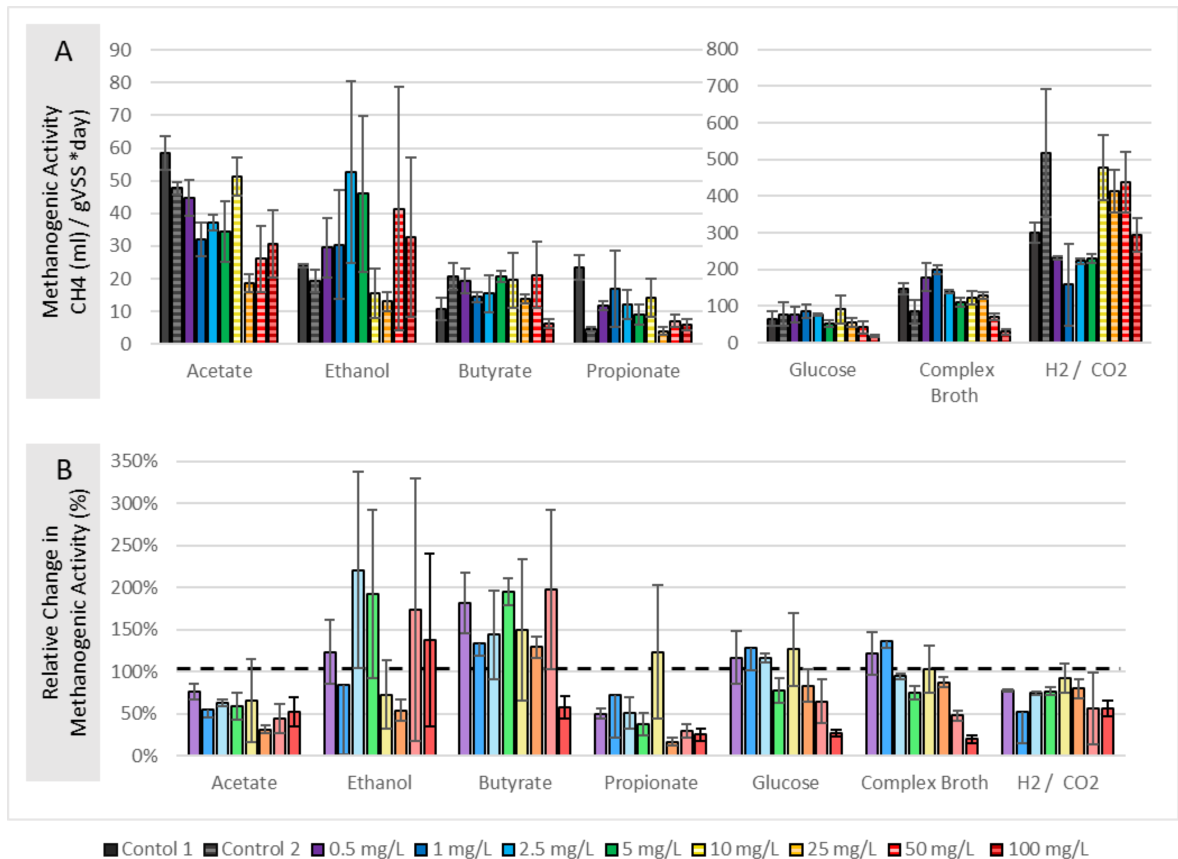



***Figure 6-6** - Changes in methanogenic activity in response to the different amoxicillin exposure. Figure A represents the calculated rates using 5 points. Both the left and right axis represent methanogenic activity (CH4 (ml)/gVSS *day) on different scales. Figure B represents the percent change from the mean control values. All bars report the mean value for the three replicates. The error bars report one standard deviation from the mean.*

286

287 ### *6.3.2.3 Acidogens – Glucose*

288 The presence of amoxicillin had a clear inhibitory effect on the rate of methanogenesis in the glucose
289 fed bottles. The rate of methanogenesis in Run 1 (0.05 – 2.5 mg/L) was as faster than the control
290 values (116 – 129%). However, within Run 2, when amoxicillin levels were 25 mg/L or greater, the
291 rate of amoxicillin was less than the control for that run (**Figure 6-6**). While the first run was not
292 found to be statistically significant, the increase in amoxicillin was statistically correlated to a
293 decrease in the rate of methanogenesis (Appendix C).

294 ### *6.3.2.4 Hydrolysers – Complex Broth*

295 The presence of amoxicillin has had a marked difference on the timing and the rate of methanogenic
296 activity within the complex broth fed bottles (**Figure 6-6**). Like the glucose fed bottles, the bottles
297 fed complex broth has a slight increase in the rate of methanogenesis for the low amoxicillin exposure
298 conditions in which the 1mg/L amoxicillin test bottles produced an average of 136% of the control.

However, above this point there is a steady decrease in the rate of methanogenesis until 100mg/L which produced methane at 20% of the control. The correlation between the increase in amoxicillin and a decrease in methane was found to be statistically significant for both runs ($p<0.05$) (Appendix C).

### 6.3.3 Total Volume of Methane

#### *6.3.3.1 Methanogens – Acetate and $H_2/CO_2$*

The addition of amoxicillin inhibited the production of methane for all amoxicillin concentrations above 0.05mg/L (**Figure 6-7**). The average volume of methane produced by the 1mg/L amoxicillin concentration was only 87% of that produced in the control. The total methane volume slowly decreased until 100mg/L which produced only 56% of the total methane compared to the control by the end of the study. While the correlation between the addition of amoxicillin and the production of methane was statistically significant for the first run ($p<0.05$) this correlation was not significant in the second run (Appendix D).

The total methane produced in the $H_2/CO_2$ bottles decreased compared to the control for the amoxicillin conditions of 10 mg/L and higher. While the 10mg/L condition produced an average of 92% of the control, the 100mg/L conditions only produced an average of 52% compared to the control conditions. There was no statistical correlation between the addition of amoxicillin and a change in total methane for the first run, however there was a significant correlation for the second run ($p<0.05$) (Appendix D).

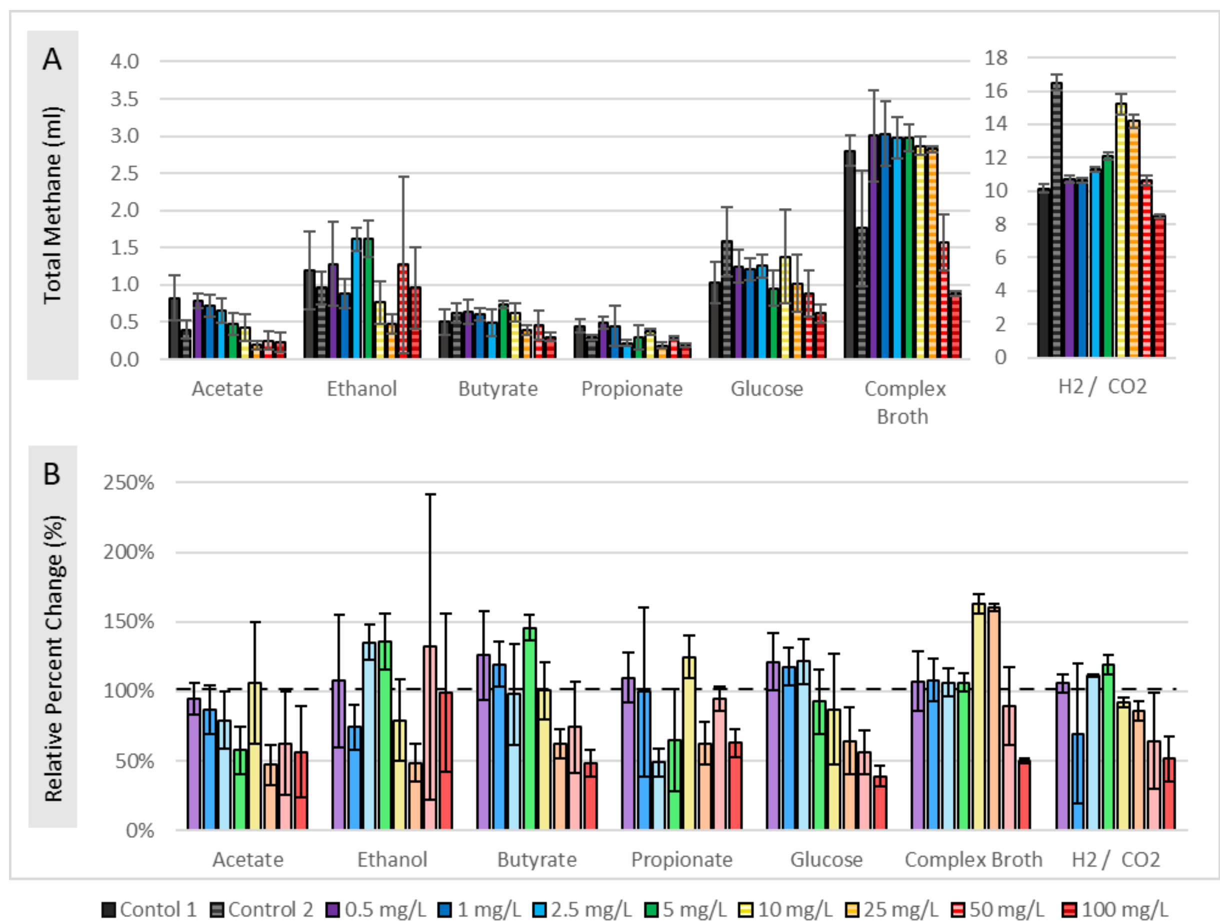



***Figure 6-7*** *- Total Methane produced at 66 hours. Figure A represents the calculated rates using 5 points. Both the left and right axis represent methane volume (ml) on different scales. Figure B represents the percent change from the mean control values. All bars report the mean value for the three replicates. The error bars report one standard deviation from the mean.*

319

320 ### *6.3.3.2 Acetogens - ethanol, propionate, and butyrate as substrate*

321 There was no clear trend in the addition of amoxicillin and a change in the total volume of methane
322 for the bottles fed with ethanol or propionate (**Figure 6-7**). The methane produced in the ethanol fed
323 bottles was highly variable between each condition and between the replicates. None of these values
324 were statistically different from the control. As was observed in the rate of methanogenesis in the
325 propionate bottles, the changes in methane production appear to be more linked to the set than to the
326 amoxicillin concentration. While the 0.5, 1.0, and 10mg/L were each roughly the same at the
327 respective set control, the 2.5, 5.0, 25, and 100 mg/L conditions each produced less total volume than
328 the respective set control. The addition of amoxicillin was not correlated with changes in total
329 methane production for either run when ethanol was the primary feed. No statistical corelation was

found for the first run when propionate was fed, however there was a statistical correlation in the second run ($p<0.05$) (Appendix D).

The addition of amoxicillin decreased the total methane produced for the butyrate fed bottles for the 25mg/L and above. While the rate of amoxicillin was observed to be similar for most conditions, amoxicillin exposure conditions of 10 mg/L and below, methane production continued after the initial 8 hours of incubation. For the higher conditions (25 through 100mg/L) methane production stopped shortly after 10 hours. This resulted in a decrease in the net volume of methane produced for these values. No statistical correlation between the addition of amoxicillin and an increase in methane was found for Run 1, though there was one found for Run 2 ($p<0.05$) (Appendix D).

#### *6.3.3.3 Acidogens – Glucose*

The increase in amoxicillin resulted in a decreased in the total volume of methane in the glucose fed bottles. In the concentrations below 2.5mg/L, however, there was slight increase in the total methane produced, each producing roughly 121% of the control volume (**Figure 6-7**). For conditions of 5mg/L and above, there was a steady decrease in the total volume produced until the 100mg/L condition, which had a mean methane production of just 39% of the control values. These changes coincided with a reduction in the rate and an increase in lag of methanogenic activity observed. However, due to the variation in the replicates, none of these differences were statistically significant ($p > 0.05$) for Run 1. However, a correlation between the addition of amoxicillin and a reduction in total methane was found for Run 2 ($p < 0.05$) (Appendix D).

#### *6.3.3.4 Hydrolysers – Complex Broth*

In addition to the delay in timing and reduction in the rate of methanogenic activity with the addition of amoxicillin, there was a reduction in the total methane produced in the complex broth fed bottles as amoxicillin concentrations increased (**Figure 6-7**). For the amoxicillin concentrations of 5mg/L and below, there was no observable difference in the total volume of methane produced. While the 10mg/L and 25mg/L conditions both proceed the same volumes of methane as the lower concentrations, when compared to the set 2 control, they were a net increase in total methane of approximately 160% for each. However, the 50mg/L and 100mg/L conditions, there was a decrease

in total methane matched by a decrease in the rate of methane produced. Due to the variation in the replicates, none of these differences were statistically significant ($p > 0.05$) for Run 1. However, a correlation between the addition of amoxicillin and a reduction in total methane was found for Run 2 ($p < 0.05$) (Appendix D).

### 6.3.4 Amoxicillin and Timing of Methanogenesis (Lag time)

Although there was some variation in the timing of the maximum rate of methanogenesis, no lag in activity was observed for the methanogenic or acetogenic test conditions. However, there was a clear trend between the addition of amoxicillin and a lag in activity observed for the glucose and complex broth fed test conditions (**Figure 6-5 and Figure 6-8**).

When glucose served as substrate, the addition of amoxicillin at all concentrations produced a noticeable increase in the lag time before the maximum rate of methanogenic activity **(Figure 6-8)**. No lag was observed in the controls. The 0.5mg/L amoxicillin conditions had an average lag time of 18 hours of incubation, while in the 100mg/L amoxicillin conditions the lag in activity increased to 38 hours of incubation. These values corresponded with a decrease in methanogenic activity above 2.5mg/L. All lag times were statistically different from the control value ($p<0.02$).

For complex broth fed bottles the addition of amoxicillin was also associated with increased lag time before methanogenic activity **(Figure 6-8)**. No lag was observed in the controls, however by the 2.5mg/L amoxicillin conditions the lag in activity was an average of 4.67 hours of incubation. The 100mg/L conditions has an average lag of 15 hours. Lag times for amoxicillin test conditions over 2.5mg/L were statistically different from the control value ($p<0.02$).

The increase in lag time associated with an increase in the addition of amoxicillin is likely the result of cell death associated with an increase in amoxicillin concentrations in the feed. Research by Trego et al. (2020) suggests that microbes within the anaerobic granules which are responsible for hydrolysis and fermentation are located on the outside of the granule. As such they might come into closest contact with amoxicillin. This could result in a lag as there would be fewer members of the community breaking down the available substrate. The lag in the lag associated with fermentation may be more severe than the lag associated with hydrolysis, as fermentation is conducted by enzymes

384 inside the cell, while hydrolysis is conducted extracellularly (Madigan, 2014) . Therefore, even in
385 the case of the death of the cell, the enzymes performing hydrolysis may remain active.

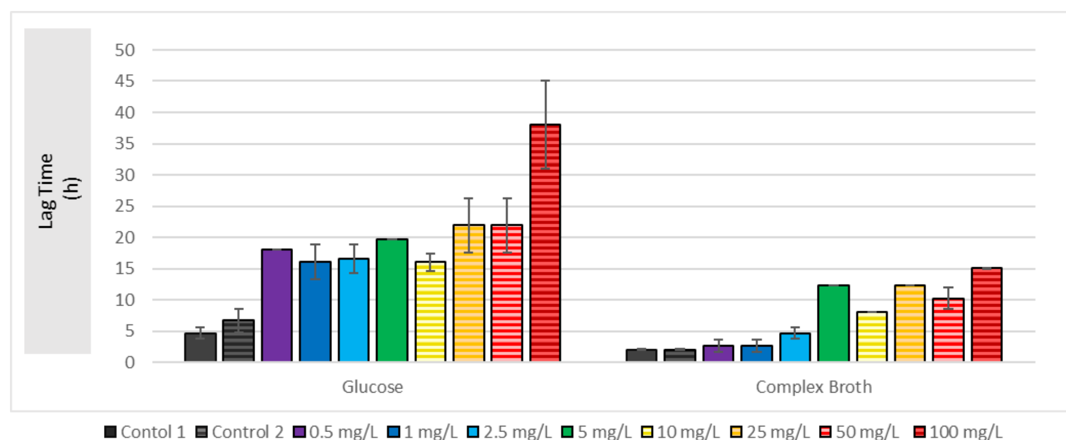



***Figure 6-8** – Lag time.*
*Lag time as defined as the timing of the first point used to calculate maximum methanogenic activity rate. All bars report the mean value for the three replicates. The error bars report one standard deviation from the mean.*

386

387 ## 6.4 DISCUSSION

388 The primary aim of this study was to measure how acute exposure to amoxicillin impacts the activity
389 for each of the trophic groups within an anaerobic community in terms of the rate and timing (lag
390 time) of methanogenesis and the total volume of methane produced from specific substrates. It was
391 established that for all feed types tested the presence of amoxicillin resulted in a measurable change
392 in the methanogenic activity, though the sensitivity of the microbes to amoxicillin differed between
393 test conditions.

394 All methanogens belong to the domain of archaea and are assumed to be naturally resistant to the
395 effects of beta-lactam antibiotics due to the lack of peptidoglycan in their cell walls (Kandler and
396 König, 1998). This study hypothesised therefore that the addition of amoxicillin to the substrate
397 would not inhibit the production of methane from either acetate or hydrogen / $CO_2$. However,
398 exposure to amoxicillin had a measurable impact on the activity of both methanogenic substrates.
399 While there was no observed change in the rate of methanogenesis for either the hydrogenotrophic
400 or the acetoclastic methanogens, there was a reduction in the total methane produced. Although the
401 amoxicillin theoretically would not have affected the methane forming archaea present, there are

402 many bacterial species which are capable of utilising acetate, hydrogen, and carbon dioxide (Ferry,
403 2015; Mutungwazi, Ijoma and Matambo, 2021). It is possible that some of the substrate would have
404 been used by those organisms towards repairing structural damage resulting from amoxicillin and
405 may not have been converted into methane.

406 The presence of amoxicillin had a minimal effect on the utilisation of the provided VFAs within this
407 study. However, it should be noted that the methanogenic activity was markedly smaller for all
408 conditions, including the control, than as would have been expected given previous studies using the
409 same seed community (see Method Development Chapters 4 and 5). For example, 0.1ml of 3M
410 butyrate produced an average of 11.25 ml methane at a rate of 184.7 ($CH_4$ (ml)/gVSS*day) in the
411 pilot study compared to an average of 0.56 ml of methane produced at a rate a maximum rate of
412 15.70 ($CH_4$ (ml)/gVSS*day) in this study. There was nearly a year between these studies. During that
413 time, the culture which had previously been acclimated to distillery waste as its primary substrate
414 (ethanol and other VFAs) was instead primarily fed a glucose and nutrient mixture. This could have
415 instead acclimated the community towards sugar fermentation as well as the hydrolysis of dead
416 cellular matter as its primary substrates. As a result, the community would have a higher rate of
417 methanogenesis when fed glucose fermentation and complex organic hydrolysis as was observed. As
418 a result, the lack of response to the presence of amoxicillin could be a result of this lack of activity
419 rather than resistance to beta-lactam antibiotics per se.

420 The presence of amoxicillin had a clear dose/response effect on the rate, timing, and overall volume
421 of methane produced by acidogenic and hydrolytic microbes. Furthermore, they were the only feed
422 types associated with a clear increase in lag phase as the result of the presence of amoxicillin,
423 particularly in the case of glucose fermentation. The fermentation of glucose can take a variety of
424 different pathways which are conducted by a variety of bacterial types, though the most common
425 products are propionate, butyrate, ethanol, acetate, formate, and $CO_2$ (Gerardi, 2003; Madigan, 2014).
426 The full hydrolysis of complex broth into methane requires additional steps to break down the lipids
427 and proteins within the mix. Hydrolysis itself is undertaken using exoenzymes on the outer
428 membrane of the cell often, by the same bacterial types which produced the hydrolysing enzymes

429 (Mutungwazi, Ijoma and Matambo, 2021). The changes in the rate of methane production are likely
430 the result in cellular damage because of the presence of amoxicillin within the substrate. The
431 reduction in total methane produced could be the result of the substrates being utilised to build more
432 cellular materials in response to the physical damage done to the cell walls by the amoxicillin.

433 That lag phase increases due to acute exposure to amoxicillin in the treatment of complex substrates
434 is an important finding in relation to wastewater treatments in that the lag could result in temporal
435 decreases in treatment efficiency in anaerobic systems. Whilst so, the cause of the increased lag phase
436 was not ascertained in this study. Tentatively, the lag in methanogenic activity observed for the
437 glucose and complex broth fed bottles could be caused by the time required for repopulation within
438 the trophic cascade. If key members were inhibited, damaged, or killed by the presence of
439 amoxicillin, this could have prevented the methane from being completely formed until that part of
440 the community could repopulate. Additionally, if the provided substrates were required for the
441 reconstruction, this could also explain the delay in activity. Furthermore, as was discussed in section
442 6.3.4, the members of the community responsible for fermentation and hydrolysis are physically
443 located on the outside of the granule where they would be most exposed to any amoxicillin within
444 the surrounding medium (Trego *et al.*, 2020)s. Therefore, they would potentially the most sensitive
445 as they have the most contact with the amoxicillin. While fermentation and hydrolysis are often
446 conducted by the same microbes (de Lemos Chernicharo, 2015) as hydrolysis is conducted
447 extracellularly, it is possible that enzymes could still breakdown substrates after the cell died. Finally,
448 if antimicrobial resistance genes were latent in the community, this lag could be caused by the time
449 required for these genes to be activated and the enzymes required to inactivate the amoxicillin to be
450 produced. To confirm these hypotheses, future studies which track the VFA make up within the
451 effluent throughout the study could determine which point the in the trophic cascade was most
452 inhibited by the amoxicillin. The build-up of a single VFA product could indicate inhibition of the
453 microbes responsible for their degradation. Genetic sequencing of the community using next
454 generation sequencing (NGS) could indicate which members of the community are promoted or
455 inhibited after amoxicillin exposure. qPCR targeting genes associated with amoxicillin resistance
456 could also be used to determine any resistance genes present. A temporal analysis throughout an

457 exposure event / SMA which analyses these hypotheses could indicate the causes and activity during
458 the lag time caused by the presence of amoxicillin.

459 Despite the wide use of beta lactams in human health, and the widespread use of anaerobic
460 communities to treat sewage, there are few studies which specifically look at the effects of
461 amoxicillin on anaerobic microbial communities. The different studies don't always agree and
462 variations in study design make it difficult to compare these studies directly. For example, a study
463 on antibiotics effect on net methane production found that 10 mg/L penicillin led to a partial
464 inhibition of 25-45% of total methane produced (Sanz, Rodríguez and Amils, 1996). Another study
465 which assessed different antibiotics effect on nitrogen removal rate reported that anaerobic systems
466 acclimated to amoxicillin could withstand concentrations up to 60mg/L without inhibition (Zhang *et*
467 *al.*, 2015). A study by Su et al (2019) did not report any inhibition of methane in anaerobic granules
468 exposed to 100mg/L amoxicillin (Su *et al.*, 2019). This study has shown that the potential sensitivity
469 of amoxicillin on the activity in anaerobic system is highly dependent on the makeup and activity of
470 the culture being tested and the feed being used. For example, amoxicillin in a highly proteinaceous
471 feed could reduce the rate of breakdown, and in the case of wastewater, the treatment efficiency of
472 the sewage. However, amoxicillin appeared to have little influence on the degradation of gaseous
473 substrates. As such, this research supports the findings of the other research currently available as
474 well as providing potential insight to the variation in findings between the studies as each of these
475 studies contained different feed substrates.

476 Furthermore, there is very little research into the concentrations of pharmaceuticals in septic tanks
477 environments. Reported amoxicillin concentrations in WWTP influent range greatly. While most
478 western countries may see an average amoxicillin in influent around 13 ng/L (Zuccato, Castiglioni
479 and Fanelli, 2005) extreme cases have been reported as high as 43,980 ng/L (Peña-Guzmán *et al.*,
480 2019). The influent into septic tanks can be might more concentrated in terms of COD as well as the
481 contaminants it contains. Conn (2010) showed that pharmaceuticals and organics in septic tanks can
482 be 1-2 orders of magnitude higher than those found in centralised water treatment facilities (Conn,
483 Siegrist, *et al.*, 2010). Therefore, it is not unreasonable to think that concentrations as high as 5mg/L

484 in extreme cases may be found in septic tanks. Based on the results of this study, as well as those
485 conducted by others (**Table 6-2**), it is reasonable to think that an acute exposure event of amoxicillin,
486 as might be found on a rural septic tank, or at a medical facility, might partially inhibit the microbial
487 community in receiving septic tank and thus reduce the capacity of the system to process waste.
488 However, the scale of the inhibition will depend on the study design.

489 *Table 6-2 - Average inhibition comparison from different feed complexities for concentration ranges.*
490 *Percentages are expressed as a proportion of the average of the control.*
491

| Feed | Measurement | Antibiotic | 0.5-2.5mg/L | 5-25mg/L | 50-100mg/L | Source |
|---|---|---|---|---|---|---|
| Acetate | Total Methane (ml) | Amoxicillin | 86.82% | 70.28% | 59.57% | This study |
| Ethanol | Total Methane (ml) | Amoxicillin | 105.60% | 87.95% | 115.60% | This study |
| Propionate | Total Methane (ml) | Amoxicillin | 114.39% | 102.93% | 61.32% | This study |
| Butyrate | Total Methane (ml) | Amoxicillin | 86.10% | 84.09% | 78.82% | This study |
| Glucose | Total Methane (ml) | Amoxicillin | 120.04% | 81.32% | 47.34% | This study |
| Complex Broth | Total Methane (ml) | Amoxicillin | 107.23% | 143.26% | 69.77% | This study |
| Acetate, propionate, butyrate | Total Methane (ml) | Penicillin | - | -25.45 | - | (Sanz, Rodríguez and Amils, 1996) |
| Synthetic wastewater | Nitrogen Removal | Amoxicillin | 0% | 0% | 0% | (Zhang *et al.*, 2015) |

492

493 While SMAs are a simple and inexpensive method for determining the activity of microbes in
494 response to substrates, they do not provide an exact identification of microbes within the culture.
495 Molecular methods would provide a greater window into the specific changes in the population in
496 response to the presence of amoxicillin on a species level. However, genetic sequencing would be
497 sensitive enough to measure changes not assessed by this SMA, such as specific changes to the
498 methanogenic pathways. Furthermore, whilst resistance to beta-lactam antibiotics within hospitals
499 and wastewater treatment facilities is well documented (Laht *et al.*, 2014; Hultman *et al.*, 2018), at
500 the time of writing there are no studies which specifically measure the proliferation of AMR genes
501 within an anaerobic community in response to added amoxicillin. A future study which measures
502 changes in the resistance genes throughout an anaerobic community would demonstrate an additional
503 way that these communities would change in response to the presence of amoxicillin. This is
504 particularly important to understand the spread of antimicrobial resistance genes through anaerobic

water treatment technologies as contaminated effluents may flow into the water environment where they can pose a danger to human and environmental health.

Finally, this study focused exclusively on shock exposure to amoxicillin on anaerobic communities. However, in many anaerobic systems, such as septic tanks treating hospital waste or natural anaerobic environments, exposure would be chronic. This would allow for adaptation to the regular presence of the antibiotic. As the microbial community composition and / or resistome shifts it is possible that sensitivity to amoxicillin will shift as well. Studies comparing the chronic and acute exposure have been conducted for other pharmaceuticals such as tetracycline (Cetecioglu *et al.*, 2013). Therefore, an additional study which examines long term chronic effects of amoxicillin would be useful to understand chronic as well as acute exposure.

## 6.5 CONCLUSION

It is increasingly important to understand how the antibiotics that we use impact our water treatment systems. Amoxicillin is one of the most prescribed antibiotics in the world. In areas which are not reliant on centralised water treatment systems, septic tanks provide most of the waste treatment though anaerobic digestion of waste and conversion of organics into methane. Nevertheless, the effects of amoxicillin and other beta-lactams have on these small water treatment systems is rarely studied. **This research showed that low concentrations of amoxicillin have the potential to increase methane production in these systems though at concentrations of 5mg/L -100mg/L amoxicillin, the treatment efficiency of these systems drops with the severity of inhibition dependant on the substrate type. Further, the impact of amoxicillin on activity is not uniform across trophic groups in an anaerobic microbial community.** While the presence of amoxicillin appears to result in minimal inhibition in the rate or volume of methane produced by the VFA fed test conditions, the methane production requiring hydrolysis or fermentation appear to be sensitive to amoxicillin at all concentrations tested. Additionally, the presence of amoxicillin resulted in an increase in the lag time for maximum methanogenic activity within the hydrolytic and glucose degradation pathways. This study shows that there is a measurable effect of amoxicillin in the range of concentrations that might be found in septic tanks. Given that decreased treatment rates and

532 increased lag time prior to commencement of treatment of complex wastes could result in decreased
533 treatment efficiency in septic systems, this finding is important therefore more research is needed to
534 better understand this relationship for both acute effects on a longer time scale.

# Chapter 7
# Changes In Community Structure and AMR Genes in Anaerobic Granules After Acute Amoxicillin Exposure

## 7.1 Introduction

Antibiotic resistance in the wastewater sector is an issue of global importance. Long term chronic exposure of highly active microbial communities to sub-lethal concentrations of a variety of antibiotics, as are found in wastewater treatment plants (WWTPs), generates ideal conditions for the development and proliferation of antimicrobial resistance genes (Rodriguez-Mozaz *et al.*, 2015; Wu *et al.*, 2016; Wang *et al.*, 2020). Centralised wastewater treatment facilities have been long observed to be point sources of the spread of antimicrobial resistance genes into the water environment (Kümmerer, 2009b). As such there are many studies exploring antimicrobial resistance wastewater microbial communities in long term bench scale studies (Aydin, Ince, *et al.*, 2015; Aydin, Ince and Ince, 2015a).

However, much of the world is reliant on decentralised wastewater treatment (Libralato, Volpi Ghirardini and Avezzù, 2012). As influent into these systems is more sporadic and concentrated than those entering centralised WWTPs, so too is the antibiotic within those influents resulting in acute rather than chronic antibiotic exposure. Whilst there are studies which focus on how acute exposure of antibiotics within an anaerobic microbial community changes activity (Cetecioglu *et al.*, 2012; Ozbayram *et al.*, 2015), there are very few which study the effect of beta lactams. Chapter 6 of this

25 thesis demonstrated that acute exposure to amoxicillin can result in immediate changes to microbial
26 activity in terms of the methane production rate and lag time before which substrates were
27 metabolised into biogas and methane. A reduction in activity implies the inhibition in activity and or
28 outright mortality of some members of the community. Amoxicillin is a broad range antibiotic which
29 inhibits the generation of penicillin binding protein (PBP) found within the cell walls of prokaryotes
30 (Macheboeuf *et al.*, 2006; Madigan, 2014) though susceptibility to amoxicillin varies from species
31 to species (Gartiser *et al.*, 2007; Shi, Leong and Ng, 2017). Furthermore, archaea, which don't
32 contain any PBP in their cell walls, are generally resistant to all beta-lactams. Anaerobic microbial
33 communities, such as those found in anaerobic granules, are highly syntrophic whereby the food for
34 some members of the community are produced (as metabolites) by other members within the trophic
35 cascade. A reduction in numbers or metabolic rate of one part of the community, therefore, could
36 result in a decline in activity and robustness of the community as a whole.

37 The presence of sublethal concentrations of antibiotics has shown to correlate to an increase in the
38 presence of antimicrobial resistance genes within the anaerobic communities. For example, an
39 increase in tetracycline resistance gene counts was observed in anaerobic microbial communities
40 when exposed to erythromycin, tetracycline and sulfamethoxazole (Aydin, Ince and Ince, 2015b).
41 Similar studies focusing on the development of antibiotic resistance to beta-lactam antibiotics in
42 anaerobic microbial communities are not available. Nevertheless, medical research, which focuses
43 on single strains of anaerobic bacteria have demonstrated beta-lactam resistance is present in many
44 species. For example anaerobic Bacteroides, Fusobacterium, and Clostridium species have long been
45 shown to produce beta-lactamase enzymes (Nord and Hedberg, 1990).

46 This study aimed to expand upon the work conducted in Chapter 6 of this thesis. As the cultures fed
47 glucose in that study appeared to be the most sensitive to the presence of amoxicillin, glucose was
48 used as the primary substrate in this study. The previous work established that increasing amoxicillin
49 exposure inhibited methanogenesis within anaerobic granular microbial communities. The
50 hypotheses tested are summarised in **Table 7-1**.

51

52 *Table 7-1 - Hypotheses*

| | Hypotheses |
|---|---|
| 1. Microbial activity | • Increasing amoxicillin would inhibit methanogenic activity.<br>• And hence the sCOD removal within the microbial communities would decrease with an increase in amoxicillin exposure. |
| 2. Community structure | • There will be a mixed microbial consortia for AD within the granules.<br>• The presence of amoxicillin will drive a change in microbial community composition which is distinct to that observed in the control (no-amoxicillin group).<br>• The changes in the community will have a resulting reduction in the overall robustness of the community as a whole and its ability to tolerate more change. |
| 3. Antibiotic resistance. | • Anti-microbial resistance genes would be present in the seed community at the offset.<br>• Exposure to amoxicillin will increase the relative abundance of beta-lactamase and other resistance associated genes. |

53

54
## 7.2 METHODS

55
### 7.2.1 Experimental design

56 This study aimed to monitor changes in community structure and the proliferation of resistance genes
57 within granular anaerobic microbial communities exposed to beta-lactam antibiotics. The analytical
58 methods summarised in this section are explained in further detail below (**Figure 7-1 and 7-2**). To
59 test how the microbial communities within anaerobic granules changed in response to an acute
60 exposure to amoxicillin, sub-samples of a common seed sludge were exposed to three different
61 concentrations of amoxicillin (1mg/L, 10 mg/L, and 50 mg/L). The sub-samples were incubated
62 using glucose as substrate along with fed control (no amoxicillin) and unfed control sub-samples and
63 were monitored over 48 hours during which time a series of sacrificial bottles were removed for
64 analysis to capture temporal changes **(Figure 7-3)**. All test conditions were conducted in triplicate.
65 The amoxicillin concentrations selected for this study were informed by the results of Chapter 6 in
66 which measurable suppression of methanogenic activity as well variations in the lag in the start of
67 activity were observed when amoxicillin in the substrate was at 5 mg/L and greater. Conversely the
68 inhibitory effects of 1 mg/L of amoxicillin on the timing and rate of amoxicillin were not statistically

69 significant. As it is possible that there may have been microbial changes in the resistome that were
70 independent of methanogenesis, this 1 mg/L value was also included in this study. As the previous
71 study demonstrated the inhibitory effect was greatest for the cultures fed exclusively on glucose, the
72 cultures within this study were also fed glucose with the aim of replicating the results. The pressure
73 in the head space of the sealed serum vials in which the sludge sub-samples were incubated were
74 monitored every 2-6 hours throughout the study and headspace gas was extracted from the bottles at
75 the final time point to determine methane production for each test condition. To obtain temporal data
76 within the study, seven sets of three sacrificial bottles were prepared for each test conditions (i.e. 3
77 sacrificial bottles for each test condition at each of 7 time points). Bottles were removed (sacrificed)
78 for times correlating to **Figure 7-3**. For each set of sacrificial bottles sampled, the effluent and
79 granules were immediately removed and stored for later analysis. Microbial community composition
80 was determined using next generation sequencing of the V4 region of the 16s rRNA gene using the
81 Illumina Miseq sequencing platform (San Diego, CA). The SmartChip Real-Time PCR system
82 (Takara Bio Inc., Shiga, Japan) was used to detect and quantify the AMR genes present.

83

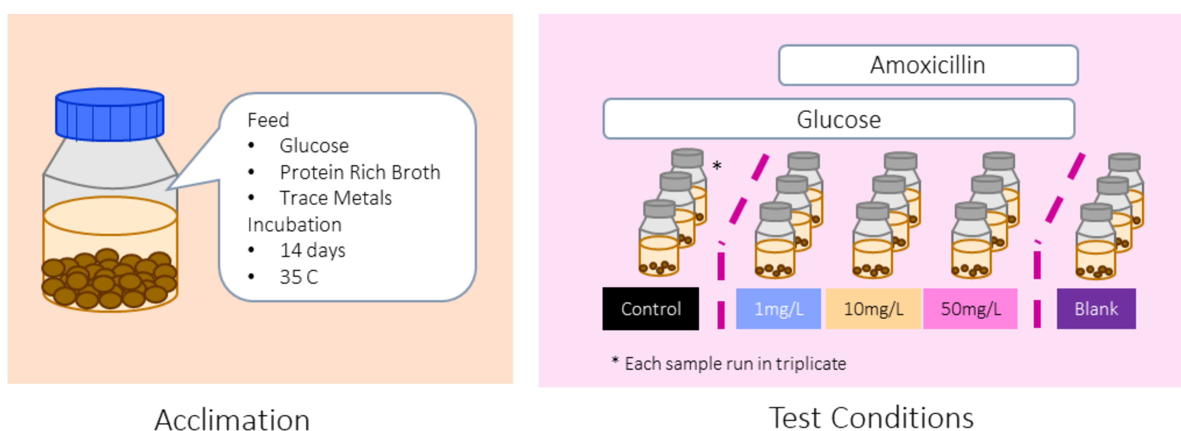



***Figure 7-1*** *- Experimental design summary.*
*The acclimation was conducted over 14 days with a protein rich broth and a trace metal nutrient added to the substrate solution. The test conditions contained three different amoxicillin feed types, as well as a control, with glucose and no amoxicillin and a blank with no glucose and no amoxicillin. All were run in triplicate.*

84

85

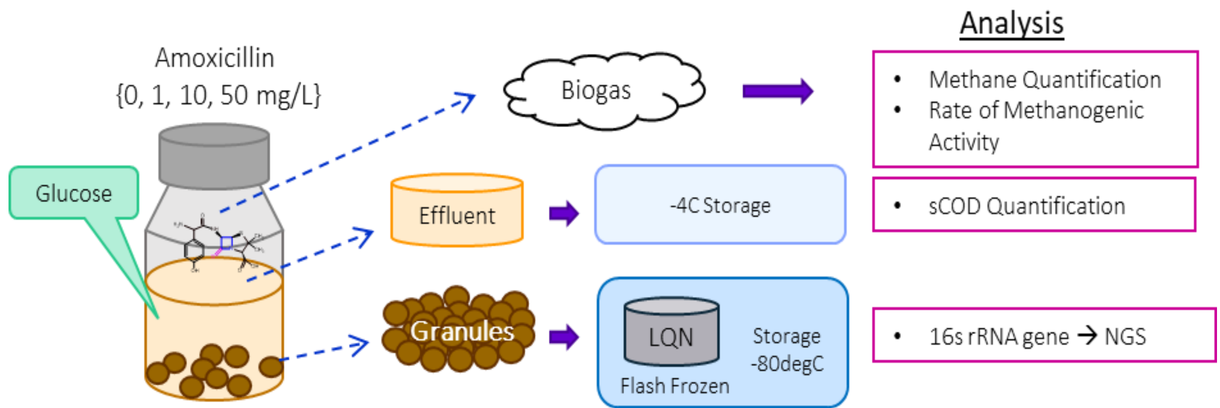



***Figure 7-2*** *Sub sampling and analyses for each sample.*
*The biogas, effluent, and granules were each sampled and stored separately. The biogas was analysed immediately for methane quantification. The effluent was stored at -4C before sCOD quantification. The granules were flash frozen in the liquid nitrogen immediately after removal and stored at -80C before DNA extraction for next generation sequencing and AMR gene quantification using qPCR array.*

86

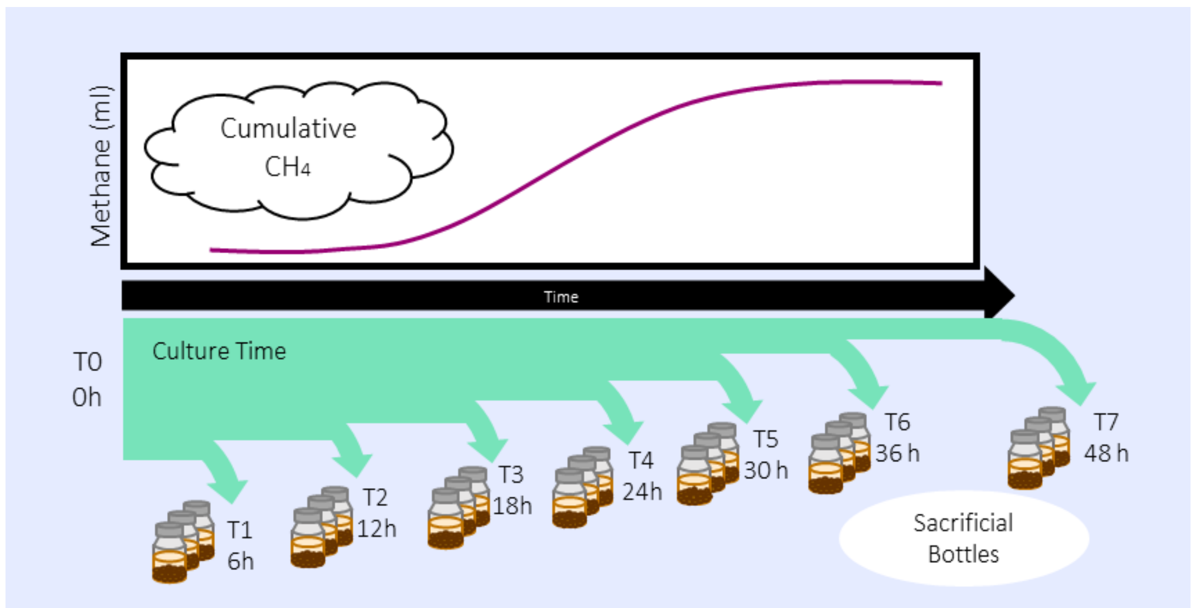



***Figure 7-3*** *- Sampling schedule.*
*Seven sub-sample time points at which triplicate bottles of each test condition were removed from the study obtain information about temporal variation in the effluent and microbial community. Triplicate samples were taken for each set of amoxicillin test conditions as well as a blank and a control for a total of 15 bottles removed at each sacrificial point.*

87

88 ### 7.2.2 Anaerobic culturing and Sampling

89 Anaerobic culturing was conducted as described in Chapter 3 section 3.1 and is summarised here. A
90 GMA was conducted using glucose as the primary substrate. One millilitre of a 1M glucose solution
91 was added to 0.5g of anaerobic granules and 9.5ml anaerobic medium containing nutrient additives,
92 for a total of 10ml. The study tested three amoxicillin concentrations with an in-bottle concentration
93 of 1 mg/L, 10 mg/L, and 50 mg/L. A total of five test conditions were run as described above.
94 Pressure readings in the headspace of each bottle were measured every 2 hours for a total duration
95 of 48 hours. The methane concentration of the headspace gas was measured at the end of the
96 experiment using a GC-FID and the rate of methane production was calculated (as described in
97 Methods Chapter Section 3.3). As the provided glucose was often consumed within 6 hours, a 4-
98 point window was used to calculate maximum activity rate. Upon removal of the sacrificial bottles,
99 the bottles were opened. The effluent was decanted and filtered through a 0.2um Whatman filter and
100 transferred to a sterile 15ml tube and stored at -20C until sCOD quantification as described in Section
101 0. Anaerobic granules were transferred to 2ml Biobanking and Cell Culture Cryogenic Tubes and
102 flash frozen using liquid nitrogen before storage at -80C until DNA extraction as described in Section
103 7.2.4.1.

104 ### 7.2.3 Measuring sCOD

105 The soluble COD (mg/l) of the filtered effluent was measured in accordance with Standard Methods
106 ISO 15705 using Hach LCK 400, (0-1000 mg/L $O_2$) and LCK 014 (1,000-10,000 mg/L $O_2$) kits and
107 quantified using a Hach DR 2800 spectrophotometer. The LCK 014 kits were initially used, however
108 when supplies ran out, samples were diluted to fall within the range of detection for the LCK 400
109 kits. The theoretical sCOD of the media at T0 was calculated from theoretical sCOD for the glucose
110 (**Equation 1**) was combined with the COD measurements of the anaerobic medium mixed and
111 nutrient mixture.

$$1920\frac{mg}{l}\,tCOD\ Glucose = \frac{6 * 32\frac{g}{mol}\ O2 * 1800\frac{mg}{L}\ Glucose}{180\frac{g}{mol}Glucose}$$

***Equation 2***

112

113 ### 7.2.4 Molecular Microbiology Methods

114 #### *7.2.4.1 DNA Extraction*

115 Anaerobic granules were flash frozen in liquid nitrogen at the time of sampling and stored at -80°C.
116 The total genomic DNA was extracted from the stored samples at the end of the experiment. Samples
117 were placed on ice and allowed to thaw for 10 min before processing. Care was taken to ensure that
118 each sample only experienced one freeze thaw cycle as part of the extraction process to minimise
119 potential DNA degradation. Once thawed, each sample was homogenised within the sampling tube
120 using a sterilised glass rod to ensure representation of the entire community in the extracted DNA.
121 DNA was extracted from 0.3g of the homogenised samples using the Fast DNA spin kit for soil (MP
122 Biomedical) and Fast Prep -24 instrument.

123 #### *7.2.4.2 Preparation of barcoded amplicons for NGS*

124 To provide insight to microbial community composition and dynamics during the study, the DNA
125 extracted from the anaerobic granules was prepared for next generation sequencing (NGS). Prior to
126 PCR preparation, the extracted DNA was thawed and quantified using the Qubit dsDNA BR and
127 normalised 1ng/ul using PCR grade water (Qiagen Nuclease free water Cat no. 129115) and stored
128 at -80°C until PCR amplification. Amplicons were prepared by PCR using barcoded primers
129 targeting the V4/V5 region of the 16s rRNA gene (F515 (Caporaso *et al.*, 2011) and R926 (Walters
130 *et al.*, 2016)) using Golay barcode primers which are each synthesized with a unique barcode gene
131 sequence and Illumina sequencing adapter (Caporaso *et al.*, 2012). PCR amplification was conducted
132 using the KAPA HiFi HotStart Ready Mix Kit (KAPA Biosystems) with the PCR conditions and
133 reactions prepared as described in **Table 7-2 and 7-3** respectively. Triplicate 25ul reactions were
134 used for each sample (for a total of 75ul per sample) to maximise amount of final PCR product while
135 minimising PCR error. Triplicate reactions were pooled and the PCR product was purified using the
136 Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's specification.
137 When the yield of the of the PCR production was below 10ng/ul for the combined triplicated PCRs,
138 the PCR would be repeated, pooled and purified. The mean library length was 411 bp.

139

140 *Table 7-2 - The PCR temperature and time conditions.*

| Phase | Time (min) | Temp (c°) | Repeat |
|---|---|---|---|
| **Initial Denaturation** | 5:00 | 95 | |
| **Denaturation** | 0:20 | 98 | X 25 |
| **Annealing** | 0:30 | 62 | |
| **Extension** | 0:40 | 72 | |
| **Final Extension** | 1:00 | 72 | |

141

142 *Table 7-3 - Volume of the PCR volumes used.*

| Reagent | Optimisation Volumes (ul) | Test Volume (ul) |
|---|---|---|
| ***PCR Grade Water*** | 6.6 | 11 |
| ***Hifi taq*** | 3 | 5 |
| ***dNTPs*** | 0.45 | 0.75 |
| ***Reverse Primer (926R) 0.3uM*** | 0.45 | 0.75 |
| ***Taq*** | 0.3 | 0.5 |
| ***Forward Primer (F515.X) 0.3uM*** | 0.45 | 0.75 |
| ***Template / NTC*** | 3.75 | 6.25 |
| ***Total*** | **15** | **25** |

148

149 The purified PCR product for each sample was then normalised to 10ng/ul DNA in nuclease free
150 water. An aliquot of 2ul was removed from each normalised sample and pooled together in a 2ml
151 tube to create a single sample for sequencing. Pooled samples were vortexed and quantified using
152 the Qubit dsDNA HS (high sensitivity) kit. Purity was assessed visually using a 2% agarose gel (as
153 described above). The pooled and multiplexed library was processed by the Earlham Institute (EI)
154 (Norwich, UK) and 300bp paired-end reads for each sample was obtained on an Illumina platform.

155 ### *7.2.4.3 Selection and Screening of AMR gene targets*

156 The quantification of antimicrobial resistant genes was conducted using the SmartChip Real-Time
157 PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). This system contains
158 5184 x 100nl reaction wells which allow for 14 different sample and assay configurations allowing
159 for up to 384 samples or assays to be run simultaneously on a single chip. To determine the selection
160 of gene targets used, the quantification of antimicrobial resistance genes was conducted in two
161 phases. First, an initial screening chip was prepared which surveyed five samples subset from the
162 total sample set for a wide variety of AMR gene targets to establish which resistance genes were

present in the exposed as well as unexposed populations. The screening chip analysed five samples for 248 possible gene targets using primers previously validated by Muziasari *et al.*, (2016, 2017). The five samples screened were:

- Two samples from T0 to establish which genes were present in the seed population.
- A single sample from time point T1 which, had been exposed to glucose but no amoxicillin, was chosen to represent conditions at the beginning of the experiment.
- Two samples were chosen from T7 which were exposed to glucose and 50 mg/L of amoxicillin. These samples were chosen as they contain the highest amoxicillin concentrations as well as longest exposure time within this study. It was hypothesised that these samples would have the greatest relative increase in amoxicillin resistance within the study.

The screening chip contained gene targets from across 6 classes: 16s rRNA (2 targets), beta-lactamases (106 targets), integrons (8 targets), multidrug resistance genes (MDR) (73 targets), mobile genetic elements (MGE) 53 targets, and tetracycline (6 targets). A detailed list of the gene targets including their forward and reverse primers can be found in Appendix A and B. Beta-lactamases and MDR class targets were both chosen to track changes in genetic resistance to beta-lactam antibiotics in response to an acute exposure to amoxicillin. Integrons and MGEs concentrations have been shown to correlate to an increase the count of resistance genes elsewhere and were chosen to reflect this relationship (Subirats *et al.*, 2019). As no tetracycline was added to any of the cultures, genes associated with tetracycline resistant genes would not be expected to increase during this experiment. As such genes associated with tetracycline were included to measure genes without selection pressure.

The results produced by the screening survey were used to narrow the scope of gene targets. The relative abundance of genes detected were averaged for all samples within the screening chip to generate an average relative abundance (ARA) score for each gene. The 36 most abundant genes (i.e. those with targets with the highest ARA scores, **Table 7-4**) were then used as targets for quantification in the remaining 96 samples from the study.

*Table 7-4 - The final 36 genes chosen for the second screening*

| Beta Lactam | Tetracycline | Integrons | MGE | MDR | 16S rRNA |
|---|---|---|---|---|---|
| cfxA | tetQ | intl3 | IS6100 | mexF | 16S rRNA |
| fox5 | | intI1_2 | ISEcp1 | oprJ | 16S rRNA2 |
| blaSFO | | intI1_1 | tnpA_2 | mepA | |
| blaTMB | | | IS1247_1 | oprD | |
| blaAIM | | | tnpA_5 | tolC_1 | |
| blaSHV_1 | | | repA | acrA_5 | |
| penA | | | tnpA_3 | acrR_3 | |
| blaACT | | | trbC | arsA | |
| blaMIR | | | orf37-IS26 | | |
| blaOXA48_2 | | | IS1111 | | |
| | | | IS630 | | |
| | | | Tn5403 | | |

#### *7.2.4.4 Smart Chip qPCR Array*

The extracted genomic DNA was quantified using the Qubit dsDNA BR kit and sample concentration was normalised by dilution to produce 100ul aliquots of 10ng/ul final concentration using nuclease free water before shipping to Resistomap for processing. PCR conditions were as described in previous publications (Muziasari *et al.*, 2016, 2017). The reaction was conducted in the SmartChip™ by the SmartChip™ Multisample Nano dispenser (TakaraBio, CA, USA). Each 100nl reaction was comprised of SmartChip TB Green Gene Expression Master Mix (TakaraBio, CA, USA), nuclease free PCR- grade water, 300 nM of each primer and a DNA template at 2 ng/µL (Muziasari *et al.*, 2016, 2017). The cycling conditions were conducted as described in Wang *et al.*, (2014). Melting curve analysis was performed for each primer set for all the samples. Melting curve analysis was processed using the SmartChip™ qPCR software. Amplicons with unspecific melting curves and multiple peaks based on the slope of melting profiles were discarded from the analysis as they were considered to be false positive data. The threshold cycle (CT) of 27 was set as the detection limit (Muziasari *et al.*, 2016, 2017). In addition to the experimental triplicates, three technical replicates were analysed for each qPCR reaction. The genes were reported as total counts as well as relative gene abundance. Relative gene abundance was calculated in proportion to the 16s rRNA gene in which relative gene abundance = $2-\Delta CT$ where $\Delta CT = \Delta CT(\text{detected gene}) - \Delta CT(\text{16S rRNA})$

215 (Muziasari *et al.*, 2016, 2017). Data processing and data analysis were performed using python
216 program by Resistomap Oy (Helsinki, Finland).

### 217 7.2.5 Bioinformatics and Statistical Methods

218 All statistical analyses were performed using R statistical package and RStudio 4.1. All analyses
219 were performed under the supervision and guidance of Dr. Umer Ijaz.

#### 220 *7.2.5.1 Sequencing Analysis*

221 Abundance tables were generated by constructing amplicon sequencing variants (ASVs) using the
222 Qiime2 workflow with DADA2 denoising algorithm (Kozich et al., 2013)
223 (https://github.com/umerijaz/tutorials/blob/master/qiime2_tutorial.md). ASV's were taxonomically
224 classified using SILVA SSU database v138. Additionally, Qiime2 was used to generate a rooted
225 phylogenetic tree of the observed ASVs and a final BIOM file was generated which combined
226 abundance information with taxonomy. This produced a 100 (samples) X 1,518 (ASVs) abundance
227 table. Furthermore, as a prefiltering step, we removed typical contaminants such as those matching
228 Chloroplast and Mitochondria and those that are unassigned
229 (https://docs.qiime2.org/2022.2/tutorials/filtering/) and dropping samples with less than 5000
230 cumulative read counts, resulted in a 97 (samples) x 1,491 (ASVs) abundance table on which we
231 performed statistical analyses. The summary statistics of reads per samples is as follows: [Minimum:
232 11,568; 1st Quartile: 59,012; Median: 74,391; Mean: 84,010; 3rd Quartile: 92,976; Maximum:
233 790,867].All further statistical analysis was carried out in R Studio (v4.0.0) using this data along
234 with metadata associated with the study.

#### 235 *7.2.5.2 Methanogenic Activity and sCOD Removal*

236 Differences in activity (methanogenic activity rate, lag time, and total biogas production volume)
237 were assessed using a Student's T-Test which compared each amoxicillin treatment condition with
238 the control. Soluble COD removal for each time point was assessed using a linear model which
239 compared the sCOD value with the time of incubation associated with that sub sample.

240 *7.2.5.3 Microbial community Analysis*

241 Microbial community analysis was conducted using the 'Vegan' package within R (Oksanen et al.
242 2013). The standard alpha diversity indices, such as Shannon entropy, Richness, Fishers Alpha, and
243 Pielous Evenness were calculated using the standard approach given in the Vegan package. Note that
244 these samples were rarefied to minimum sample size. This is particularly useful for the richness
245 estimate which gives the estimated species based on the minimum samples size. Differences in alpha
246 diversity measurements between time points (or test conditions) were assessed using an ANOVA
247 (Vegan's aov() script) to calculate pairwise ANOVA P-values.

248 Community composition variation between treatments and incubation time (Beta diversity) were
249 assessed using pairwise Permutational Multivariate Analysis of Variance (PERMANOVA).
250 Principal Coordinate Analysis (PCoA) plots were used to visualise variances in dissimilarity based
251 on Weighted UniFrac distance matrices using different distance measures (Vegan's capscale()
252 function). The data set was grouped in terms of time points as well as amoxicillin conditions and
253 ellipses were drawn using Vegan's ordiellipse() function that represent the 95% confidence interval
254 of the standard errors.

255 The core microbiome of the total community was calculated utilising all samples using R's
256 microbiome package as described in (McKenna *et al.*, 2020) and defined as taxa which are prevalent
257 in 85% of samples. Analysis was performed for relative abundance at genus level.

258 *7.2.5.4 Predicted taxa-function robustness*

259 The taxa-function robustness proposed by Eng and Borenstein (2018) is a measure of the microbial
260 communities structure and metabolic pathways. This was used to estimate microbial community
261 resilience and stability with the aim of quantifying an inherent component of the structure-function
262 relationship between taxonomic and functional profiles using community composition and of the
263 distribution of genes across genomes (Eng and Borenstein, 2018). For each sample, a perturbation
264 model (100 perturbations / sample) was applied to produce artificial microbial communities within
265 the vicinity of original microbial communities observed in each sample. The taxonomic shift of these
266 artificial perturbations were then compared to original community using phylogeny-aware weighted

267 UniFrac dissimilarity metric. For each perturbation, we also compared its functional shift from the
268 original community using cosine dissimilarity of the predicted functional profiles. To obtain
269 predicted functional profiles, the ASVs were taxonomically assigned using Greengenes database
270 (gg_13_5) for which functional KEGG orthologs and their corresponding copy numbers were
271 available. Next, the relationship between taxonomic perturbation magnitude and functional profile
272 shift for each sample (after 100 perturbations) were fitted using the linear regression model on natural
273 log-transformed data: $\ln(f) = -a + b \ln(t)$, where f is the functional shift obtained from cosine
274 dissimilarity, and t is the taxonomic shift obtained from the weighted UniFrac dissimilarity metric.
275 The two unknown coefficients: attenuation $a$**,** and buffering *b* are the community level robustness
276 proxies which then give the estimate of functional robustness. Two measurements are used:
277 attenuation and buffering. Attenuation is described as the expected rate at which increases in the
278 taxonomic perturbation magnitude are expected to increase functional shifts. Buffering describes
279 how large a perturbation must be before a functional profile shift becomes apparent. The attenuation
280 parameter in particular is important which is based on the slope of the response curve, with a larger
281 value suggesting that taxonomic perturbations have little impact on functional shifts. It should be
282 noted that there are practical and theoretical limitations to functional prediction methodologies.
283 Primarily, functional assignments are for *potential* function not the *actual* function as not all these
284 relationships have been validated. As our ability to sequence ever more genomes outpaces our ability
285 to verify functions practically within the laboratory, the direct relationship between sequence and
286 function has only been partially assessed and our confidence in functional predictions relied on the
287 robustness and validity of our databases (Devos and Valencia, 2000). Nevertheless, for this study we
288 are perturbing the sampling space to see how much relative shift occurs in terms of function. Because
289 we are focusing on the overall shift at community level in which the comparison is in relative terms,
290 even if a few taxa are not well resolved, the results should not be meaningfully affected. Similar
291 applications of taxa function robustness and have been recently successfully published (Nikolova et
292 al., 2021). The method uses Greengenes database (n=203,452) to resolve function instead of the
293 recent version of SILVA MOD (v 138 has n=436,680). This decision by the Eng and Borenstein
294 (2018) to use Greengenes could be the result of using 16S rRNA copy numbers (a requirement for

the algorithm for normalisation purposes), which are either easy to calculate for Greengenes, or are readily available.

#### 7.2.5.5 Taxa differential analysis

8 To find ASVs that are significantly different between multiple conditions, we used DESeqDataSetFromMatrix() function from DESeq2 (Love, Huber and Anders, 2014) package with the adjusted p-value significance cut-off of 0.05 and log2 fold change cut-off of 2. This function uses negative binomial general linear model (GLM) to obtain maximum likelihood estimates for ASVs log fold change between two conditions. Then Bayesian shrinkage is applied to obtain shrunken log fold changes subsequently employing the Wald test for obtaining significances. Although relative abundance between sample groups is widely used when investigating microbiomes, there is little consensus on best practices for differential analysis methods (Calgaro *et al.*, 2020; Nearing *et al.*, 2022). Every method has analytical biases and underlying assumption. For example, rarefaction can introduce false positives, it is commonly used because it simplifies analyses with various in read depth. McMurdie and Holmes, (2014) argue that both proportions and rarefied counts results in a high rate of false positives. Although functions such as metagenomeSeq can performs well then there are a high number of replicates, they can still tend towards high false positive rates. Therefore, the paper advocates that investigators avoid rarefying altogether. Nevertheless, analysis conducted by Calgaro *et al.*, (2020) which compared differences between commonly available differential analysis techniques has suggested that limma voom, corncob, and DESeq2 (used in this study) had the best overall performance.

#### 8.1.1.1 Environmental Fitting

To see if changes in covariates (AMR qPCR genes relative abundance) has an impact on microbial community structure, we fitted smooth surfaces of the covariates on an ordination plots (PCoA in this case) using penalised splines by employing ordisurf() function from R's Vegan package (Oksanen, 2018). The method uses a generalised additive model by regressing the covariate as C ~ S(Dim1,Dim2), where C is the covariate and Dim1 and Dim2 are the ordination scores extracted

from PCoA and S() is a spline function. We have only shown those covariates where the model fits i.e., $p < 0.05$.

#### *8.1.1.2 Antimicrobial Resistance*

To determine if the relative abundance of AMR genes in each sample correlated to incubation time, a general linear model was run correlating the relative abundance of each resistance or associated genes with incubation time. This was run separately for each amoxicillin conditions. To determine if there was a linear relationship between the resistance genes and the mobile genetic elements a GLM was run correlating the relative abundance of the resistance genes with the mobile elements and time. Samples were reported to a p-value threshold of $<0.05$.

## 8.2 RESULTS

### 8.2.1 Methanogenic Activity and sCOD Removal

Methane production proceeded to approximate steady state for all conditions within the 48-hour window of the study (**Figure 7-4**). Methane was generated at an average rate of 71.4 (SD 24.5), 56.3 (SD 15.2), 89.7 (SD 14.7), and 83.6 (SD 14.5) for the control, 1 mg/l, 10 mg/L and 50 mg/L amoxicillin respectively (**Table 7-4**). Based on the results from Chapter 3, it was assumed that the presence of amoxicillin would result in measurable changes to the methanogenic activity. However, no clear relationship between the addition of amoxicillin and changes in the rate of methanogenic activity were observed for the different amoxicillin concentrations provided in this study. T-tests comparing the rate of methane production in each test condition against the control were not found to be significant ($P > 0.05$) (**Figure 7-5**) for any of the amoxicillin concentrations used. However, differences in the time of maximum activity was found to be statistically different from the control value for each of the amoxicillin test conditions though there was no relationship between the increase in amoxicillin and the time of maximum methanogenic activity **(Figure 7-5)**. These results differ from similar analysis as reported in Chapter 6 in which the presence of an increase in amoxicillin resulted in a decrease in methanogenic activity of the acidogenic microbial subpopulation as well as an increase in the time between the addition of substrate and the generation of methane. Differences in the results between these studies could be the result of community drift due to differences in culture acclimatization. While each study utilised granules from the same source, the granules within this study were fed and incubated with a nutrient rich broth for two weeks before the beginning of the study.  Additionally, the sludge granules had been stored with intermittent feeding between the two studies which could also have contributed to community drift.

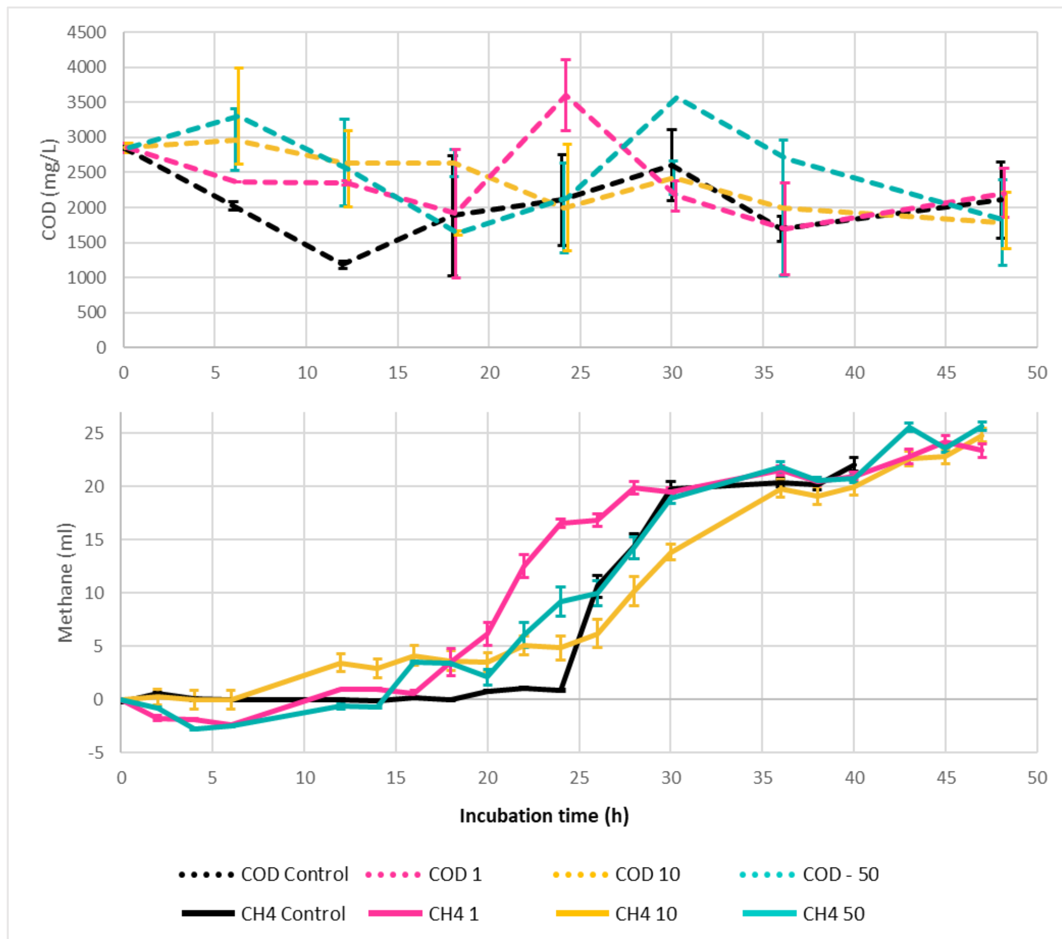



***Figure 7-3 -*** *COD and methane production throughout the study.*
*The cumulative methane reported has been blank adjusted. All points report the mean value for the six replicates. The error bars report one standard deviation from the mean.*

353

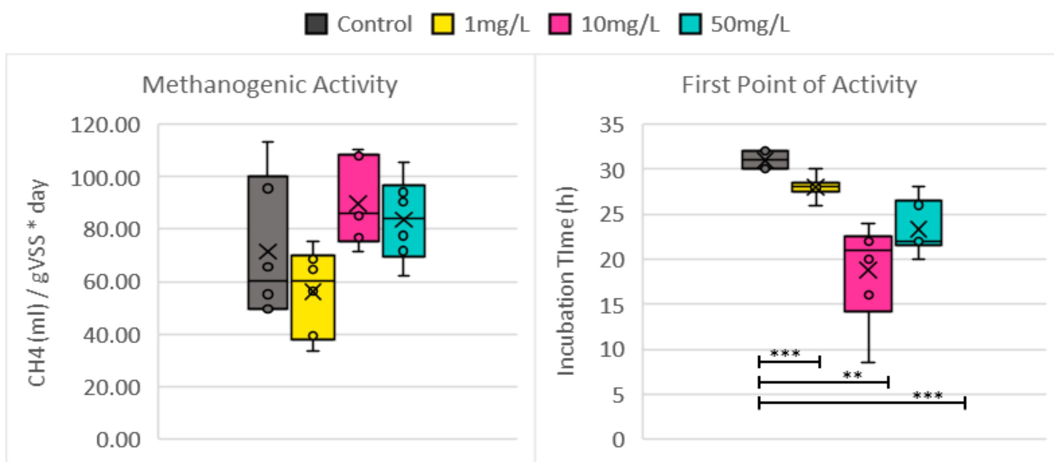



***Figure 7-2 -*** *Boxplots comparing the methanogenic activity and the first point in the activity.*
*No statistical differences were found in the methanogenic activity. However, statistical differences were found in the first point of activity. (*** $p \leq 0.001$, ** $0.001 < p \leq 0.01$, * $0.01 < p \leq 0.05$.) The x represents the mean value for the three replicates. The error bars report one standard deviation from the mean.*

354 355 356 *Table 7-5 - The mean and standard deviation for the rate of methanogenesis and the timing of the first activity of methane production.*
*P values are for t-tests comparing each of amoxicillin test conditions to the control.*

| | | Control | 1 mg/L | 10 mg/L | 50 mg/L |
|---|---|---|---|---|---|
| | *n* | 6 | 6 | 6 | 6 |
| Rate (CH4 ml /gVSS*Day) | *Mean* | 71.39 | 56.26 | 89.66 | 83.58 |
| | *SD* | 24.50 | 15.17 | 14.70 | 14.53 |
| | *p* | | 0.273 | 0.190 | 0.366 |
| First Activity (hours) | *Mean* | 31.00 | 28.00 | 18.76 | 23.33 |
| | *SD* | 1.00 | 1.15 | 5.18 | 2.75 |
| | *p* | | 0.001 | 0.003 | 0.001 |

357

358 The sCOD of the effluent was quantified for each of the sacrificial vials during the study to generate
359 a time series. A t-test comparing the final sCOD values recorded for each test condition found that
360 each of the amoxicillin exposed cultures were not statistically different from the control (p-value >
361 0.05) (**Table 7-5**). Although there was an overall drop in sCOD in the effluent from T0 to T6, the
362 sCOD values throughout the study fluctuated over time (**Figure 7-4**). Broadly, effluent sCOD
363 occurred in three phases: initial drop in sCOD, followed by an increase, after which the effluent
364 sCOD began to fall again. The timing, rapidity, and scale of these fluctuations were different for each
365 of the feed conditions. Fluctuation in sCOD values during the observational period could be the result
366 of cell death due to the presence of amoxicillin and non-optimal substrate usage in the presence of
367 amoxicillin. As the amoxicillin was used up and the community recovered, the available substrate
368 was utilised and the overall sCOD levels converged. The initial drop in sCOD was slower for each
369 of the test conditions and even increased initially for the 50 mg/L conditions, suggesting that the
370 presence of amoxicillin inhibited glucose uptake. The sCOD of the control conditions increased
371 slowly after 18 hours and began to fall again after 30 hours. Conversely the sCOD in 10 and 50 mg/L
372 conditions both rose and fell much faster than the second and third phases than the control conditions.

373 374 *Table 7-6 - Table comparing the mean and standard deviation of the total sCOD at T7 (48 hour).*
*The p-value reports the difference from the test condition from the control.*
375

| | ***G - 0*** *mg/L* | ***G - 1*** *mg/L* | ***G – 10*** *mg/L* | ***G - 50*** *mg/L* |
|---|---|---|---|---|
| *Mean* | 2107.67 | 1786.00 | 2205.00 | 1814.00 |
| *SD* | 547.45 | 610.57 | 348.53 | 405.25 |
| *p-value* | | 0.61 | 0.84 | 0.58 |

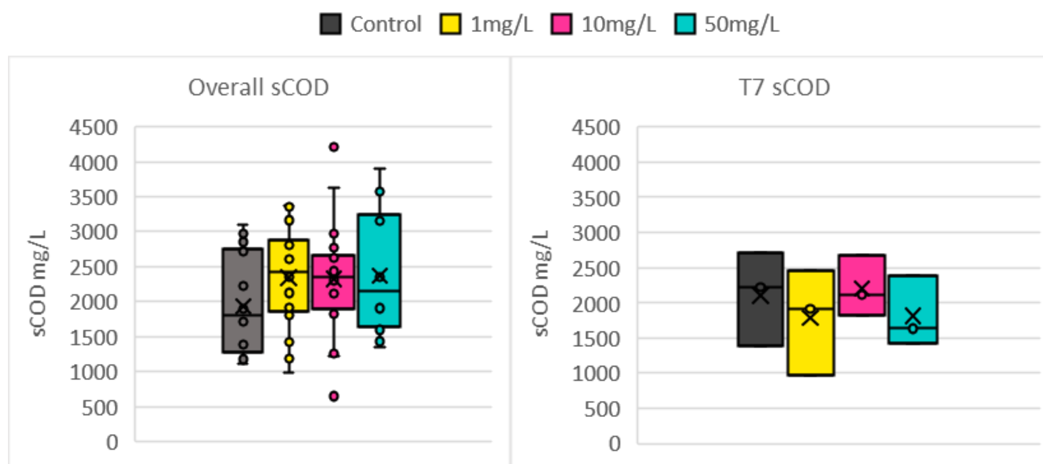



***Figure 7-4*** *- Differences in the sCOD.*
*The overall sCOD contains all the values within the study while the T7 sCOD refers only to the values at the end of the study.*

376

377 ### 8.2.2 Microbial Community Composition

378 The microbial community make up was revealed using next generation sequencing of the 16s rRNA
379 genes targeting the V4 region. Three primary questions were asked of the data set: 1) Does the
380 presence of amoxicillin significantly change the community structure compared to the seed
381 community? 2) Who is present in the community? 3) Which microbes are changing in response to
382 the presence of amoxicillin?

383 ### 8.2.3 Community Diversity

384 The alpha diversity indices: Pielous evenness, richness, and Shannon diversity, were calculated for
385 each of the amoxicillin test conditions. This calculation included all time points for each amoxicillin
386 condition. Although it was hypothesised that the presence of amoxicillin would reduce the diversity
387 when compared to the control and seed community, this was not found to be the case. Conversely,
388 statistical differences were detected amongst the richness and evenness metrics. In both cases, the
389 greatest statistical difference from the seed community was the control condition for which a drop in
390 richness and diversity and an increase in evenness was observed (**Figure 7-7**). While none of the test
391 conditions are statistically different from the initial community at seed, each of the test microbial

392 communities are statistically different from the control condition (glucose fed bottles without
393 amoxicillin).

394 The microbial composition between groups was compared by plotting samples on Principle
395 coordinate analysis (PCoA) using weighted UniFrac distance measure. When grouped by amoxicillin
396 and feed conditions, the control (glucose only, no amoxicillin) samples clustered distinctly from the
397 seed community. By contrast, both the amoxicillin fed conditions and the unfed blank conditions
398 clustered close to the seed community with the amoxicillin conditions all clustering together. There

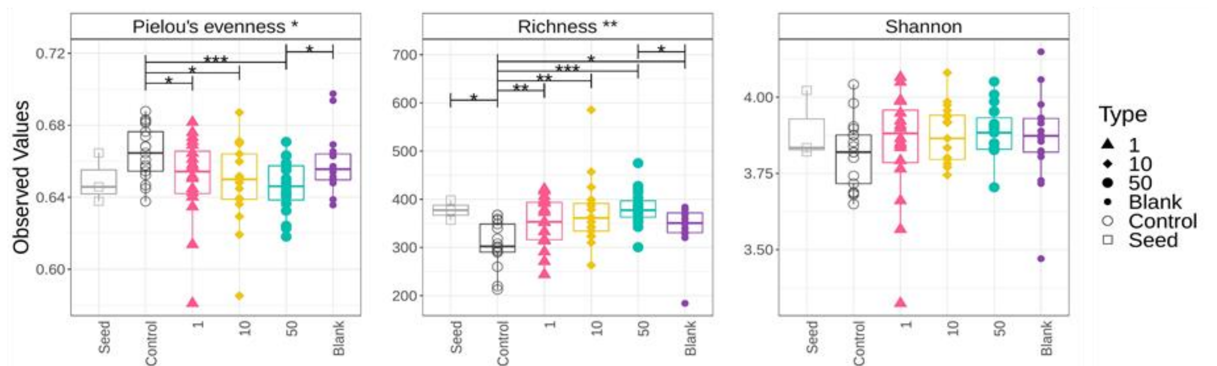
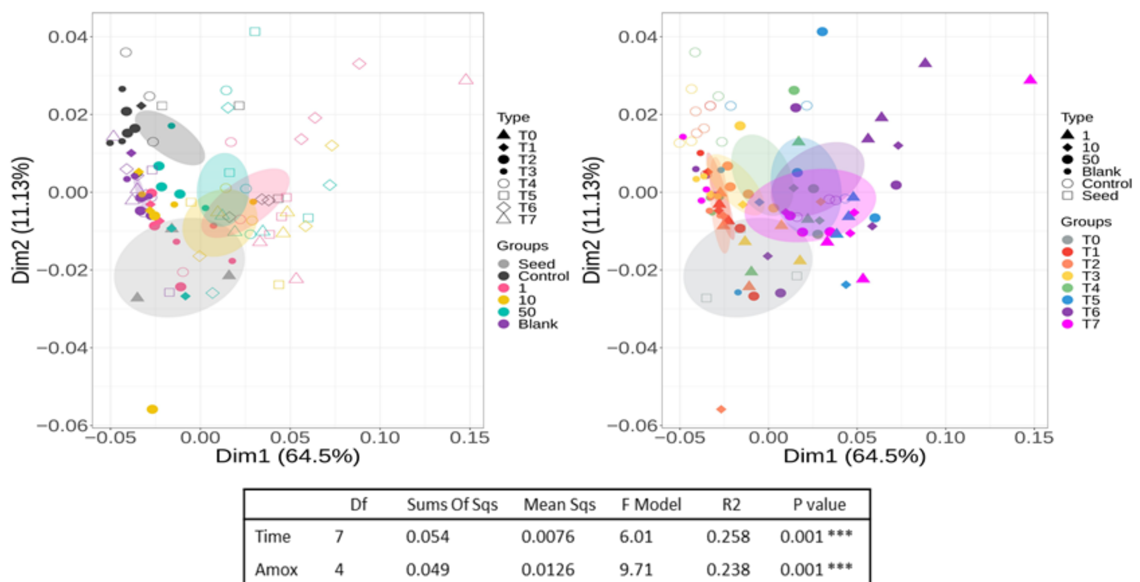



***Figure 7-6*** *- Alpha diversity figures.*
*The line in the box plots represents the mean of the values. The boxes are for the first and third quartile (Q1 and Q3) of the data. The lines at the top represent statistically significant differences between each run. (*** p≤0.001, ** 0.001 < p ≤ 0.01, * 0.01< p ≤ 0.05.). Amoxicillin concentrations are in mg/L.*




| | Df | Sums Of Sqs | Mean Sqs | F Model | R2 | P value |
|---|---|---|---|---|---|---|
| Time | 7 | 0.054 | 0.0076 | 6.01 | 0.258 | 0.001 *** |
| Amox | 4 | 0.049 | 0.0126 | 9.71 | 0.238 | 0.001 *** |

***Figure 7-6*** *- Beta diversity figures.*
*The principal coordinate analyses (PCoA) were generated using weighted UniFrac similarities. The ellipses represent 95% confidence interval of the standard errors.*

399 did not appear to be any gradation of effect with concentration of amoxicillin exposure (**Figure 7-**
400 **8).** Both this result and the results of the alpha diversity indices were unexpected. It was assumed
401 that the presence of amoxicillin would drive changes in microbial community composition. That the
402 opposite trend was observed suggests that the presence of glucose is the primary driver of changes
403 in community composition, whilst the presence of amoxicillin inhibits adaptation. When the samples
404 on the PCoA are grouped by time, a clear progression is observed showing that adaptation through
405 time progresses stepwise from the seed community. PERMANOVA analysis found that both
406 amoxicillin condition and time combined explained nearly 50% of the variation in the data set and
407 were both statistically significant (p <0.001) (**Figure 7-8**). It should be noted that the variation on
408 beta diversity **Figure 7-8**, Dim 1 on the x-axis and Dim 2 on the y-axis represents the variability as
409 explained within the first two dimensions. The PCOA does not reduce the dimensions of multivariate
410 data sets as is done in other methods such as non-metric distance scaling (NMDS). However, this
411 procedure transforms the data in such a way that the majority of the variability lies in the first few
412 dimensions. This simplified visualisation in the 2D/3D space by ignoring other dimensions.
413 Therefore, in the 2D plot, the percentage values should add up to the variability explained by these
414 two dimensions. The remining variability (50.4%) are explained by the dimensions not visualized.

### 415 8.2.4 Community make-up

416 The microbial community composition was largely stable and did not vary greatly between test
417 conditions such that the top 25 families consistently comprised nearly 80-90% of taxa present **(Table**
418 **7-6**). Core microbiome analysis (**Figure 7-10**), which reports the families present in at least 85% of
419 all samples found that all samples contained the same three most abundant families: *Spirochaetaceae,*
420 *Aminicenantales*, and *Bacteroidetes vadinHA17* which were each found at roughly 18-20% relative
421 abundance. Other dominant families, which appeared in all samples, though to varying degrees of
422 dominance, were: *Dysgonomonadaceae, Syntrophobacteraceae, Methanobacteriaceae,*
423 *Synergistaceae, Clostridiaceae, Caldatribacteriaceae*, and *Kosmotogaceae*. The putative function of
424 these families is described in **Table** . The dominant archaeal species observed for all samples where
425 the Bathyarchaeia, Methanobacterium, Methanofastidiosales, Methanosaeta, and
426 Methanomethylovorans.

427 The alpha and diversity metrics (**Section 8.2.3**) established that while the change in the structure of
428 the community was statistically significant, this difference was subtle. To determine which species
429 were most closely associated with the changes in the community, a taxa differential analysis was run
430 in which statistically significant changes in species counts were calculated from pairwise
431 comparisons of each test condition and the seed community **(Figure 7-11)**. Species which had at
432 least a 2- log fold change between conditions and adjusted p value $< 0.05$ were deemed significant.
433 Two species were found to increase in relative abundance in the seed community compared to the
434 seed community. *Clostridium sensu stricto 1*, which is responsible for the generation of $H_2$ and $CO_2$
435 from formate, increased in relative abundance for all samples, and *Petrimonas*, a hydrolytic member
436 of the *Dysgonomonadaceae* family capable of degrading recalcitrant polysaccharides to liberate
437 oligosaccharides or monosaccharides (Khamis et al., 2020), increased in relative abundance in the 1
438 mg/L and 50 mg/L conditions. Both species have been positively correlated to the expression of the
439 ARG sul2 and has been suggested as potential host bacteria for ARGs (Li *et al.*, 2021). However,
440 *Clostridium sensu stricto 1*, was found to be downregulated – in which the number of total counts
441 detected decreases - in the blank samples, again suggesting that the change was driven more by the
442 presence of glucose than the presence of the amoxicillin. Increased relative abundance was
443 exclusively observed in taxa which were already abundant whilst decreasing relative abundance (less
444 abundant in the test condition than in the seed community), was observed in comparatively rarer taxa
445 (dark blue bars in Figure 7-11). In general, there were more taxa downregulated in the control and
446 the 1 mg/L conditions than in the 10 mg/L and 50 mg/L conditions, however there was little overlap
447 within these changes.

448 *Table 7-6 – The most prominent families in the top 25 taxa within the community.*

| Family | Description |
|---|---|
| *Spirochaetaceae* | A facultative saccharolytic rod shaped bacteria (Karami *et al.*, 2014) |
| *Aminicenantales* | A hydrolytic bacteria which is able to degrade carbohydrate and protein matrixes and can provide sulphate-reducing and sulphurising bacteria with biochemical metabolism substrates. (Tao *et al.*, 2021; Fan *et al.*, 2022) |
| *Bacteroidetes_vadinHA17* | A hydrolytic and fermentative bacteria producing $H_2$, $CO_2$, fatty acids and alcohols (Wang *et al.*, 2019) |
| *Dysgonomonadaceae* | A strict facultative anaerobic and fermentative bacteria capable of fermenting saccharides into organic acids. (Shamurad *et al.*, 2020) |
| *Syntrophobacteraceae* | *Syntrophobacteraceae* is a member of the Class Syntrophobacterales. They are sulfate reducing strict anaerobes. (Waite *et al.*, 2020) |
| *Methanobacteriaceae* | *Methanobacteriaceae are a rod to fillementous archea* are able to grow on $H_2$+$CO_2$ and formate 2-propanol/ $CO_2$. (Garcia, Patel and Ollivier, 2000) |
| *Synergistaceae* | *Synergistaceae are* Gram-negative, nonmotile rods who are strictly anaerobic. They are chemoorganotroph capable of fermenting amino acids into formate, acetate, propionate, $H_2$, and ammonia. They are not able to ferment carbohydrates. (Allison, MacGregor and Stahl, 2015) |
| *Clostridiaceae,* | Involved in acetate production / consumption. (Esquivel-Elizondo *et al.*, 2017) |
| *Caldatribacteriaceae*, | VFA-oxidizing family of anaerobic bacteria known to metabolize fatty acids as well as oxidize propionate and butyrate (Khoei *et al.*, 2021) |
| *Kosmotogaceae* | Mesophilic bacteria capable of $H_2$ oxidation and thiosulfate reduction using a sulfide dehydrogenase. (Nesbø *et al.*, 2019) |

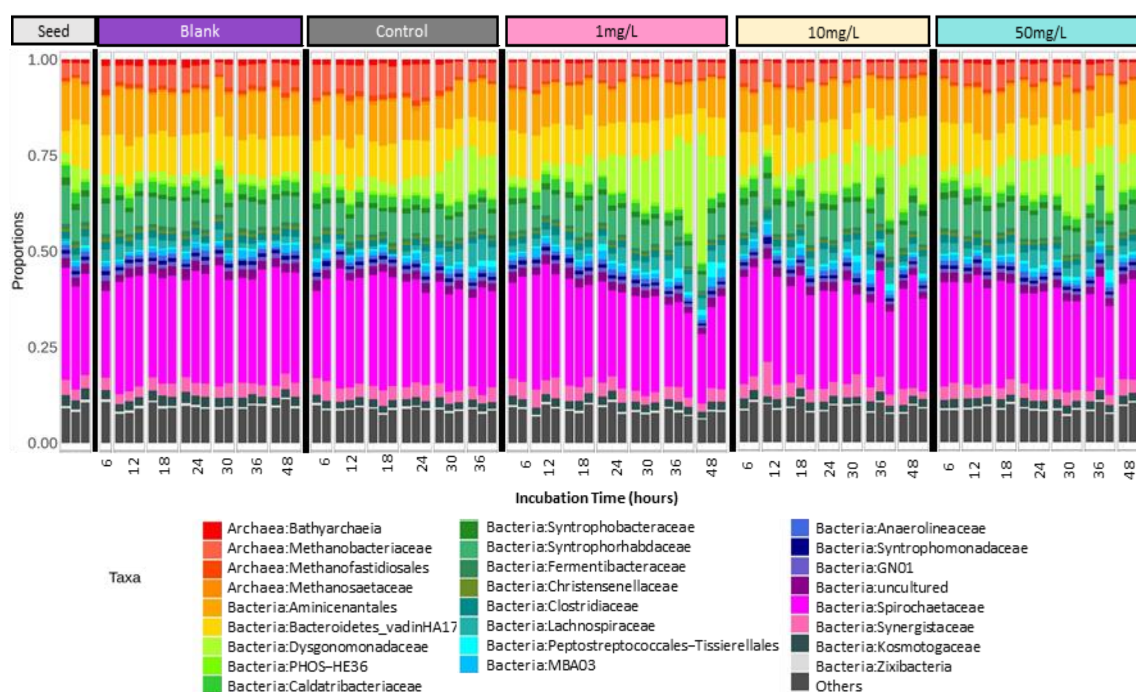



***Figure 7-7*** *- The top 25 taxa found throughout the entire sample set.*
*Samples are grouped by substrate and amoxicillin type. The x-axis represents the hours of incubation. Each of the replicates are grouped together for each time point.*

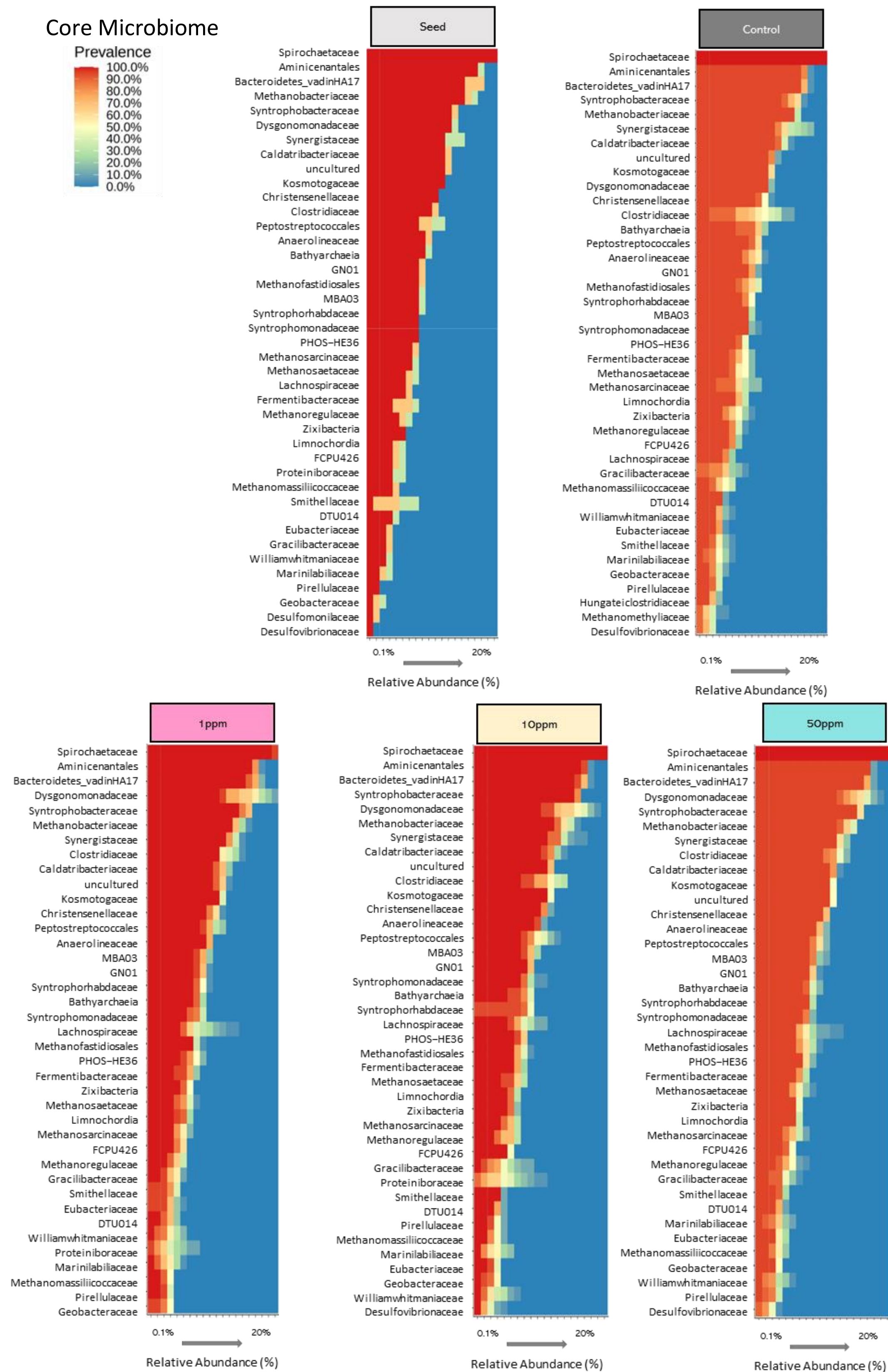



*Figure 7-8 – The core microbiome.*

*The core microbiome as represented by the genera found in at least 85% of samples for each of the test conditions. The x-axis represents the detection limit at varying relative abundances. Each sample contains the same most abundant genera.*

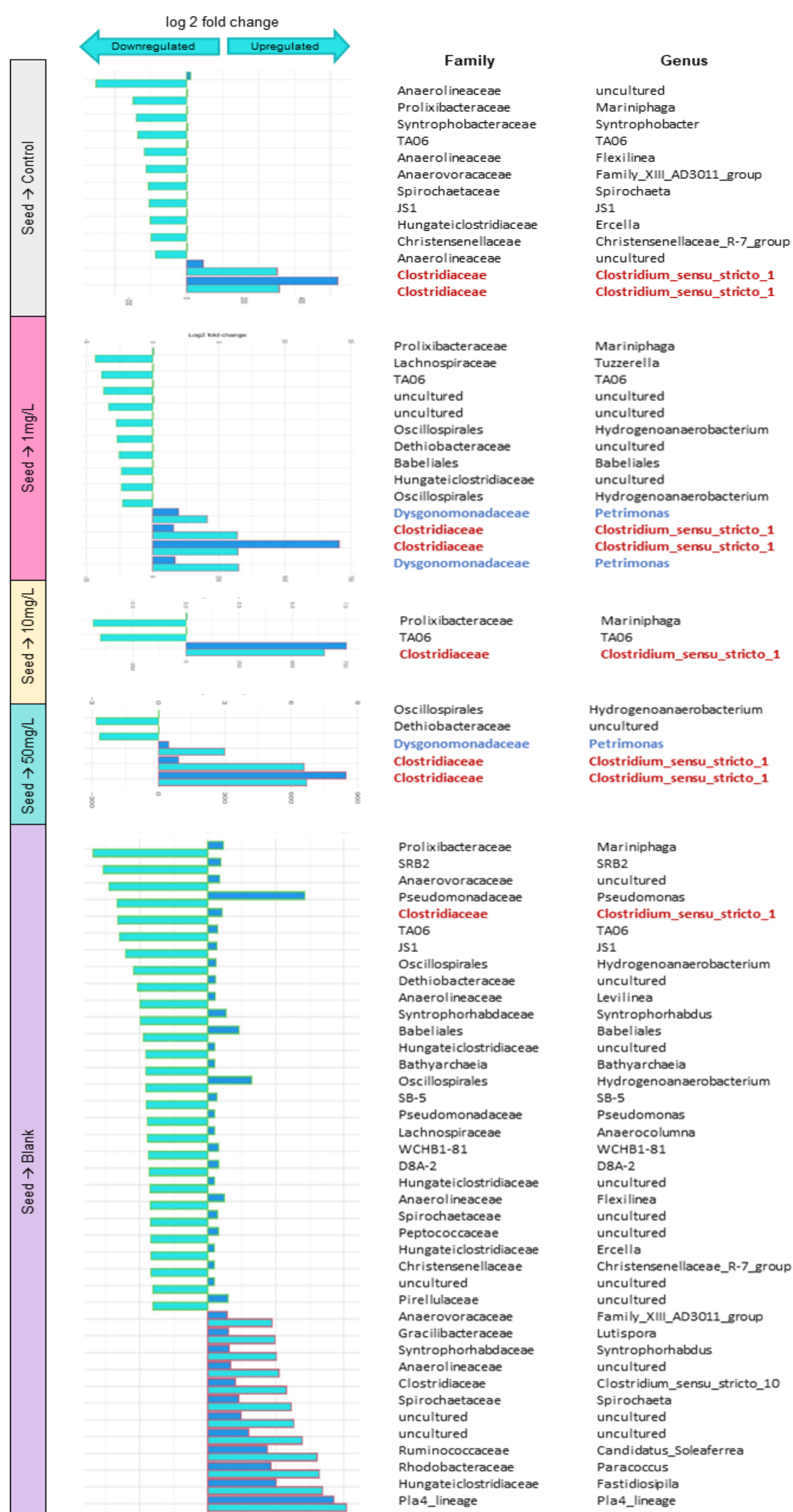



***Figure 7-9** – Taxa differential.*
*Taxa differential which identifies the taxa with the greatest differences from the mean. The plot shows both logfold changes (green bars) as well as mean abundances (blue bars).*

451 ### 8.2.5 Taxa-Functional Robustness

452 Taxa-function robustness estimates community resilience and stability through measuring
453 distribution of genes associated with the structure-function relationship between taxonomic and
454 functional profiles which are reported via attenuation values with an increase in attenuation
455 representing an increase in community robustness (Eng and Borenstein, 2018). As anaerobic
456 community structures are highly syntrophic, changes in community structure can potentially disrupt
457 functionality and overall taxa-function robustness particularly when those changes target highly
458 related species. To provide a quantitative comparison of the difference in taxa-function robustness
459 between the microbial communities exposed to amoxicillin over time, the attenuation values for each
460 treatment were assessed over time as defined by the taxa-function response curves. Attenuation
461 values report how much change a community can withstand before the overall robustness of the
462 community is compromised in which low values represent low robustness and high values represent
463 high robustness. Within this study, the robustness of the control community was found to remain
464 broadly similar throughout the study. Conversely, in the test conditions as well as the unfed blank
465 conditions a drop in overall robustness was observed between the beginning and end of the study.
466 However, the 1 mg/L condition resulted in an initial drop in robustness followed by a stabilising
467 effect after T4 (24 hours). These results indicate that although methanogenic activity in the
468 community was not observed to change, the presence of amoxicillin in all conditions resulted in a
469 less robust community which may be more susceptible to other forms of disturbance such as
470 temperature fluctuation or changes in loading rate.

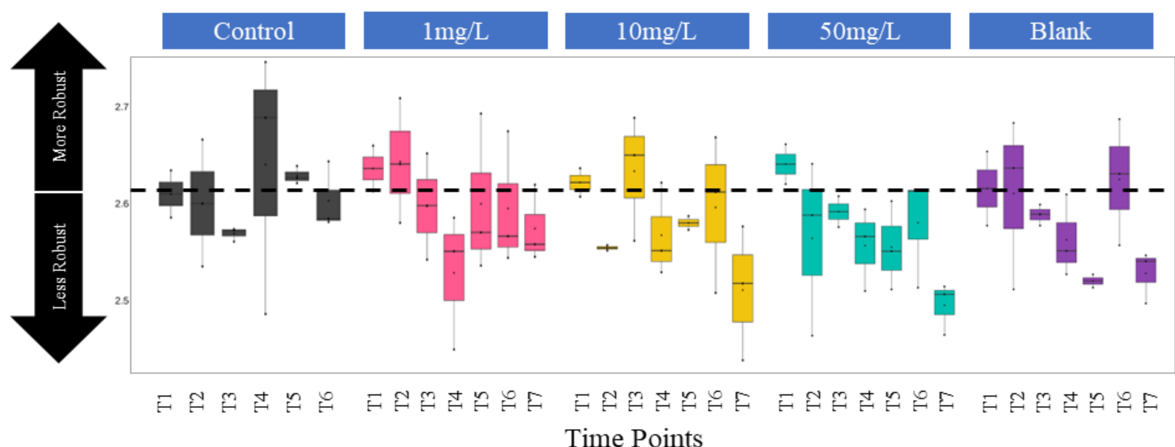



*Figure 7-10 – Robustness (attenuation parameters) through time for each of the samples. Greater values indicated more robustness while lower values indicate less robustness.*

471 ## 8.3 ANTIMICROBIAL RESISTANCE

472 As the fundamental activities of antibiotics are naturally occurring, so too are the genes associated
473 with resistance mechanisms. As such, microbial communities often harbour a natural pool of
474 antibiotic resistance genes within the community, though they may not be activated. This study aimed
475 to establish which genes were present in the genome of the seed community and to detect the relative
476 increase in abundance of any antimicrobial resistance genes within the anaerobic granules when
477 exposed to a shock load of amoxicillin. Detection was conducted in two phases of qPCR array (as
478 described in Methods Section 0). In the initial screening phase a suite of 248 genes were tested using
479 a subset of five samples. This initial set contained genes that encode for resistance directly (beta-
480 lactamases, tetracycline resistance, and multi-drug resistant genes) as well as genes that facilitate the
481 spread of resistance (mobile genetic elements and integrons). The remaining samples were quantified
482 for the 34 most abundant genes identified in the first screening and two 16s rRNA primers.

483 In the second screening run, 11 genes were found in most samples (**Table 7-7**): four antimicrobial
484 resistance genes (*cfxA, tetQ, mexF,* and *oprJ*), and seven genes associated with mobile genetic
485 elements and integrons (*intl3, intI1_2, IS6100, IS1247_1, ISEcp1, tnpA_2,* and *tnpA_5*). Of these,
486 nine were found in all samples. *Cfxa* is a class A cephalosporinase which inactivates beta-lactams
487 through a deactivation of the beta lactam ring. The detection and proliferation of *cfxA* in anaerobic
488 bacteria has been associated with the presence of amoxicillin (Iwahara et al., 2006). The tetQ
489 resistance gene confers resistance against tetracycline antibiotics through modification of the
490 ribosome. The mexF and oprJ are associated with the tripartite efflux pump systems MexCD-OprJ
491 and MexEF-OprN. These systems are found within the gram-negative bacteria Pseudomonas
492 aeruginosa (Terzi, Kulah and Ciftci, 2014). The mex pumps move compounds from the inner
493 membrane into the pump while the opr pump moves compounds from the outer membrane out of the
494 cell (Aeschlimann, 2003; Terzi, Kulah and Ciftci, 2014). While the *mexF* and *oprJ* genes are
495 associated with different systems, they are closely related, and each system has been found to confer
496 resistance for beta-lactam antibiotics (Aeschlimann, 2003).

497

498 *Table 7-7 - The antibiotic resistance genes detected in the qPCR array.*

| Gene | Type | Function |
|---|---|---|
| *cfxA* | Beta-lactamase | AMR Gene - Class A cephalosporinase which enzymatically deactivate the beta-lactam ring. (https://card.mcmaster.ca/home) |
| *tetQ* | Tetracycline Resistance | AMR Gene - Ribosomal protection protein. Its gene is associated with a conjugative transposon and has been found in both Gram-positive and Gram-negative bacteria. (https://card.mcmaster.ca/home) |
| *mexF* | MDR | AMR Gene - multidrug efflux system MexF is the multidrug inner membrane transporter of the MexEF-OprN complex (https://www.uniprot.org/) |
| *oprJ* | | AMR Gene - OprJ is the outer membrane channel component of the MexCD-OprJ multidrug efflux complex. (https://www.uniprot.org/) |
| *intl3* | Integron | Integron-integrase gene which catalyses the insertion of gene cassettes into and enables the capture and insertion of exogenous genes in to the genome (Gillings *et al.*, 2014) |
| *intI1_2* | | |
| *IS6100* | MGE | Insertion sequence (IS) – transposable elements found in bacterial species and plasmids that encode gene for mobilisation and insertion (Varani *et al.*, 2021) |
| *IS1247_1* | | |
| *ISEcp1* | | |
| *tnpA_2* | | Transposon - Mediates transposition of transposon tnp by a 'cut and paste' mechanism (Steiniger-White *et al.*, 2002). TnpA transposons are associated with IS21, IS6, IS6100, IS1216, and ISEcp1 insertion genes (Muziasari *et al.*, 2017) |
| *tnpA_5* | | |

499

500 Mobile genetic elements (MGEs) are DNA segments that encode enzymes to copy and transport
501 genetic material both intracellularly and extracellularly promoting the horizontal transfer of genetic
502 material including the spread of ARGs (Pazda *et al.*, 2019). Insertion sequences (IS) and transposons
503 (Tn) are small portions of DNA that transport themselves (and associated genes) to new locations.
504 Integrons (int) use site-specific recombination to catalyse the insertion of gene cassettes into DNA
505 and enables the capture and insertion of exogenous genes in to the genome (Partridge *et al.*, 2018).
506 This study detected the insertion sequences *IS6100, IS1247_1,* and *ISEcp1* as well as the transposons
507 *tnpA_2* and *tnpA_5*. The *tnpA* transposons have been found to be associated with *IS21, IS6, IS6100,*
508 *IS1216*, and *ISEcp1* insertion genes (Muziasari *et al.*, 2017). Furthermore, the *intl3* and *intI1_2* genes

associated with the Integron-integrase gene were detected in the samples. The presence of *intI1* genes has been suggested as a proxy for the presence of antimicrobial resistance (Gillings *et al.*, 2014).

As expected, AMR genes were detected in the seed community despite no known previous exposure to amoxicillin or any other known antibiotics. Within these samples, the beta-lactamase gene *cfxA* was found to be the most prevalent comprising 75.39% of all the detected gene counts and was observed to increase in relative abundance in amoxicillin exposed microbial communities. The second most abundant gene detected was the multi drug resistant *mexF* gene at 6.82%. There appeared to be a general increase in the total number of AMR genes detected relative to the 16S gene though there was a peak at T6 (30 hours) followed by a decline in relative abundance which was most notable for *cfxA* (**Figure 7-13**).

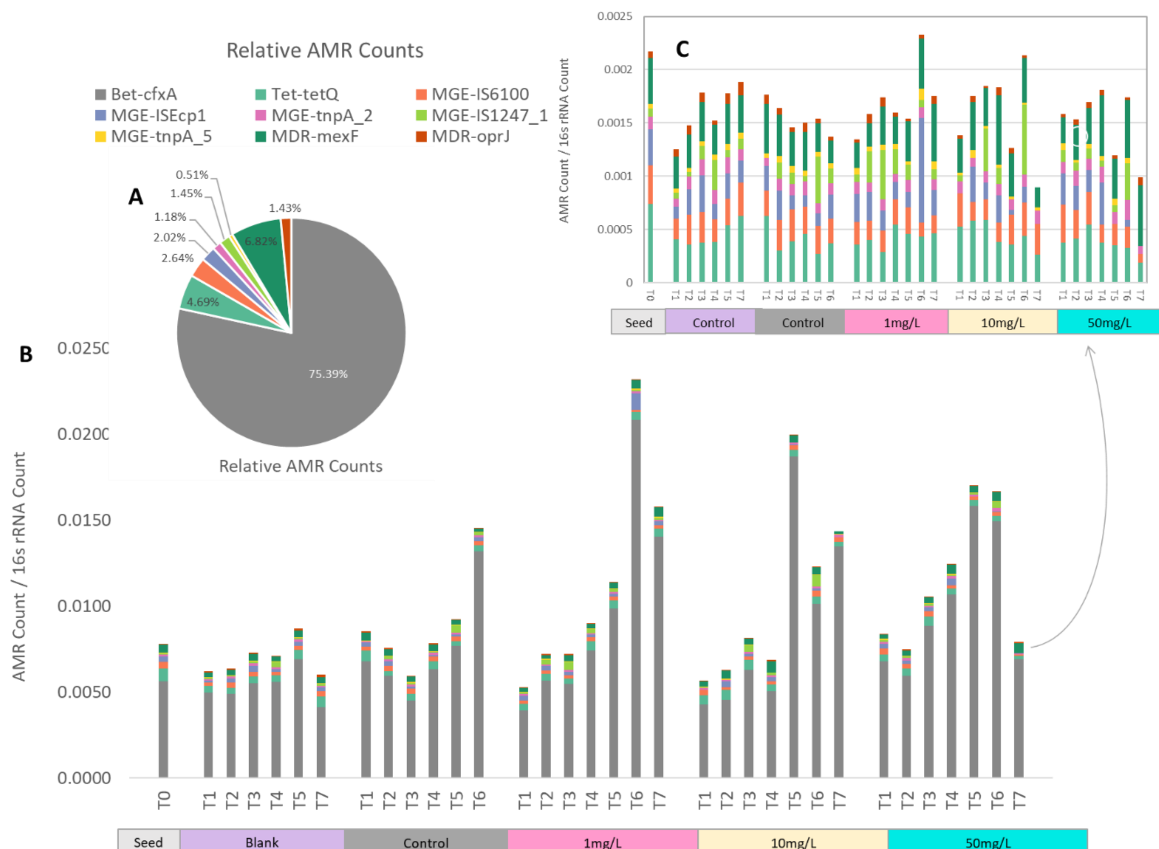



***Figure 7-11** – Abundance of Genes Relative the 16S Gene Over Time*
*(A) The pie chart represents the proportion of the total AMR gene counts detected in all samples as a percentage of all counts. Bet – beta lactam genes, MGE – mobile gene element, Tet – tetracycline resistance gene, MDR – multidrug resistance gene. Each stacked bar chart shows the AMR gene counts relative to total 16s gene counts through time for each test conditions. (B) Bar chart B includes all the genes detected. (C) Bar chart C represents contains all genes except the cfxA gene.*

519 ## 8.4 DISCUSSION

520 This study aims to assess whether a shock exposure to the antibiotic amoxicillin within the influent
521 of anaerobic systems, as might be found within decentralised wastewater treatment, could inhibit
522 methanogenesis and sCOD removal. As previous work presented in Chapter 3 demonstrated that 1
523 mg/L, 10 mg/L, and 50 mg/L of amoxicillin concentrations can decrease methanogenic activity, it
524 was assumed that an increase in amoxicillin exposure would result in a corresponding reduction in
525 sCOD removal throughout the study. The sCOD over time was observed to increase and decrease
526 from the beginning and end of the study. However, the sCOD of the final effluent values taken at 48
527 hours were statistically similar to eachother, indicating that although overall sCOD concentration
528 during the study was different between test conditions (**Figure 7-6**), the overall ability to remove
529 sCOD at 48 hours was not greatly impacted by the presence of the amoxicillin. It should be noted
530 that these results agree with the work conducted by Massé *et al.*, (2000) which reported that the
531 presence of penicillin did not have a significant effect of sCOD removal over several fill draw cycles
532 of an anaerobic SBR.

533 There are two possible drivers for the fluctuations in sCOD observed in this study. The initial drop
534 in sCOD in the control samples is likely caused by the utilisation of the glucose substrate in the
535 absence of amoxicillin. However, the presence of amoxicillin inhibited the full conversion of glucose
536 to methane in which intermediate products would remain in solution. This, along with damaged
537 cellular material, could have resulted in an increase in the sCOD observed. Amoxicillin inhibits
538 growth by irreversibly bonding with the peptidoglycan in the cellular membrane (Madigan, 2014).
539 This damages cell walls, inhibits the repair of the damage, and prevents the production of new cells
540 until all the free amoxicillin is used or destroyed and new PBP enzymes can be created. Once the
541 free amoxicillin is no longer active, the microbes would be available to utilise any free substrates,
542 which may explain the delayed drop in sCOD in the amoxicillin test conditions. The conversion of
543 glucose into methane requires a wide variety of microbes able to both utilise glucose as well as all
544 its by-products. As this damage inhibits cellular function, the glucose present would not be able to
545 be fully converted into methane as efficiently. Further study measuring variations in RNA or

546 exclusively measuring living cellular materials within the effluent can confirm this hypothesis.
547 Ultimately, the cause of fluctuating sCOD over the course of this experiment is unknown. This
548 warrants further study which monitors changes in sCOD over time during amoxicillin exposure
549 events. To determine if the increase in sCOD is due to a build-up of methane production
550 intermediates, a study which includes VFA quantification in the effluent can determine which trophic
551 classes are being inhibited.

552 Anaerobic microbial communities are known to grow at a slower pace than aerobic microbial
553 communities as the anaerobic metabolic pathways yield less energy for growth using alternative
554 electron acceptors to oxygen (Gerardi, 2003). As such, many studies of anaerobic microbial
555 communities are conducted over days and weeks with highly intermittent sampling conducted over
556 days. This misses the any immediate effects within the few hours of exposure. The hydraulic
557 retention time of decentralised WWT systems such as septic tanks can be on the order of days.
558 Therefore, the immediate aftereffects within the first few hours of amoxicillin can have knock on
559 impacts on treatment efficiency during that time. Thus, studying response over short time intervals
560 is important.

561 This study showed that changes in the community can be detected within hours after an amoxicillin
562 exposure event. A two-to-three-fold increase in the relative net presence of AMR resistance genes
563 within effluent can be detected when amoxicillin in the influent is as low as 1 mg/L (**Figure 7-13**).
564 Changes to the overall community structure resulting from the presence of amoxicillin was
565 observable from the first point at 6 hours from inoculation (**Figure 7-8**). However, as this study
566 focused on the use of 16s rRNA, which both captures the DNA within live as well as dead cells the
567 full extent of the variation may not have been captured. As such, the community shifts observed may
568 underestimate the real changes within the community, ie, only those living after an acute exposure
569 to amoxicillin. Future work which focuses on the RNA within the active communities could further
570 develop these results. In the absence of amoxicillin, the community shifted with the presence of
571 glucose substrate, which was not seen to the same extent when amoxicillin was present (**Figure 7-**
572 **7**). This suggests, that even if methanogenic activity occurs at the same rate with and without

573 amoxicillin, small concentrations (1mg/L and less) can have an effect on the community make up.
574 Finally, a decrease in community robustness was observed within 24 hours of exposure to amoxicillin
575 for all tested conditions indicating communities exposed to amoxicillin can be sensitive to further
576 stresses (**Figure 7-12**).

577 Changes in the resistome were also detectable within the first 48 hours. As predicted, AMR genes
578 were detected within the seed community which had not been exposed to any known antibiotics
579 within the three years prior to the study. A linear model found that beta-lactam resistance gene *cfxA*
580 was positively correlated to the diversity and time within study. The relative abundance of the efflux
581 pump encoding *mexF* and *orpJ* were also found to linearly correlate to time for the 50 mg/L exposure
582 condition. Although *mexF* and *orpJ* each encode for different efflux pumps they both belong to the
583 RND efflux pump family which are capable of conferring resistance to amoxicillin (Iman Islamieh
584 *et al.*, 2018). Despite net increases in the relative AMR gene counts for T5 (30h) and T6 (36h), the
585 total AMR counts appeared to decrease for T7 (48h). This suggests that while AMR genes might be
586 produced in response to the presence of amoxicillin, once the antibiotic is no longer active (through
587 decay or non-reversable PBP bonding) these genes are no longer selected for. Further research over
588 a longer time period or the inclusion of RNA sequencing for activated genes could explain the
589 changes in AMR genes observed in T7.

590 In conclusion, this research suggests that acute exposure to concentrations of amoxicillin used in this
591 study have a detrimental effect on an anaerobic activity and structure of microbial communities.
592 While there wasn't a change in the methanogenesis or sCOD removal within the first 48 hours, there
593 were fluctuations in sCOD removal within the first 12 hours appeared to be inhibited. As septic tanks
594 are continuous flow, even a temporary decrease in treatment efficiency can result in the discharge of
595 untreated waste into uncontrolled environments. Furthermore, even small concentrations of
596 amoxicillin (1 mg/L) within the influent have been shown to have a measurable effect on the
597 microbial community. Most notably this includes the presence of AMR genes in the community and
598 potentially the effluent, as well as a decrease in community robustness making the system more
599 susceptible to knock on effects on performance. If effluent quality decreased, such than an increase

600 in organics are released in the effluent, these could expose the surrounding environment to AMR 601 genes. It should be noted that there were several specific differences in the design between this study 602 and an in-situ septic tank. For example, the temperature in traditional septic tanks, composition of 603 the substrate in the influent, and the pattern of exposure. However, research involving heated septic 604 tanks (Koottatep *et al.*, 2018) are currently being conducted, for which the results of this study would 605 be relevant.

## 606 8.5 CONCLUSIONS

607 While many studies looking into the effects that antibiotics can have on anaerobic communities look 608 at longer time scales with data points on the scale of days (Cetecioglu, 2014; Aydin, Ince and Ince, 609 2015b), this study demonstrates that changes in the treatment efficiency, microbial community, and 610 relative abundance of AMR genes can be detected within the first 48 hours of exposure. Changes in 611 the patterns of sCOD removal was detected within the first 12 hours with an increasing effect with 612 increasing amoxicillin exposure conditions. There was a measurable effect on the microbial 613 community for all amoxicillin concentrations present (1mg/L – 50mg/l). Furthermore, a two-to-614 three-fold increase in the relative net presence of AMR resistance genes within effluent can be 615 detected in when amoxicillin in the influent is as low as 1 mg/L within the first 36h of incubation. 616 Consequently, decentralised waste treatment technologies relying on anaerobic microbial 617 communities for treatment, such as septic tanks, should be considered potential point source 618 pollutants for the spread of antibiotics and AMR genes into the wider environment, particularly 619 within the first 48 hours after exposure.

## 620 8.6 FUTURE WORK

621 The results of this research suggest that the presence of amoxicillin can make septic tanks more 622 vulnerable to additional stresses, and therefore, further work is required before drawing concrete 623 conclusions and generalisations of these on different scenarios with varying environmental 624 conditions. Based on our analyses, future studies could include:

- While this study focused on the first 48 hours after acute exposure, studies which focus on the effects during longer time scales can capture recovery, and determine if there is a point in which amoxicillin exposed conditions and the control converge in terms of community and waste treatment efficiency. It would be important for these studies to include observations on an hourly rather than daily basis.
- Septic tanks insitu can have a variety of temperatures depending on region and time of year. This study utilised mesophilic temperatures, which are rarely observed in insitu septic tanks (though some research is currently underway developing heated septic tanks). As biochemistry can change with different temperature ranges, future studies could repeat this work for communities adapted to different temperature ranges, closer to those as would be found in operating septic tanks.
- This work demonstrated that there were changes in the sCOD during the first 12 hours and to the community for all amoxicillin exposure conditions. Future studies which focused on the analysing changes in function through KEGG ortholog analysis would indicate specifically which pathways are inhibited by the presence of amoxicillin within the community.

# Chapter 8

# The Structural Stability of Amoxicillin within Anaerobic Culture Medium

## 9.1 INTRODUCTION

Pharmaceutical micropollutants entering water treatment facilities and waterways is an issue of global concern. Beta-lactam antibiotics, including penicillin and amoxicillin, are amongst the most widely used antibiotics in the world comprising 50-70% of total antibiotics used in most countries (Surridge and Watson, 2012; Loos *et al.*, 2018). However, this widespread usage also leads to increased risks to the aquatic environment through water discharge from treatment facilities as well as runoff from agricultural settings (Kümmerer, 2009a, 2009b). The spread of amoxicillin within the environment can lead to the proliferation of antimicrobial resistance (Zhang, Zhang and Fang, 2009; Rizzo et al., 2013) in turn leading to a decrease in the therapeutic potential of these lifesaving drugs (Zaman et al., 2017). It has been suggested that if antimicrobial resistance is allowed to spread, the resulting loss of our antimicrobial drugs could be as large a threat to humanity as climate change (Torjesen, 2013). To curb the spread of amoxicillin and other pharmaceuticals into the environment and the resulting wave of antibiotic resistance which follows, it is important to understand both how these drugs behave as they move through our water treatment systems and the resident microbes responsible for treatment.

The toxicity of pharmaceuticals on culturable microbes is measured in a variety of ways. Techniques such as disc diffusion and tube dilution are traditionally used when studying the antibiotic effect on single organisms capable of growing on agar medium (Madigan, 2014). However, many microbes are not readily grown on plates including those who grow in syntrophic pairs, groups, or in complex microbial communities found such as those found in wastewater treatment processes. In these cases, the toxicity is measured by changes in microbial activity when microbes or microbial communities are exposed to known concentrations of the target compound (OECD, 2007). Anaerobic microbial communities, which underpin processing in anaerobic digesters for wastewater treatment, require specialised culturing conditions in which methanogenic activity inhibition is measured through relative changes in the volume and rate of methane formation (Alexy, Kümpel and Kümmerer, 2004; OECD, 2007; Cetecioglu *et al.*, 2013; Aydin, Ince, *et al.*, 2015).

The degradation pathways of amoxicillin have been understood as far back as 1971 (Hou and Poole, 1971). The antibiotic activity of beta-lactams operates primarily through its characteristic four-membered lactam ring structure (Loos *et al.*, 2018) (**Figure 8-1**). When this structure is hydrolysed, either through enzymatic actions - which are the primary mechanism of biological beta lactam resistance - or through chemical or photodegradation, the beta lactam ring opens and deactivates the molecule. (Vahdat and Sunderland, 2007; Längin *et al.*, 2009). Research into the stability of beta lactam drugs have shown that the rate of decay of the beta lactam ring is influenced by factors such as pH, concentration and conductivity of the buffer, and the temperature of the matrix as a whole (Chadha, Kashid and Jain, 2003; Pratama, Peterson and Palmese, 2012; Braschi *et al.*, 2013). As the focus of this work is drug delivery, most of this work primarily utilised buffers with the aim of pH modification.

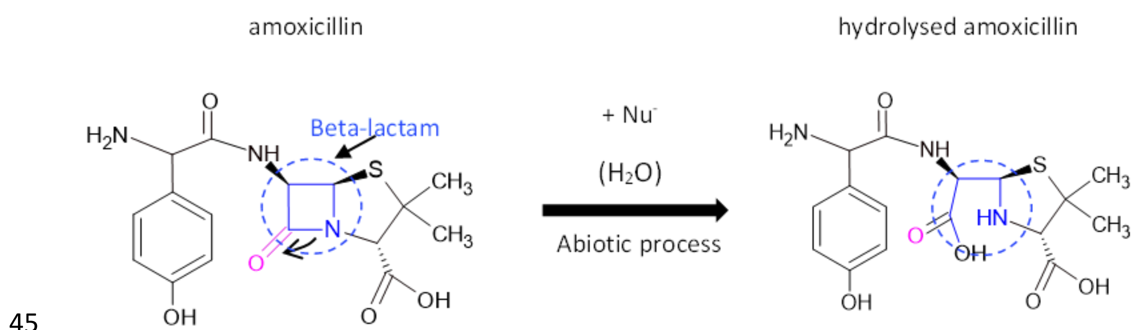



*Figure 9-1 - Amoxicillin is abiotically degraded through the hydrolysis of the beta-lactam ring (blue). This ring is the active site of the molecule which interacts with the peptidoglycan within the microbial cell wall. It can abiotically decay through a nucleophilic attack on the carbonyl group within the beta-lactam ring (pink). The example above shows hydrolysis with a water molecule, though other nucleophilic compounds can attack using the same mechanism.*

However, studies which measure the antibiotics efficacy and potency in terms of growth inhibition rarely consider the impact of abiotic decay. A wide variety of compounds are added to culture media used to sustain anaerobic microbial communities in laboratory cultures during inhibition tests. Anaerobic culture medium can include oxygen scavengers, salt nutrients, indicators, stabilisers, as well as buffers (Shelton and Tiedje, 1984; Aydin, Shahi, *et al.*, 2015). Yet, the influence of media components on the structural stability of the analyte of interest within culture-based toxicity studies are rarely if ever considered. When decay is discussed, it is assumed that degradation is primarily biological in nature (Alexy, Kümpel and Kümmerer, 2004; Hijosa-Valsero *et al.*, 2011). Therefore, the influence of the individual components within culture media on the decay rate of beta-lactam antibiotics independent from the biological degradation is unknown.

**The aim of this study was to determine the effect of commonly used chemical components of anaerobic culture medium on the decay rate of amoxicillin in solution at concentrations used in other chapters of this thesis.** Understanding the role that commonly used additives within anaerobic culture medium have on the degradation rates of amoxicillin will help to disambiguate between the biological and chemical degradation of amoxicillin within these studies. This study theorised that the rate of amoxicillin degradation is determined by the chemical structures of other solutes in the solution.

69 ## 9.2 Materials and Methods

70 ### 9.2.1 Overview of experiment Design

71 To determine how anaerobic culture medium components influence abiotic amoxicillin decay, the
72 decay rate of amoxicillin was tested in combination with each component individually. The chemical
73 components tested included commonly used buffers, organic and inorganic nutrients and an oxygen
74 scavenger (**Figure 8-2**). Amoxicillin, at a range of initial concentrations 0.0008, 0.0040, 0.0080 mM
75 which correlates to 1 mg/l, 5 mg/l, and 10 mg/l to reflect concentrations that are used in Chapters 6
76 and 7, was stored in solution with individual components of the anaerobic medium at (i) at 35°C, the
77 most common incubation temperature for anaerobic culture of wastewater microbial communities
78 (**Figure 8-3**), and (ii) at -20°C, a common temperature for storage of samples arising from such
79 studies (**Figure 8-4**). To determine decay rates, samples were taken periodically, and amoxicillin
80 concentration was quantified using LC-UV (**Figure 8-2**).

81

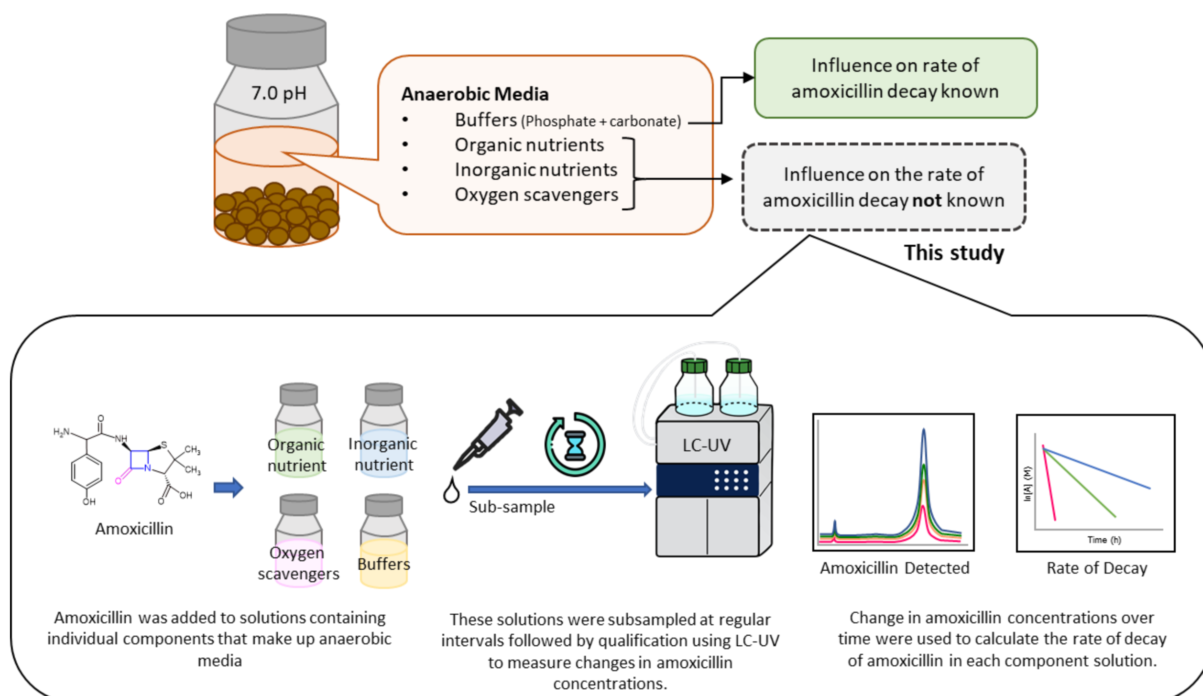



*Figure 9-2 - Summary of the experimental design and logical flow of this study.*
*It is often assumed that as anaerobic media is pH neutral (7.0 pH) that chemical degradation of amoxicillin within culture media would be minimal. However, this assumption has not been thoroughly tested. This study measures the rate of amoxicillin decay in the presence of the individual components of anaerobic media.*

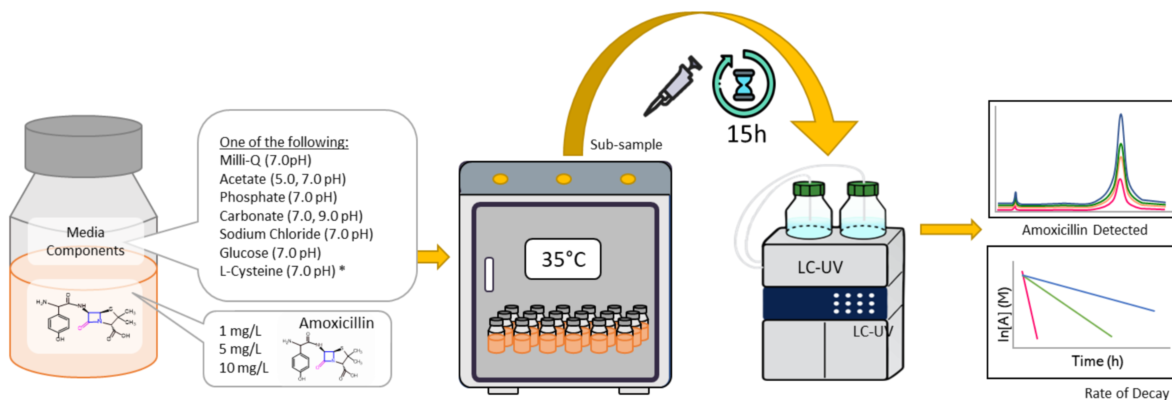



***Figure 9-3*** *- Experiment No 1 – Decay of amoxicillin at 35°C.*

*This experiment measures the decay of amoxicillin when incubated in the presence of one of the components found in anaerobic media adjusted to a pH of 7.0. Two additional buffers were also added to show how pH variation can alter amoxicillin stability and change the rate of decay.*

82

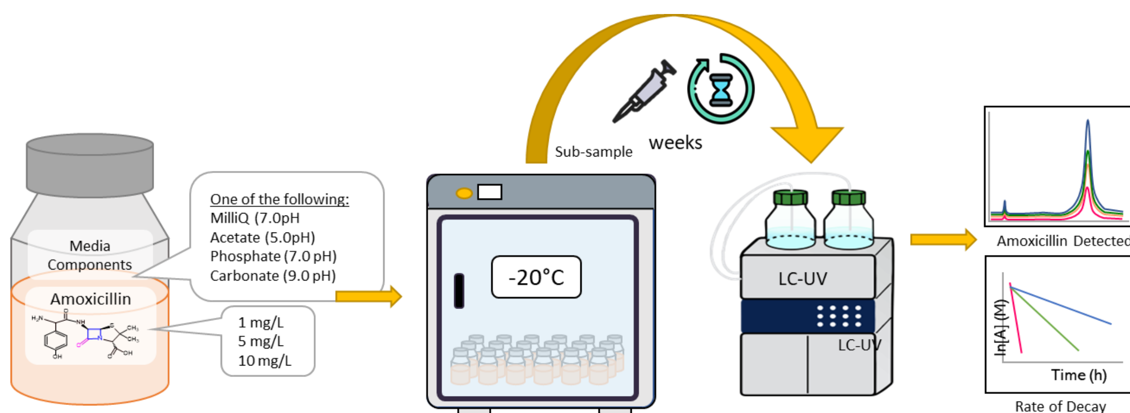



***Figure 9-4*** *- Experiment No 2 – Decay of amoxicillin when stored at -20°C.*
*This experiment measured the decay of amoxicillin at different pH values at temperatures used for storage.*

83

84 During the course of experimentation, it was established that when l-cysteine was in excess,
85 amoxicillin decayed at a greater rate than could be detected under the initial experimental conditions.
86 Therefore, an additional experiment was conducted in which varied l-cysteine concentrations (0.125-
87 8mg/l) were incubated with amoxicillin (at 10mg/l) (**Figure 8-5**). The aim of this work was to
88 determine the concentration of l-cysteine at which amoxicillin decay rates could be monitored using

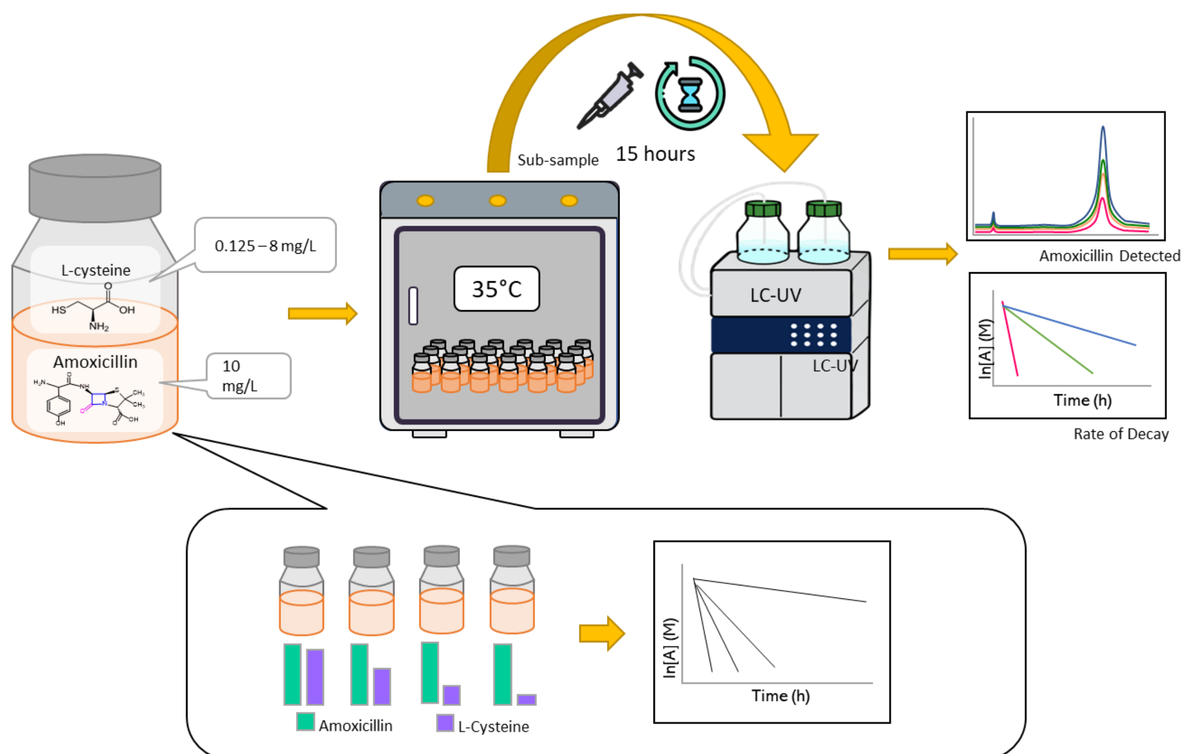



*Figure 9-5 - Experiment No 3 – Measuring the rate of decay of amoxicillin in the presence of differing concentrations of l-cysteine.*
*Experiment no 1 found that the decay of amoxicillin was instantaneous in the presence of 8mM of l-cysteine and an accurate rate measurement was not able to be taken. As the rate of amoxicillin decay is dependent on the concentration of l-cysteine, lowering the concentration of a l-cysteine should also slow the rate of decay of amoxicillin. As such, this experiment aims to determine the ratio of amoxicillin to l-cysteine in which amoxicillin decay can be measured.*

89 manual sampling and amoxicillin detected using an HPLC. This work was conducted at 35°C to
90 mimic the incubation temperatures preferred by mesophilic microbes.

## 91 9.2.2 Reagents

### 92 *9.2.2.1 Test compounds selected from anaerobic growth medium*

93 The test compounds and concentrations used in this study were chosen to reflect those used in
94 anaerobic medium specific to the SMA test and common to biological activity inhibition assays
95 reported in the literature (Colleran *et al.*, 1992; D. Coates, F. Coughlan and Colleran, 1996;
96 Cetecioglu, 2014). In total, the effect of seven compounds on amoxicillin decay was tested in this
97 study **(Table 8-1)** at a concentration of 40mM each with the exception of glucose and l-cysteine
98 which were used at concentrations relevant to culturing methods. Three of the seven compounds
99 were buffers. Carbonate and phosphate are both used in anaerobic medium for the purpose of pH
100 buffering (Shelton and Tiedje, 1984; OECD, 1997). Acetate is commonly used as a buffer in
101 chromatography (Marco *et al.*, 2017), though it is readily degradable by methanogens during

102 anaerobic culturing and used as a feed source in experimentation (Heidari, Fatemeh Nabavi, Saffari
103 Khouzani and Mehdi Amin, 2012; Aydin, Ince, *et al.*, 2015). L-Cysteine is an oxygen scavenger used
104 in anaerobic culturing (Rymovicz *et al.*, 2011). Sodium chloride was used to represent inorganic
105 nutrients added to cultures. The standard trace nutrient mix was not used for this experiment as the
106 metal salts could potentially damage the LC column. Glucose is highly bioavailable and a commonly
107 used substrate. Milli-Q water was used as control. This study hypothesised that as the Milli-Q water
108 has few dissolved substances, that this would produce the slowest decay rate. All seven compounds
109 were adjusted to a pH of 7.0 to remove pH as a variable and hence enable direct comparison.
110 Additionally, acetate and carbonate buffers were used at pH=5.0 and pH=9.0 respectively. This pH
111 range represents that typical of anaerobic systems used for wastewater treatment, and enabled
112 observation of the influence of pH on the rate of decay. All mixes were autoclaved before the addition
113 of amoxicillin stock. All conditions were tested at a temperature of 35°C, though Milli-Q® Type 1
114 Ultrapure Water, acetate (5.0pH), phosphate (7.0pH), and carbonate (9.0pH) were additionally tested
115 at -20°C (**Figure 8-3 and 8-4**).

116 ***Table 9-1*** *- Test compounds and solutions used during the experiment. Conductivity tested at 20°C.*

| Compound | pH | Function | Molarity | Conductivity (uS) | Mix | |
|---|---|---|---|---|---|---|
| **Water** | 7.0 | Primary Matrix | NA | 4.0 | NA | |
| **Acetate Buffer** | 5.0 | Buffer<br>Food source | 40 mM | 1619 | $NaC_2H_4O_2$<br>$C_2H_4O_2$<br>MilliQ | 2.209 g<br>0.785 g<br>1000ml |
| **Acetate Buffer** | 7.0 | Buffer<br>Food source | 40 mM | 2580 | $NaC_2H_4O_2$<br>MilliQ | 3.28 g<br>1000ml |
| **Phosphate Buffer** | 7.0 | Buffer | 40 mM | 4460 | $K_2HPO_4$<br>$KH_2PO_4$<br>MilliQ | 3.737 g<br>2.523 g<br>1000ml |
| **Carbonate Buffer** | 7.0 | Buffer | 40 mM | 2528 | $NaHCO_3$<br>MilliQ | 3.36 g<br>1000ml |
| **Carbonate Buffer** | 9.0 | Buffer | 40 mM | 3140 | $NaHCO_3$<br>$Na_2CO_3$<br>MilliQ | 3.822 g<br>0.477 g<br>1000ml |
| **Glucose** | 7.0 | Food Source | 1 M | 9200 | $C_6H_{12}O_6$<br>MilliQ | 180g<br>1000ml |
| **NaCl** | 7.0 | Nutrient additive | 40 mM | 5.6 | NaCl<br>MilliQ | 2.34<br>1000ml |
| **L-Cysteine** | 7.0 | Oxygen Scavenger | 8 mM | 37 | L-Cysetine0.969g<br>MilliQ | <br>1000ml |

117

118 #### 9.2.2.2 Amoxicillin solution preparation

119 The concentrations of amoxicillin (1mg/l, 5 mg/l, and 10mg/l) used in this study were chosen to
120 reflect concentrations used in other chapters (Chapter 6 and Chapter 7) in this thesis. Working
121 solutions were prepared by mixing pure amoxicillin (Sigma Aldrich) with autoclaved Milli-Q water
122 to 100 mg/l then diluted with the appropriate test solution to obtain 1.0 mg/l, 5.0 mg/l, and 10 mg/l.
123 If necessary, solutions were pH adjusted dropwise using dilute HCl or KOH until reaching the desired
124 pH. All bottles used during mixing were rinsed with methanol and MilliQ before use.

125 #### 9.2.2.3 L-Cysteine solution preparation

126 To measure the interactions between l-cysteine and amoxicillin, an additional study was run with
127 seven different amoxicillin concentrations. The aim of this work was not to determine the rate of
128 decay, but rather to determine the maximum ratio of l-cysteine to amoxicillin which will produce a
129 decay curve that is detectable on the LC-UV. Therefore, l-cysteine was prepared as an 8.0 mg/l stock
130 solution by dilution in MilliQ water and serial dilutions were made from the stock again using MilliQ
131 water to produce the concentrations given in **Table 8-2**.

132 ***Table 9-2****- L-Cysteine and amoxicillin concentrations and the ratios of cysteine to amoxicillin used during*
133 *experimentation.*

| **L-Cysteine** | | **Amoxicillin** | | **Ratio** |
|---|---|---|---|---|
| *Concentration* | *Molarity (mM)* | *Concentration* | *Molarity (mM)* | *LC : AMOX* |
| 0.125 mg/l | 0.0001 | 10 mg/l | 0.0027 | 0.04 : 1 |
| 0.25 mg/l | 0.0002 | 10 mg/l | 0.0027 | 0.08 : 1 |
| 0.5 mg/l | 0.0004 | 10 mg/l | 0.0027 | 0.15 : 1 |
| 1.0 mg/l | 0.0008 | 10 mg/l | 0.0027 | 0.30 : 1 |
| 2.0 mg/l | 0.0017 | 10 mg/l | 0.0027 | 0.60 : 1 |
| 4.0 mg/l | 0.0033 | 10 mg/l | 0.0027 | 1.21 : 1 |
| 8.0 mg/l | 0.0066 | 10 mg/l | 0.0027 | 2.41 : 1 |

134

135 ### 9.2.3 Incubation, storage and sampling

136 The experiments were conducted at two different temperatures, 35°C to simulate culturing
137 conditions, and -20°C to simulate frozen storage conditions. For experiments incubated at 35°C, a

138 45 ml working solution was prepared for each test (containing the test compound plus amoxicillin).
139 Incubation bottles (30mL serum vials) were soaked in Virkon ™ (Lanxess) before use, after which
140 they were rinsed three times and dried. The 45mL working solution was then sub-divided between 3
141 bottles (15mL in each) such that each test condition was prepared in triplicate. Sealed bottles were
142 placed in a 35°C incubator and sampled approximately every 8 - 12 hours until 7 timepoints were
143 measured. To sample, a 1.0 ml aliquot was removed through the septa and placed in a sample vial
144 and immediately quantified using the LC-UV.

145 To test frozen storage conditions, 30ml of each dilution condition was prepared. A set of the freshly
146 prepared mixtures were immediately analysed. The rest was distributed into 1.5ml aliquots and
147 placed in a -20°C freezer. Samples were removed, thawed, and quantified using an LC-UV on days
148 0, 15, 147, and 177. Each condition was tested in triplicate.

### 149 9.2.4 Chromatographic Analysis

150 The amoxicillin was quantified using a Shimadzu LC20AT liquid chromatograph (Kyoto, Japan)
151 equipped with an SIL 20AHT Autosampler and a DGU-20 A Degassing unit. Chromatographic
152 separation was achieved using a Purospher® STAR RP-18 end capped column (5µm, 150mm x 4.6
153 mm) Merck KGaA, Darmstadt, Germany. Detection was conducted using an SPD-10 UV Detector
154 using wavelengths 295nm. The system was controlled by LabSolutions (Agilent Technologies). The
155 injection volume was 100µl. The flow rate was 0.9 ml/min. The mobile phases used were A: acetate
156 buffer (0.01M, pH 5.0) and B: HPLC grade methanol. The elution gradient: 0 - 4 min 10% B, 4 - 18
157 min 10 - 30% B, 18 - 20 min 30% B, 20 - 25 min 10%.

158 The optimisation and method validation for the LC-UV analysis is detailed in the Methods chapter.
159 Briefly, the validation was based on protocols described by the International Conference of
160 Harmonization (ICH) Guidelines (FDA, 1995; US FDA, 1996) and Liquid Chromatography method
161 development methods by Injac et al, (2009) and Martínez-Huelamo et al. (2009). The method validity
162 was determined by preparing and quantifying a range of standard solutions of amoxicillin in MilliQ
163 (0.1 – 10mg/l) in which linearity $r^2 > 0.999$. The method LOD was 0.123 mgL-1 and LOQ was 0.236
164 mgL-1 as defined by S/N≥2 for LOD and S/N ≥ 10 for LOQ.

165 ### 9.2.5 Statistical Analysis

166 The decay of chemical compounds can be described using two interrelated variables, (1) the decay
167 constant *k* and (2) half-life. (Ebbing and Gammon, 2016). Test compound concentrations used were
168 normalised to 40mM to maintain consistency across conditions (except for l-cysteine which was
169 8mM and glucose which used 1M to mimic concentrations used in other studies within this
170 dissertation. Thus, the concentrations of medium were several orders of magnitude more
171 concentrated than the amoxicillin concentrations used. It has been established both by theory (Ebbing
172 and Gammon, 2016) as well as by observation (Vahdat, 2000; Chadha, Kashid and Jain, 2003;
173 Kakimoto and Funamizu, 2007; Vahdat and Sunderland, 2007) that while the decay of amoxicillin
174 in the presence of other compounds with which it can react, the decay reaction as a whole is a second
175 order reaction such that the decay of depends both on the concentration of reactant as well as the
176 concentration of buffer. However, when the concentration of one of the reacting materials is in great
177 excess relative to the other (as occurs in this context) a second order reaction will behave like a first
178 order reaction as the concentration of the other solutes is effectively constant. As a result, the decay
179 in this study is said to be pseudo first order and reported as k'. As such, all values for the decay
180 constant k are truly for the pseudo first order decay of k' and will be calculated using first order
181 decay calculations.

182 The second order rate reactions is calculated as follows:

$$Rate = -\frac{d[A]}{dt} = k[A][B] \qquad \textit{Equation 1}$$

183

184 However, when B is >> than A, it can be treated as a constant. This produces a pseudo first order
185 reaction. Letting k' = [B]k give you the pseudo first order reaction. For this reason, the calculation
186 of the decay constant k and the half-life times will treat the decay as first order. The pseudo first
187 order rate reaction can be calculated as follows:

$$Rate = -\frac{d[A]}{dt} = k'[A] \qquad \textit{Equation 3}$$

188

189 Rearranging this give you,

$$\frac{d[A]}{[A]} = -k'dt$$ *Equation 4*

190

191 Integrating this with respect to time gives,

$$\ln[A] - \ln[A_0] = -k't$$ *Equation 5*

192

193 Equation 4 can be used to determine the rate at any given time in which *t* is the amount of time passed
194 since the initial measurement, [*A*] is the concentration at that time, and [$A_0$] is the initial
195 concentration. In this equation, *k* is the rate constant. While this constant should not change as the
196 concentration of the target compound decays, *k* will change with temperature and will not be constant
197 between the 35°C and -20°C studies and will need to be calculated separately for each temperature.

198 The value for *k* can be determined experimentally by plotting the natural log of the concentration
199 against time. When the decay is first order, this relationship should produce a straight line (**Equation**
200 **5**) in which *k* is the slope of the line and $ln[A_0]$ is the constant b.

$$\ln[A] = -k't + \ln[A_0]$$ *Equation 6*

201

202 For this study, the slope of this line, and therefore *k'*, was determined by calculating the best fit line
203 from the observed data. To assess the linearity of this line, a regression analysis was conducted for
204 each of the test conditions and the r-square value was determined for the triplicate runs of each of
205 the decay conditions at all time points. This was obtained separately for each of the different
206 amoxicillin concentrations used.

207 The half-life was calculated using the value of *k* obtained using the following equation:

$$half\ life = \frac{\ln 2}{k'}$$ *Equation 7*

208

209 ## 9.3 RESULTS

210 ### 9.3.1 Amoxicillin Decay on 35°C

211 This research found that the rate of decay of amoxicillin was different depending on the differing
212 composition of each of the matrices utilised in the study (**Figures 8-6, 8-7,** and **8-8, Table 8-3**). The
213 slowest decay observed was in the glucose solution, though the decay results were highly variable in
214 which the 10 mg/L (27.367 uM) amoxicillin had a k' of 0.0012 and a half-life of 563.31h, while the
215 1mg/L (13.69 uM) amoxicillin had a k' of 0.0004 and a half-life of 1804.22 hours. The amoxicillin
216 concentration detected within glucose solution was highly variable both during the study, as
217 determined by the low $R^2$ values, as well as between replicates resulting in high p-values (**Table 8-**
218 **8**).

219 Amoxicillin in MilliQ water, acetate (7pH), acetate (5pH), and NaCl all had similar rates of decay
220 for each of the amoxicillin concentrations used. Amoxicillin in MilliQ decayed with a half-life of
221 544.09, 454.44, and 393.85 for 1 mg/L (27.37 uM), 5 mg/L (13.68 uM), and 10mg/L (2.74 uM)
222 respectively. Amoxicillin in 7pH acetate solution decayed with a half-life of 463.01, 466.71and
223 289.96 for 1 mg/L (27.37 uM), 5 mg/L (13.68 uM), and 10mg/L (2.74 uM) respectively. Amoxicillin
224 in acetate at 5pH decayed with a half-life of 415.20, 415.20, and 354.38 hours for 1 mg/L, 5 mg/L,
225 and 10mg/L respectively. Amoxicillin in NaCl decayed with a half-life of 241.93, 325.42, and 288.15
226 hours for 1 mg/L ,5 mg/L, and 10mg/L respectively. Of these, the 5pH acetate solutions has the least
227 variance between replicates and samples ($R^2$ = 0.697 - 0.928) while the NaCl Solution had the
228 greatest in which there was variation within replicates ($R^2$ = 0.520 - 0.749).

229 Amoxicillin decayed at a similar rate in the 7pH carbonate solution and the phosphate solutions with
230 half-lives ranging from 103.82-129.53 hours and 71.41-124.64 hours for the 7pH carbonate and
231 phosphate solutions respectively. In both cases, the rate of decay was the slowest for the 1mg/L (2.74
232 uM) amoxicillin concentration, though this difference was most pronounced within the phosphate
233 solutions in which the 1mg/L (2.74 uM). Amoxicillin in the 9pH carbonate solution decayed rapidly
234 with mean half-lives ranging from 23.85 - 34.38 hours.

235 Amoxicillin in l-cysteine decayed so rapidly that an accurate measurement was not able to be taken.
236 By the time the first sample was taken at 2 hours, the amoxicillin concentrations were less than 10%
237 of the initial concentration and were below the limit of detection for some of the samples. After this
238 point, the amoxicillin concentrations remained somewhat stable though, many samples were below
239 the lower limit of detection.

240

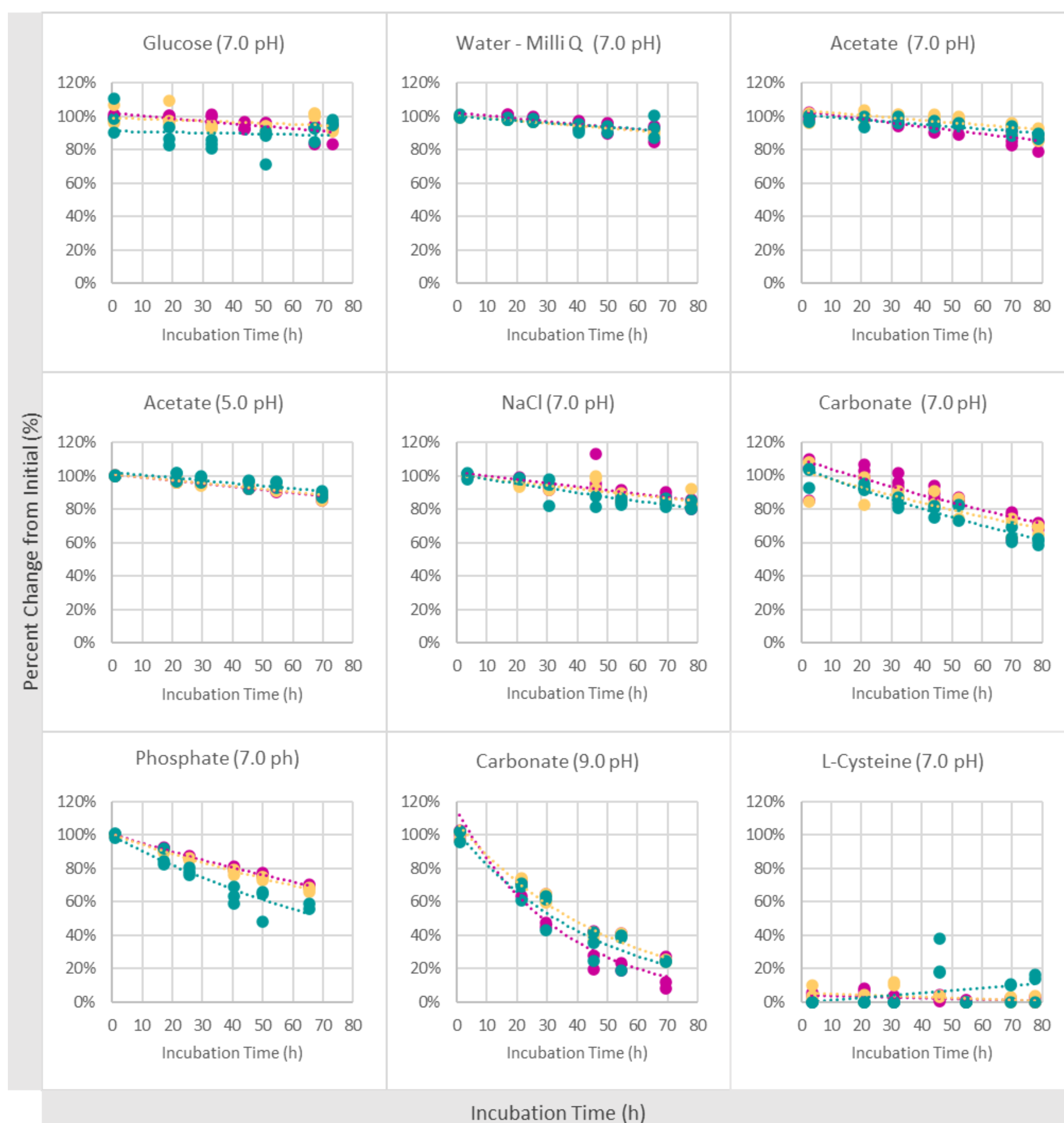



***Figure 9-6*** *- Amoxicillin decay at 35°C.*
*The Y axis for each figure measures the percent change from the initial concentration. The x-axis measures time. The trend lines measure exponential decay.*

***Table 9-3*** *- Decay rates for amoxicillin in each of the solutions provided.*
*P-values are for liner regression analysis of the natural log of amoxicillin concentrations through time (hours). The slope of this was used to calculate the k' and half-life (*** $p \leq 0.001$, ** $0.001 < p \leq 0.01$, * $0.01 < p \leq 0.05$.)*

| Buffer type (pH) | Amoxicillin Mg/L | uM | Half-Life (M/ hour) | K' | $R^2$ | Standard Error | P-value |
|---|---|---|---|---|---|---|---|
| **Glucose (7.0 pH)** | 10 | 27.37 | 563.31 | 0.0012 | 0.477 | 0.0003 | 0.001 ** |
| | 5 | 13.68 | 1047.90 | 0.0007 | 0.109 | 0.0005 | 0.182 |
| | 1 | 2.74 | 1804.22 | 0.0004 | 0.012 | 0.0009 | 0.671 |
| **MlliQ (7.0 pH)** | 10 | 27.37 | 393.85 | 0.0018 | 0.634 | 0.0003 | < 0.001 *** |
| | 5 | 13.68 | 454.44 | 0.0015 | 0.965 | 0.0001 | < 0.001 *** |
| | 1 | 2.74 | 544.09 | 0.0013 | 0.450 | 0.0004 | 0.002 ** |
| **Acetate (7.0 pH)** | 10 | 27.37 | 289.96 | 0.0024 | 0.731 | 0.0003 | < 0.001 *** |
| | 5 | 13.68 | 466.71 | 0.0015 | 0.537 | 0.0003 | < 0.001 *** |
| | 1 | 2.74 | 463.01 | 0.0015 | 0.723 | 0.0002 | < 0.001 *** |
| **Acetate (5.0 pH)** | 10 | 27.37 | 354.38 | 0.0020 | 0.928 | 0.0001 | < 0.001 *** |
| | 5 | 13.68 | 373.11 | 0.0019 | 0.877 | 0.0002 | < 0.001 *** |
| | 1 | 2.74 | 415.20 | 0.0017 | 0.697 | 0.0003 | < 0.001 *** |
| **Carbonate (7.0 pH)** | 10 | 27.37 | 129.53 | 0.0054 | 0.731 | 0.0007 | < 0.001 *** |
| | 5 | 13.68 | 134.42 | 0.0052 | 0.758 | 0.0007 | < 0.001 *** |
| | 1 | 2.74 | 103.82 | 0.0067 | 0.905 | 0.0005 | < 0.001 *** |
| **Phosphate (7.0 pH)** | 10 | 27.37 | 124.64 | 0.0056 | 0.997 | 0.0001 | < 0.001 *** |
| | 5 | 13.68 | 114.63 | 0.0060 | 0.991 | 0.0001 | < 0.001 *** |
| | 1 | 2.74 | 71.41 | 0.0097 | 0.856 | 0.0010 | < 0.001 *** |
| **Carbonate (9.0 pH)** | 10 | 27.37 | 23.85 | 0.0291 | 0.822 | 0.0034 | < 0.001 *** |
| | 5 | 13.68 | 34.38 | 0.0202 | 0.970 | 0.0009 | < 0.001 *** |
| | 1 | 2.74 | 31.52 | 0.0220 | 0.853 | 0.0024 | < 0.001 *** |
| **NaCl (7.0 pH)** | 10 | 27.40 | 288.15 | 0.0024 | 0.520 | 0.0005 | < 0.001 *** |
| | 5 | 13.70 | 325.42 | 0.0021 | 0.668 | 0.0003 | < 0.001 *** |
| | 1 | 2.70 | 241.93 | 0.0029 | 0.749 | 0.0004 | < 0.001 *** |

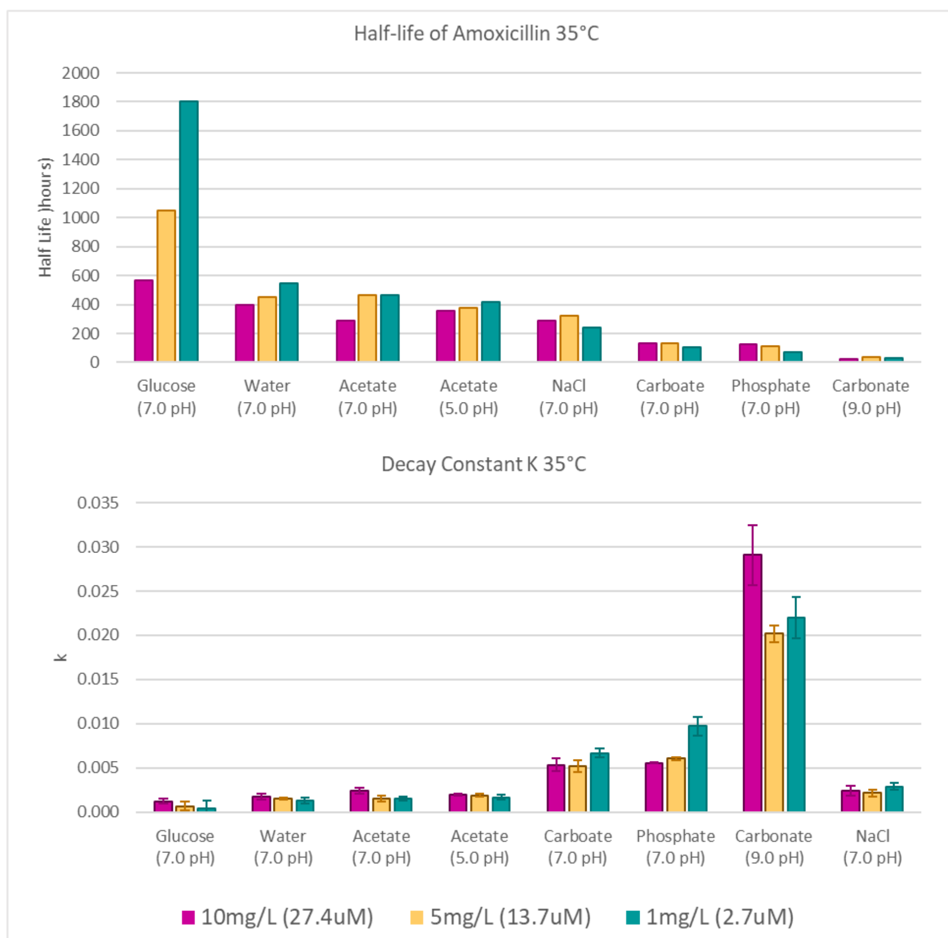



***Figure 9-8*** *- The top figure plots the half-lives of amoxicillin in each of the conditions. Each bar represents a different concentration of amoxicillin used in the experiment. The bottom figure plots of the calculated k' values. The error bars in the bottle figure represent the standard error within the regression analysis used to calculate k'.*

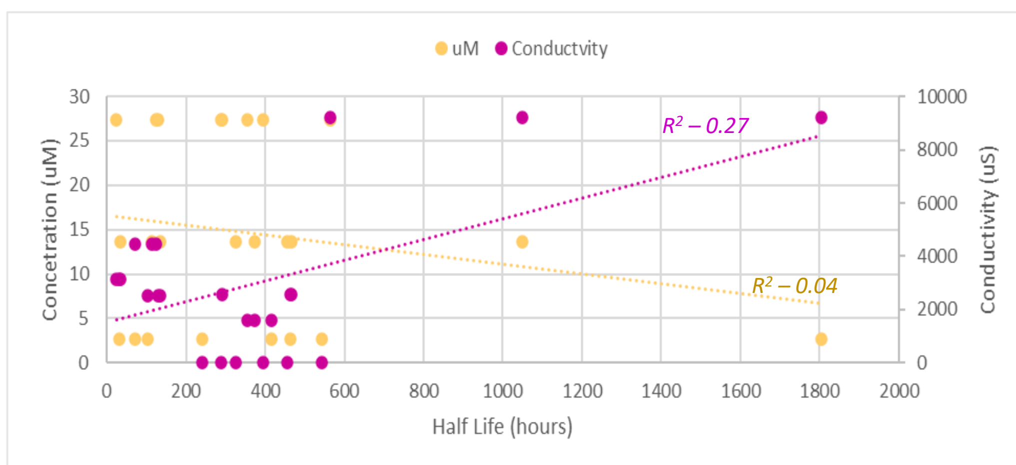



***Figure 9-7*** *- Regression plot analysing the conductivity and concentration of the each of the solutions used in the experiment against the half life in amoxicillin within those solutions.*
*This graph uses all the different solutions as well as all the half life calculations in a single pool.*

248 To determine if the conductivity or concentration of the solutions used were correlated to the rate of
249 amoxicillin decay, a regression model was run between these values and half live for all amoxicillin
250 concentrations and solution types. These models found that conductivity ($R^2$ – 0.27) and
251 concentration ($R^2$ – 0.04) were both poor predicters for the rate of amoxicillin decay (**Figure 8-8**).

252 #### *9.3.1.1 Amoxicillin Decay at -20 C*

253 The results show that when frozen at -20°C, amoxicillin in pure milliQ water had the maximum half-
254 life of 475.90 days ($R^2$ - 0.704) (**Figure 8-9, Table 8-4**). The decay rate of amoxicillin in acetate was
255 slightly shorter with an average half-life of 400.84 days. As with the decay at 35°C described in the
256 previous section, the decay rate of amoxicillin in the presence of phosphate and carbonate buffers
257 was much more rapid than for the other conditions studied. The decay of amoxicillin within
258 phosphate buffer was consistent between the three different concentrations in which half-lives ranged
259 from 32.49-33.34 days. Amoxicillin within carbonate buffer decayed at a similar rate, though the
260 runs were not as consistent between the different concentrations used and half-lives ranged from 14.8
261 – 25.16 days.

262 ***Table 9-4*** *- Decay rates for amoxicillin in each of the solutions provided at -20°C.*
263 *P-values are for liner regression analysis of the natural log of amoxicillin concentrations through time (hours). The*
264 *slope of this was used to calculate the k' and half-life (*** $p \leq 0.001$, ** $0.001 < p \leq 0.01$, * $0.01 < p \leq 0.05$.)*
265

| Buffer type (pH) | ppm | uM | Half-Life (days) | k | $R^2$ | Standard Error | P-value |
|---|---|---|---|---|---|---|---|
| **Water (7.0 pH)** | 10 | 27.37 | 422.307 | 0.002 | 0.936 | 0.000 | 0.000 *** |
| | 5 | 13.68 | 466.442 | 0.001 | 0.963 | 0.000 | 0.000 *** |
| | 1 | 2.74 | 476.907 | 0.001 | 0.704 | 0.000 | 0.001 *** |
| **Acetate (5.0 pH)** | 10 | 27.37 | 366.196 | 0.002 | 0.930 | 0.000 | 0.000 *** |
| | 5 | 13.68 | 400.840 | 0.002 | 0.987 | 0.000 | 0.000 *** |
| | 1 | 2.74 | 331.808 | 0.002 | 0.747 | 0.000 | 0.000 *** |
| **Phosphate (7.0 pH)** | 10 | 27.37 | 33.336 | 0.021 | 0.999 | 0.000 | 0.000 *** |
| | 5 | 13.68 | 32.961 | 0.021 | 0.998 | 0.000 | 0.000 *** |
| | 1 | 2.74 | 332.492 | 0.002 | 0.748 | 0.000 | 0.000 *** |
| **Carbonate (9.0 pH)** | 10 | 27.37 | 25.161 | 0.028 | 0.994 | 0.001 | 0.000 *** |
| | 5 | 13.68 | 20.936 | 0.033 | 0.962 | 0.003 | 0.001 *** |
| | 1 | 2.74 | 14.800 | 0.047 | 0.829 | 0.011 | 0.012 * |

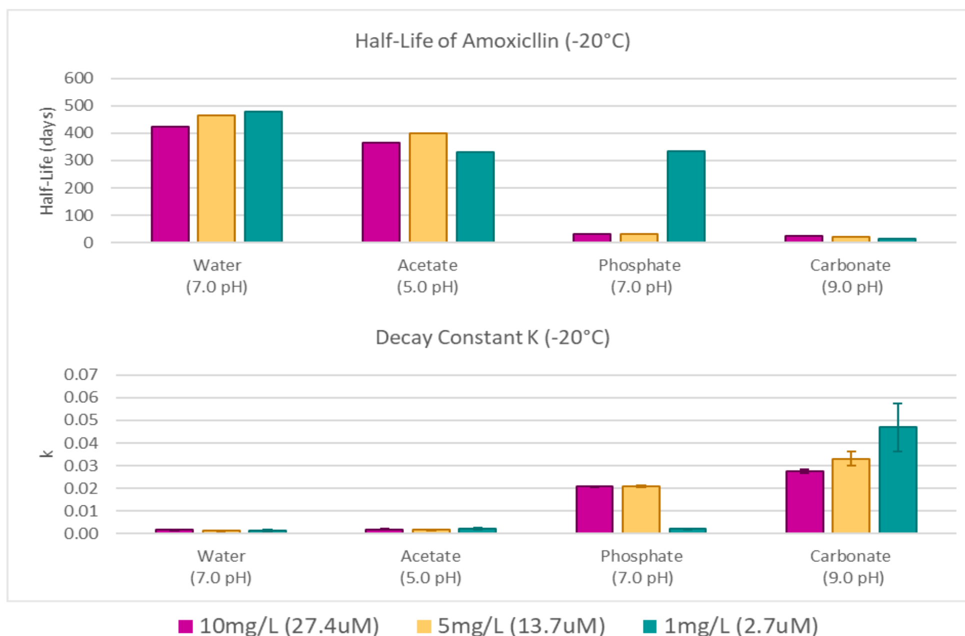



***Figure 9-10*** *- The top figure plots the half-lives of amoxicillin in each of the conditions. Each bar represents a different concentration of amoxicillin used in the experiment. The bottom figure plots of the calculated k' values. The error bars in the bottle figure represent the standard error within the regression analysis used to calculate k'.*

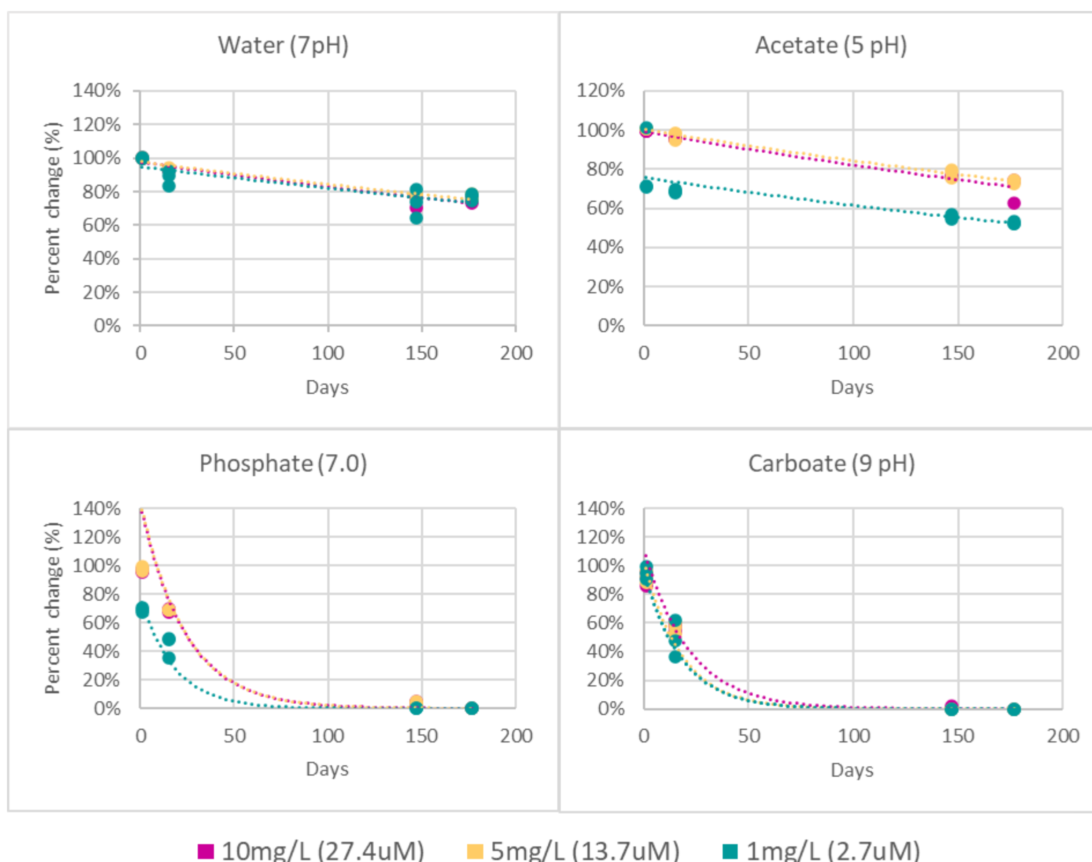



***Figure 9-9*** *- Amoxicillin decay at -20°C.*
*The Y axis for each figure measures the percent change from the initial concentration. The x-axis measures time in days. The trend lines measure exponential decay.*

267 ### *9.3.1.2 L-Cysteine Decay*

268 The results from section 8.3.1 reported that when in a solution of 8mM l-cysteine, 10mg/L (2.74
269 mM) amoxicillin decayed to the LOQ so rapidly an accurate measurement was not able to be taken.
270 It was suspected that this was the result of an interaction between the amoxicillin and the l-cysteine.
271 As such, this experiment measured changes in the decay rate of amoxicillin with varying
272 concentrations of amoxicillin.

273 The decay rate of amoxicillin was observed to increase with an increase in the concertation of l-
274 cysteine in solution (**Table 8-5, Figure 8-11**). When l-cysteine solution was at a concentration of
275 0.125 mg/l the half-life of amoxicillin was 452.05 hours, which was comparable to the decay rates
276 observed in MilliQ water the 35°C degradation conditions (for example, 10 mg/L of amoxicillin
277 which decayed in milliQ water with a half-life of 393.85 hours). However, the rate of amoxicillin
278 decay increased with increased l-cysteine concertation. When the l-cysteine solution was above
279 2mg/l, amoxicillin concentrations were below the limit of detection and were not able to be reliably
280 calculated. The greatest ratio tested in which amoxicillin was reliably detectable was for 1mg/l of l-
281 cysteine at a ratio of 0.3x the l-cysteine to amoxicillin. The shape of the decay curves was not linear
282 when the natural log of amoxicillin was plotted against time, suggesting that this relationship was
283 not a pseudo first order relationship, as was found in previous sections. This is likely because, unlike
284 in previous sections, the concentrations of l-cysteine solutions were not in excess of amoxicillin. It
285 is possible that the amoxicillin and l-cysteine interact with eachother during decay in a second order
286 reaction.

287 ***Table 9-5*** *– Decay rates for amoxicillin at different concentrations of l-cysteine.*
288 *P-values are for liner regression analysis of the natural log of amoxicillin concentrations through time (hours). The slope*
289 *of this was used to calculate the k' and half-life (*** p≤0.001, ** 0.001 < p ≤ 0.01, * 0.01< p ≤ 0.05.)*

| Concentration of L-Cysteine | | Amoxicillin | **Molar RATIO** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **mg/L** | **uM** | **uM** | **LC:Amox** | **K'** | **Standard Error** | **$R^2$** | **Half-life (hour)** | **P-value** |
| 0.125 ppm | 0.10 | 2.74 | 0.04 | 0.002 | 0.00 | 0.26 | 452.95 | 0.13 |
| 0.25 ppm | 0.21 | 2.74 | 0.08 | 0.003 | 0.00 | 0.33 | 208.03 | 0.08 |
| 0.5 ppm | 0.41 | 2.74 | 0.15 | 0.018 | 0.00 | 0.79 | 38.34 | 0.00 *** |
| 1 ppm | 0.83 | 2.74 | 0.30 | 0.035 | 0.01 | 0.74 | 19.65 | 0.00 ** |
| 2 ppm | 1.65 | 2.74 | 0.60 | 0.192 | 0.04 | 0.91 | 3.61 | 0.01 * |
| 4 ppm | 3.30 | 2.74 | 1.21 | 0.188 | 0.04 | 0.90 | 3.68 | 0.01 * |
| 8 ppm | 6.60 | 2.74 | 2.41 | Below Detection | | | | |

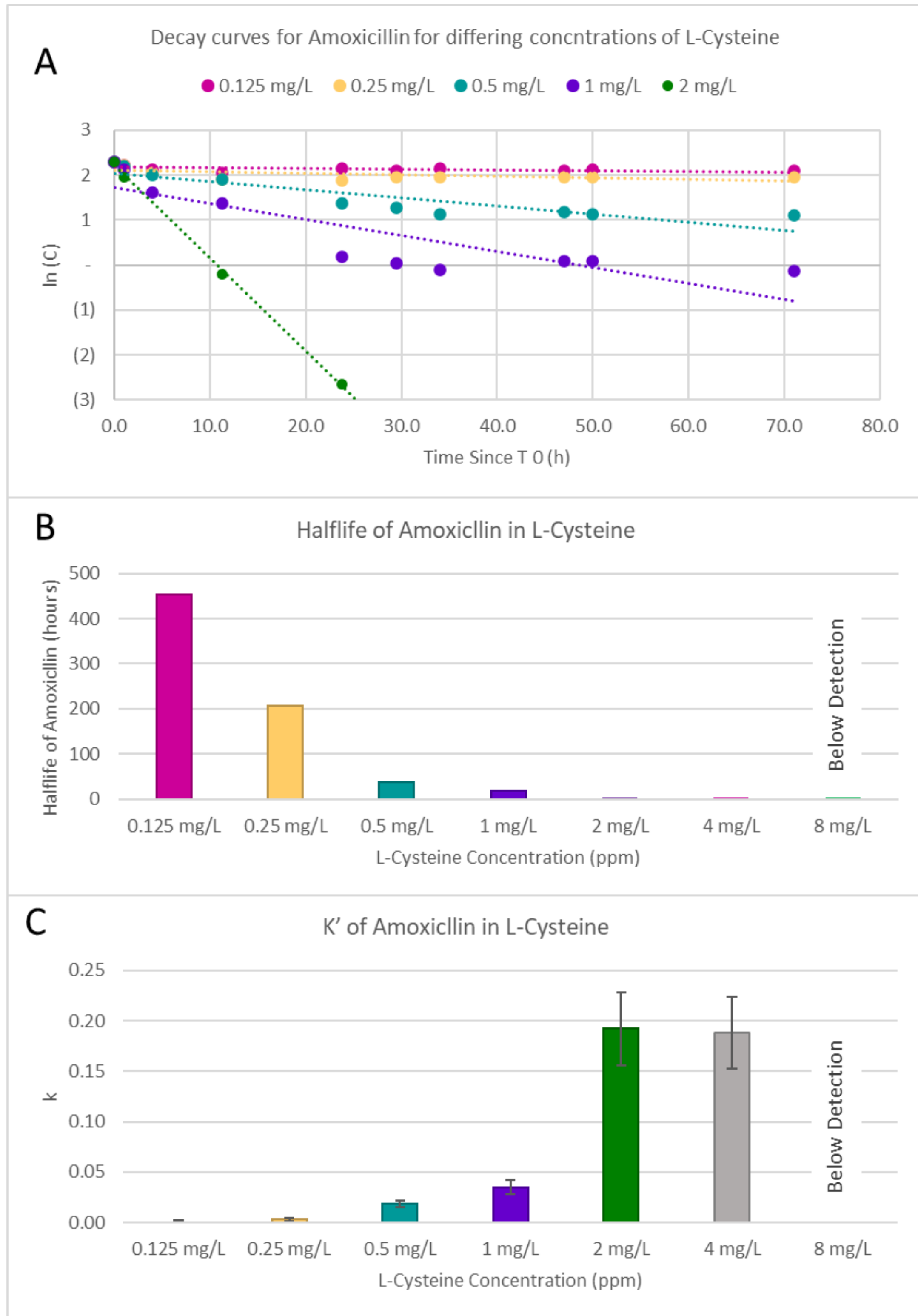



***Figure 9-11*** *– Summary of l-cysteine decay.*
*(A) The decay curves for each of the different l-cystine concentrations. The Y axis represents the natural log amoxicillin concentration (mg/L) for each time point. (B) The half-life of amoxicillin for each l-cysteine concentration used. (C) The k' values for the decay of amoxicillin in different l-cystine values. The error bars in the bottle figure represent the standard error within the regression analysis used to calculate k'.*

291 ## 9.4 DISCUSSION

292 ### 9.4.1 The decay of amoxicillin in anaerobic medium components at 35°C

293 This study found that the decay rate of amoxicillin was different when in the presence of each of the
294 individual components of anaerobic culture medium. Previous research into the rate of decay of
295 amoxicillin found a relationship between the pH of the buffer used and the rate of decay for
296 amoxicillin (Vahdat and Sunderland, 2009). However, these studies did not consider how the
297 chemical properties of the buffer medium might influence decay rate. Both enzymatic and abiotic
298 decay amoxicillin through a nucleophilic substitution on the carbonyl group of the beta-lactam (Hirte
299 *et al.*, 2016; Zango *et al.*, 2019). When decay is abiotic, the rate of this reaction is determined by the
300 concentration of the reactant and its nucleophilicity (King *et al.*, 2017). Nucleophiles are attracted to
301 positively charged electrophilic sites within a molecule (**Figure 8-12**). The nucleophilic strength of
302 a reactant is often correlated to the pKa of the deprotonated base of its acid base couple. For example,
303 the decay rate of amoxicillin increases with a rise in pH, as the concentration of nucleophilic
304 hydroxide ions also increases (Chadha, Kashid and Jain, 2003). However, the different chemical
305 structures, even at the same pH can have differing nucleophilicity. It is possible that the differences
306 in the decay rate of amoxicillin found in this study are related to the differences in the nucleophilicity
307 and pKa of each of the medium components tested.

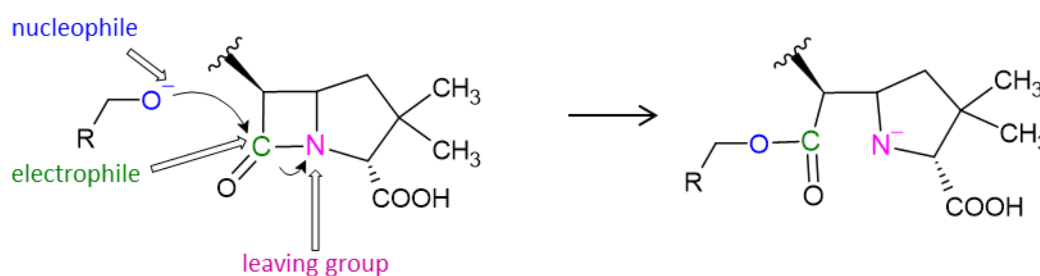


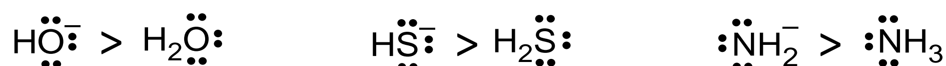

***Figure 9-12*** *- The nucleophilic decay of the beta-lactam ring.*

308

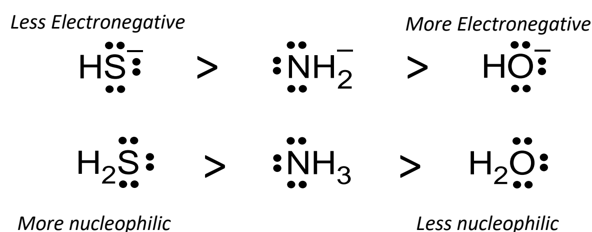
309 The nucleophilicity, or willingness of a nucleophile to donate its electrons, is determined by various
310 conditions such as charge, electronegativity, and steric hinderance (Hemming, 2001; Fu, 2017;
311 Hamlin, Swart and Bickelhaupt, 2018) (**Figure 8-13**). As atoms or molecules become more electron

rich their ability to donate electrons increases. Therefore, the negatively charged molecular forms and conjugate bases are better nucleophiles than their neutrally charged or protonated counterparts. Similarly, there is an inverse relationship between an increase in electronegativity and how tightly bound an electron pair is. Therefore, as the electronegativity increases the nucleophilicity decreases. Additionally, steric hindrance restricts the access to the protons, and therefore slows the reaction rate and effective nucleophilicity as well (Edwards and Pearson, 1962; Hemming, 2001; Mayr and Ofial, 2008).

**Charge – The conjugate base is a stronger nucleophile**




**Electronegativity – Increasing electron density decreases nucleophilicity**




**Steric Hinderance – the bulkiness of a molecule reduces accessibility and slows the rate of reactions and nucleophilicity**

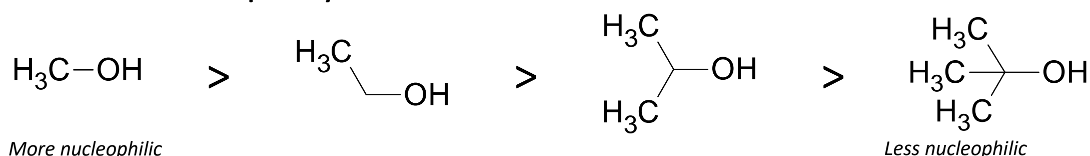



*Figure 9-13- Examples of conditions which can influence nucleophilicity.*

Acetate has a single site of potential ionisation and is moderately nucleophilic with a pKa of 4.76. At a pH of 7, the acetate ion is dominant, however at a pH of 5, the deprotonated acetic acid ion only makes up 68.9% (**Figure 8-14**). Amoxicillin decayed at a similar rate within each of the acetate buffers with half-lives ranging from 289.96-463.01 hours at a pH of 7 and 354.38-415.20 for a pH of 5. Theoretically, this result should be reversed with accelerated decay with the increased presence of the deprotonated ion. However, Vahdat (2000) has reported that the stability of amoxicillin decay

326 was U-shaped in which lower pH values also resulted in amoxicillin decay, though stability was at
327 its maximum between a pH of 5 and 7 and produced roughly similar decay rates within an acetate
328 buffer.

329 Phosphate and carbonate ions are known to be strong nucleophiles, and each contain multiple active
330 sites. Phosphate has four ionic states, though at a pH of 7.0 the ionic states $PO_4H^{2-}$ and $PO_4H_2^-$ are
331 dominant and roughly equal in concentration (**Figure 8-14**). $PO_4H^{2-}$ is only mildly nucleophilic with
332 a pKa of 2.2. However, $PO_4H_2^-$ is more nucleophilic with a pKa of 7.2. The highest charged state of
333 $PO_4^{3-}$ has a pKa of 12.4 and is highly nucleophilic, though is absent at a pH of 7.0.

334 Despite the strong nucleophilicity of the phosphate ions, the bulkiness of the tetrahedral shape of the
335 molecule sterically hinders intermolecular interactions and therefore inhibits the reason and therefore
336 the nucleophilicity. Amoxicillin was observed to decay rapidly relative to water with a maximum
337 half-life of 124.64 hours or 5.2 days. The rate of decay of amoxicillin differed between the pH 7.0
338 and pH 9.0 carbonate buffers. When in a pH of 7.0 carbonate solution amoxicillin decays at a similar
339 rate to phosphate, with a half-life of 134.42 hours or 5.6 days. However, when the pH is increased to
340 9 the relative decay rate increased resulting in a maximum half-life of 34.38 hours or 1.4 days.
341 There are three forms of the carbonate ion: $CO_3H_2$, $CO_3H^-$, and $CO_3^{2-}$ with a pKa of 6.35 and 10.3
342 for the first and second transition respectively. At a pH of 7.0 the fully protonated carbonate $CO_3H_2$,
343 the weaker nucleophile, is dominant and makes up 91% of the solution. However, at a pH of 9.0, a
344 deprotonated bicarbonate $CO_3H^-$, a stronger nucleophile, is dominant at 84.8% of the solution. The
345 increased reactivity of the ions presents at pH 9.0 explains the differences in the decay of the two pH
346 conditions for carbonate buffer.

347 The most rapid amoxicillin decay observed was amoxicillin in the l-cysteine solution which decayed
348 so rapidly that an accurate measurement was not able to be taken in the initial study as most of the
349 measurements taken were below the limit of detection. Cysteine is a complex amino acid which
350 contains both a carboxylic acid, an amine, and a thiol, each of which are strong nucleophiles. When
351 cysteine is dissolved it does not exist as a neutral species as the thiol, amine, and carboxyl groups
352 can each be ionised (**Figure 8-14**). At a pH of 7.0, the solution was dominated by the

353 $NH_3^+CHSHCOO^-$ ion which comprised 94.8% of the ions. The deprotonated hydroxyl group has a
354 pka of 1.7. deprotonating the thiol has a pKa of 8.39, and deprotonating the amine has a pKa of 10.77.
355 As such each are strong nucleophiles and would readily attack the carboxyl group on the beta lactam.
356 Additionally, hydrogen bonding between the lone pairs of electrons on the carbonyl and a protonated
357 amine group would position the two molecules in such a way to increase the reactivity of the reaction.
358 This would explain the extremely rapid rates of decay observed for the decay of amoxicillin in the
359 presence of l-cysteine.

360 Amoxicillin in NaCl solution was observed to decay with a maximum half-life of 325 hours. This is
361 faster than the decay rate of amoxicillin in water or acetate buffer conditions. While neither chloride
362 or sodium ions participate in nucleophilic substitutions, chlorine ions have been shown to catalyse
363 nucleophilic substitution reactions within seawater (Elliott and Sherwood, 1993; Hazra et al., 2019).
364 For example, Hazra et al (2019) found that the Cl- ions in sea water were effective catalysis for the
365 oxidation of alcohol to carboxylic acids or ketones and aromatic amines to imines in water. This was
366 done through a combination of radial and ionic mechanisms. It is possible that similar mechanism is
367 operating within this study to decay the alcohols and amines present within amoxicillin.

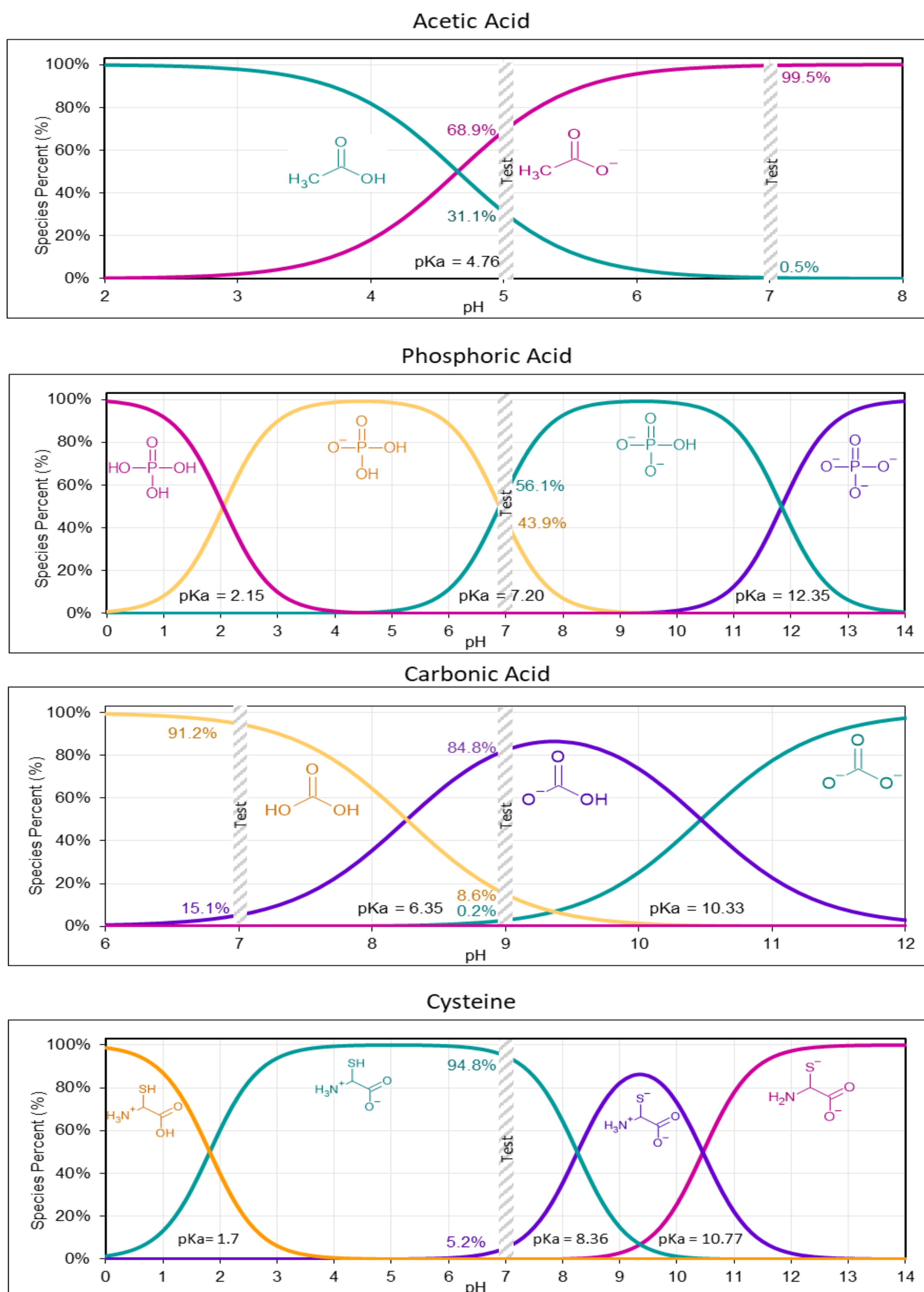



***Figure 9-14** - Species distribution plots for the different components used within the study. Each of the test conditions are indicated in hashed grey bar with the percentage of each species present at that pH indicated with the associated colour. All charts and calculations were made using the CurTiPot–pH and acid–base titration curves: analysis and simulation freeware, version 4.2 (Gutz, 2014).*

369 ### 9.4.2 The decay of amoxicillin in anaerobic medium components at -20°C

370 Previous research has established that the catalytic influence of the buffer medium behaves
371 differently when frozen than in a liquid state (Vahdat, 2000; Vahdat and Sunderland, 2009). The
372 Arrhenius equation calculates the change in decay rate constant *k* with a change in temperature.
373 Research by Vahdat (2007) which assessed changes in the decay rate of amoxicillin in acetate and
374 phosphate buffers indicated that temperatures below freezing can diverge from the rate predicted by
375 the Arrhenius equation. A similar effect was observed in this study as the rate of decay for the
376 phosphate and carbonate buffers was disproportionately shorter when frozen than for amoxicillin in
377 the acetate or MilliQ water. Several mechanisms were proposed for this variation. As the phase of
378 liquid changes during freezing, dissolved solids concentrate in the liquid fraction until a point of
379 supersaturation before complete freezing. This would create solute concentration higher than those
380 within the original solution. As the decay rate of the amoxicillin is correlated to the concentration of
381 the other solutes within solution, an increase of concentration would increase the rate of amoxicillin
382 decay as well. It is also possible that differences in the eutectic temperature of a species, the lowest
383 temperature that a solution can reach before freezing, could selectively crystalise or precipitate
384 different buffer constituents under frozen conditions (Vahdat and Sunderland, 2007). This could lead
385 to changes in the pH or concentration also changing the effective rate of decay as different parts of
386 the solution selectively freeze out of the solution causing the remaining components to change their
387 relative concentrations.

388 ### 9.4.3 Amoxicillin decay in the presence of l-cysteine

389 In the initial set of analyses (**Section 8.3.1**) amoxicillin decayed to below the LOQ before the first
390 sampling time point (6h) and therefore accurate quantification was not possible. As the amoxicillin
391 and l-cysteine could potentially interact with each other in a second order reaction, reducing the
392 concentration of l-cysteine in the solutions was hypothesised to also reduce the rate of decay for
393 amoxicillin. This study aimed to determine the maximum ratio at which an accurate measurement
394 for the decay of amoxicillin can be calculated using the LC-UV method of quantification of
395 amoxicillin. L-cysteine is generally used in anaerobic media as an oxygen scavenger however, the
396 solutions used in this experiment were not anaerobic. The effect that amoxicillin would have on the

397 functional properties of cysteine were not studied. However, if the interaction between amoxicillin
398 and cysteine changed the structure of l-cysteine it is possible that higher concentrations of amoxicillin
399 could inhibit the ability for l-cysteine to capture oxygen during culturing, in turn preferentially
400 damaging the methanogenic archaea within the culture. Furthermore, the shape of the decay curves
401 were not linear when the natural log of amoxicillin concentration was plotted against time suggesting
402 that the decay relationship was not a pseudo first order reaction. This is likely due to the fact that,
403 unlike in previous sections, the concentrations of l-cysteine solutions were not in excess of
404 amoxicillin. It is possible that the amoxicillin and l-cysteine interact with eachother during decay in
405 a second order reaction.

## 406 9.5 CONCLUSIONS

407 This study demonstrated that the decay of amoxicillin is driven more by the chemistry of the other
408 solutes than by pH alone. This study focused on compounds used in anaerobic culture media. While
409 some of the compounds tested included buffers (acetate, phosphate, and carbonate) for controlling
410 the pH during the experiment, the rest were non-buffering components within media. While there are
411 many studies which looked at the change in decay rates with a change in pH, the specifics of these
412 experiments varied in many parameters such as temperature, concentration, and buffer type. As a
413 result, it is difficult to directly compare results from this research. For example, similarly to the work
414 in this study, Chadha, Kashid and Jain (2003) and Vahdat and Sunderland (2007) also measured the
415 decay of amoxicillin in a 7.0 phosphate buffer at 35-37°C. However, each study used different
416 relative concentrations of buffer to amoxicillin. As a result, the calculated decay constant $k$ is
417 different for each study and can cannot be directly compared. Kakimoto and Funamizu (2007) found
418 that ionic strength, measured in eC conductivity, was one of the most important factors responsible
419 for the degradation of amoxicillin, however no correlation between conductivity and rate of
420 amoxicillin decay was found in this study. This is likely due to the fact that in the research conducted
421 by Kakimoto and Funamizu (2007) the changes in conductivity were correlated to differences in
422 concentration, which are known to correlate to changes in rate, while this study measured differences
423 conductivity between different compounds.

This study focused primarily on the catalytic influence of the compounds found within anaerobic culture medium on the breakdown of amoxicillin and has demonstrated that non-buffering materials can still have a catalytic effect. The compounds used in this study were not exhaustive and other media types may also result in the rapid decay of amoxicillin. For example, amino acids, particularly those containing sulphur, such as cystine, cysteine, and methionine, have the potential to aggressively degrade the beta lactam ring. Vitamin B12 has been long known to be a strong nucleophile (Bailey, 1968). While it is produced by some bacteria and archaea, it is important for the synthesis of DNA and therefore is often added to growth media. Metal ions break beta lactam rings. Though this wasn't studied in this study, this could further impact the decay (Navarro *et al.*, 2003; Alekseev *et al.*, 2006). Essential trace metals such as zinc and iron are often added to growth media (Shelton and Tiedje, 1984), which are not nucleophiles themselves, but are known to be catalysts for nucleophilic substitution reactions (Murakami, Yorimitsu and Oshima, 2009; Li *et al.*, 2018).

A review of emerging contaminants in municipal wastewater treatment plants reported that amoxicillin and other beta lactams are rarely detected in the influent of water treatment plants (Tran, Reinhard and Gin, 2018). The review believes that this is the result of rapid enzymatic decay by the resident microbes within those systems. However, the work in this study suggests that chemical decay may also be a significant part of that process. It also suggests that chemical pre-treatment of hospital wastewater before its traditional treatment methods could be useful for the reduction in exposure to pharmaceutical waste which is high in antibiotics and the proliferation of antibiotic resistance. For example, the Fenton's reagent is a mixture of ferrous iron (usually in the form of $FeSO_4$) and hydrogen peroxide ($H_2O_2$) and can catalyse the oxidation of organic contaminants in wastewater. It has been proposed as a means of decontaminating the pharmaceutical residues and resistant microbes within hospital waste (Berto *et al.*, 2009) and anaerobic wastewater containing amoxicillin (Su *et al.*, 2019).

This study shows that the stability of amoxicillin is largely dependant on its surrounding matrix, which implies that its toxicity does as well. Studies which aim to assess amoxicillin activity through culturing should keep this chemical decay in mind. For example, due to the interaction of amoxicillin

451 and the oxygen scavenger l-cysteine, it is possible that high amoxicillin concentrations could
452 deactivate the l-cysteine in turn reducing its effectiveness in removing oxygen in media prepared for
453 obligate anaerobes. In this context it is not the amoxicillin directly inhibiting growth, but rather an
454 interaction between the amoxicillin and the growth media. Therefore, the decay of amoxicillin should
455 be assessed directly as part of the experiment or should be commented on as part of the interpretation
456 of the results if direct measurements are not possible.

457 ***Table 9-6*** *- Comparison of results between this study and similar studies below freezing.*
458 *All values from this study report the decay reported for the 10 mg/L conditions.*
459

| Solute | Buffer Concentration | pH | Temperature (C°) | K | t 1/2 (d) | Source |
|---|---|---|---|---|---|---|
| **Acetate** | 2.2 M | 4.6 | -9.3 | 0.0263 | 26.38 | Vahdat et al 2007 |
| **Acetate** | 2.2 M | 4.6 | -7.3 | 0.0224 | 30.99 | Vahdat et al 2007 |
| **Acetate** | **40 mM** | **5.0** | **-20** | **0.0019** | **8788.704** | **This study** |
| **Carbonate** | **40 mM** | **9.0** | **-20** | **0.0208** | **603.864** | **This study** |
| **Phosphate** | **40 mM** | **7.0** | **-20** | **0.0275** | **800.064** | **This study** |
| **Phosphate** | 1 M | 7.0 | -13.5 | 0.0073 | 94.44 | Vahdat et al 2007 |
| **Phosphate** | 1 M | 7.0 | -9.3 | 0.0298 | 23.27 | Vahdat et al 2007 |
| **Phosphate** | 1 M | 7.0 | -7.3 | 0.0342 | 20.28 | Vahdat et al 2007 |
| **Water** | **N/A** | **7.0** | **-20** | **0.0016** | **10135.37** | **This study** |

460

461 ***Table 9-7*** *- Comparison of results between this study and similar studies with temperature ranges from 35 – 43°C.*
462 *All values from this study report the decay reported for the 10 mg/L conditions.*
463

| Solute | Buffer Concentration | pH | Temperature (C°) | K | t 1/2 (h) | Source |
|---|---|---|---|---|---|---|
| Acetate | 2.2 M | 4.6 | 35 | 0.0081 | 86.00 | Vadhat et al 2009 |
| Acetate | 2.2 M | 4.6 | 42 | 0.0146 | 47.48 | Vadhat et al 2009 |
| **Acetate** | **40 mM** | **5.0** | **35** | **0.0018** | **380.90** | **This study** |
| **Acetate** | **40 mM** | **7.0** | **35** | **0.0018** | **406.56** | **This study** |
| **Carbonate** | **40 mM** | **7.0** | **35** | **0.0057** | **122.59** | **This study** |
| **Carbonate** | **40 mM** | **9.0** | **35** | **0.0237** | **29.92** | **This study** |
| **Glucose** | **1 M** | **7.0** | **35** | **0.0012** | **563.31** | **This study** |
| **NaCl** | **40 mM** | **7.0** | **35** | **0.0025** | **285.17** | **This study** |
| Phosphate | N/A | 5.0 | 37 | 0.0038 | 183.58 | Chadha et al 2003 |
| Phosphate | 1 M | 7.0 | 35 | 0.0154 | 45.01 | Vadhat et al 2009 |
| **Phosphate** | **40 mM** | **7.0** | **35** | **0.0071** | **103.56** | **This study** |
| Phosphate | N/A | 7.0 | 37 | 0.0043 | 162.10 | Chadha et al 2003 |
| Phosphate | N/A | 7.0 | 43 | 0.0085 | 81.62 | Chadha et al 2003 |
| Phosphate | N/A | 9.0 | 37 | 0.0387 | 17.91 | Chadha et al 2003 |
| Phosphate | N/A | 9.0 | 43 | 0.0665 | 10.42 | Chadha et al 2003 |
| **Water** | **N/A** | **7.0** | **35** | **0.0015** | **464.13** | **This study** |

464

# Chapter 9
# Conclusions and Future Work

## 10.1 CONCLUSIONS

The study of antibiotics within wastewater treatment has been widely reported in the context of large wastewater treatment systems. However, much of the world is reliant on decentralised water treatment technologies which primarily rely on anaerobic communities for biological water treatment. Despite being one of the most prescribed antibiotics globally, there is very little research into the effects that amoxicillin has on anaerobic systems. In this research project I explored the effects that an acute exposure to amoxicillin would have on an anaerobic community in terms of methanogenic activity, treatment efficiency, change in the microbial ecology, and the development of antimicrobial resistance genes. Furthermore, I explored the stability of amoxicillin structure within anaerobic culture medium with the aim of measuring the rate of abiotic decay. The primary findings of this dissertation were:

**Does amoxicillin inhibit methane production and water treatment?**

Chapter 6 established that that for all feed types tested the presence of 0.5 – 100mg/L amoxicillin resulted in a measurable change in the methanogenic activity, though the sensitivity of the microbes depended on the type of substrate used. There was a strong relationship between the addition of amoxicillin and a decrease in the rate of methane production for the cultures glucose and complex broth (a mixture of proteins, lipids, and sugars). In both cases, the presence of 5ppm of amoxicillin and above resulted in a measurable decrease in the rate of methane production. Additionally, an increase in the concentration of amoxicillin was associated with an increase in the lag methane production for both substrates. This suggests that the pathways responsible for degrading sugars and

25 proteins were most sensitive to the presence of amoxicillin. However, a second experiment which
26 exposed anaerobic granules to 1, 10, and 50 mg/L of amoxicillin with glucose as the feed, did not
27 find any statistical differences in the rate of methane production for the different amoxicillin
28 conditions. The differences between these runs were likely due to changes in the community structure
29 resulting from different preparation conditions. Based on sCOD measurements the presence of
30 amoxicillin did not have a strong effect on the ability for culture to treat waste.

31 **Does amoxicillin change the microbial community structure of anaerobic systems?**

32 This study showed that changes in the anaerobic microbial community structure can be detected
33 within hours after amoxicillin was added to the culture. A decrease in relative community robustness
34 between the samples was observed within 24 hours of exposure to amoxicillin for all tested
35 conditions indicating communities exposed to amoxicillin can be sensitive to further stresses.
36 Changes to the overall community structure resulting from the presence of amoxicillin was
37 observable form the first point at 6 hours from inoculation. In the absence of amoxicillin, the
38 community composition and structure shifted with the presence of glucose substrate, which was not
39 seen to the same extent when amoxicillin was present. This suggests, that even if methanogenic
40 activity occurs at the same rate with and without amoxicillin, small concentrations (1mg/L and less)
41 can have an effect on the community make up.

42 **Does the presence of amoxicillin increase the abundance of antimicrobial resistance genes?**

43 Changes in the resistome were detectable within the first 48 hours. A two-to-three-fold increase in
44 the relative net presence of AMR resistance genes within effluent was detected even when
45 amoxicillin in the influxes was as low as 1 mg/L. Despite net increases in the relative AMR gene
46 counts for T5 (30h) and T6 (36h), the total AMR counts appeared to decrease for T7 (48h). This
47 suggests that while AMR genes might be produced in response to the presence of amoxicillin, once
48 the antibiotic is no longer active (through decay or non-reversable PBP bonding) these genes are no
49 longer selected for.

50 **How fast does amoxicillin degrade within a lab setting?**

51 This study demonstrated that the decay of amoxicillin is driven more by the chemistry of the other
52 solutes than by pH alone. The stability of amoxicillin is largely dependent on its surrounding matrix,
53 which implies that its toxicity does as well. Studies which aim to assess amoxicillin activity through
54 culturing should keep this chemical decay in mind. For example, due to the interaction of amoxicillin
55 and the oxygen scavenger l-cysteine, it is possible that high amoxicillin concentrations could
56 deactivate the l-cysteine in turn reducing its effectiveness in removing oxygen in media prepared for
57 obligate anaerobes.

## 58 10.2 FUTURE WORK

59 This work expanded our understanding of the effects of beta-lactam antibiotics in anaerobic systems.
60 However, there are still many unanswered questions. This section explores some of the questions
61 posed by this research.

62 **1. What metabolic pathways are most inhibited by the presence of amoxicillin?**

63 This work demonstrated that the presence of amoxicillin affected both the rate and volume of
64 methane produced as well as changed the community structure of anaerobic granules. Both are
65 evidence that there is disruption to the metabolic pathways within the community. However, the
66 details of which pathways were disrupted and where this disruption took place was outwith the scope
67 of this work. There are several potential methods for further studying metabolic pathways identified
68 here. One way to fill in the gaps of this work would be to incorporate additional modalities such as
69 metabolomics (targeted / untargeted) in an integrative framework. This is routinely done in human
70 microbiome studies where short chain fatty acids (SCFAs) are typically also analysed. Additionally,
71 rather than taking short read amplicon-based approaches, shotgun metagenomics could be employed
72 to recover actual function of these microbial communities. Finally, while this work focused
73 exclusively on changes to the 16s rRNA genes. This method is not able to distinguish between the
74 active and inactive or dead parts of the community. Focusing on the RNA sequencing would only
75 measure parts of the community which hare active and therefore could measure changes in activity
76 in response to the presence of amoxicillin. This is particularly important when studying anaerobic
77 communities. Recent work by De Vrieze at all (2018) found that while there was some overlap

78 between the DNA and RNA community detected at OTU level for bacteria within the community, a
79 greater difference for the archaea present in that community (De Vrieze et al., 2018). This suggest
80 that when measuring changes to anaerobic communities, DNA alone is unlikely to capture all the
81 changes in activity present. This paired with AMR data and over a longer time scale could be highly
82 elucidating for our understudying of the impact of non-lethal antibiotics within anaerobic
83 communities.

84 **2. Will a community exposed to amoxicillin "recover" from the exposure and converge**
85 **with the community distribution of the unexposed community?**

86 The work in Chapter 7 suggested that the presence of amoxicillin inhibited community development
87 such that the community makeup of the test conditions was more closely related to the control than
88 to those fed with glucose in the absence of amoxicillin. Given the short duration of the experiment,
89 it was not determined if the communities would eventually recover to be closer in composition and
90 robustness to the unexposed conditions once the pressure of amoxicillin exposure ceases, or if these
91 changes would remain in the community make up even after the amoxicillin was no longer acting
92 within the community. This could be explored through repeating the experiment with a longer
93 window of observation. This could be done either through repeat batch feeding or through use of a
94 bioreactor. The results of this work could inform knowledge of long-term impacts of acute exposure
95 events.

96 **3. How long are the presence of AMR genes within the effluent detectable after a shock**
97 **exposure to amoxicillin?**

98 Results in Chapter 7 found that AMR genes were detectable in the effluent of anaerobic systems.
99 However, the results suggested that the presence of AMR genes was only temporary as the
100 concentrations decreased in the final samples of the experiment. Running a similar test, in which
101 anaerobic granules are exposed to amoxicillin, and then monitored for a longer period of time,
102 possibly with multiple rounds of feeding would be able to determine if the decrease in AMR gene
103 concentrations continues. This would determine of the effluent from anaerobic systems could act as
104 point sources for the spread of AMR genes into the environment.

**4. Do other oxygen scavengers degrade amoxicillin at the same rate of l-cysteine? Would other formulas for anaerobic medium change the observed inhibitory effects of amoxicillin on anaerobic cultures?**

The results from Chapter 8 found that the presence of l-cysteine has a strong catalytic effect on the degradation of amoxicillin. Cysteine was used in this anaerobic culture as an oxygen scavenger. Other oxygen scavengers such as sodium sulphite and ascorbic acid are also used in anaerobic culturing. Given that these both have differing molecular structures than cysteine, it is possible that they would interact with amoxicillin in a differently. If so, could changing the composition of the anaerobic medium change the measured toxicity of the amoxicillin? To test this, another decay test like the ones conducted in Chapter 8 could determine if these oxygen scavengers have the same decay rate. If they do, running parallel studies in which, an array of amoxicillin concentrations and medium comprising the different oxygen scavengers could determine how much the medium itself influenced the measured methanogenic inhibition.

1

2 # Chapter 10

3 # References

4

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617

# Appendix A – Gene Targets Used in Screening Chip

| | Gene | Target antibiotics (major) | Forward Primer | Reverse Primer |
|---|---|---|---|---|
| 1 | 16S rRNA | 16S rRNA | GGGTTGCGCTCGTTGC | ATGGYTGTCGTCAGCTCGTG |
| 2 | 16S rRNA2 | 16S rRNA | CCTACGGGAGGCAGCAG | ATTACCGCGGCTGCTGGC |
| 3 | cfiA | Beta Lactam | GCAGCGTTGCTGGACACA | GTTCGGGATAAACGTGGTGACT |
| 4 | blaMOX/blaCMY | Beta Lactam | CTATGTCAATGTGCCGAAGCA | GGCTTGTCCTCTTTCGAATAGC |
| 5 | blaOCH | Beta Lactam | GGCGACTTGCGCCGTAT | TTTTCTGCTCGGCCATGAG |
| 6 | blaPAO | Beta Lactam | CGCCGTACAACCGGTGAT | GAAGTAATGCGGTTCTCCTTTCA |
| 7 | blaVEB | Beta Lactam | CCCGATGCAAAGCGTTATG | GAAAGATTCCCTTTATCTATCTCAGACAA |
| 8 | blaROB | Beta Lactam | GCAAAGGCATGACGATTGC | CGCGCTGTTGTCGCTAAA |
| 9 | blaOXY | Beta Lactam | CGTTCAGGCGGCAGGTT | GCCGCGATATAAGATTTGAGAATT |
| 10 | blaPSE | Beta Lactam | TTGTGACCTATTCCCCTGTAATAGAA | TGCGAAGCACGCATCATC |
| 11 | cphA_1 | Beta Lactam | GCGAGCTGCACAAGCTGAT | CGGCCCAGTCGCTCTTC |
| 12 | bla-L1 | Beta Lactam | CACCGGGTTACCAGCTGAAG | GCGAAGCTGCGCTTGTAGTC |
| 13 | cfxA | Beta Lactam | TCATTCCTCGTTCAAGTTTTCAGA | TGCAGCACCAAGAGGAGATGT |
| 14 | cepA | Beta Lactam | AGTTGCGCAGAACAGTCCTCTT | TCGTATCTTGCCCGTCGATAAT |
| 15 | ampC/blaDHA | Beta Lactam | TGGCCGCAGCAGAAAGA | CCGTTTTATGCACCCAGGAA |
| 16 | blaGES | Beta Lactam | GCAATGTGCTCAACGTTCAAG | GTGCCTGAGTCAATTCTTTCAAAG |
| 17 | blaSFO | Beta Lactam | CCGCCGCCATCCAGTA | GGGCCGCCAAGATGCT |
| 18 | blaTLA | Beta Lactam | ACACTTTGCCATTGCTGTTTATGT | TGCAAATTTCGGCAATAATCTTT |
| 19 | blaZ | Beta Lactam | GGAGATAAAGTAACAAATCCAGTTAGATATGA | TGCTTAATTTTCCATTTGCGATAAG |
| 20 | blaVIM | Beta Lactam | GCACTTCTCGCGGAGATTG | CGACGGTGATGCGTACGTT |
| 21 | pbp5 | Beta Lactam | GGCGAACTTCTAATTAATCCTATCCA | CGCCGATGACATTCTTCTTATCTT |
| 22 | pbp | Beta Lactam | CCGGTGCCATTGGTTTAGA | AAAATAGCCGCCCCAAGATT |
| 23 | mecA | Beta Lactam | GGTTACGGACAAGGTGAAATACTGAT | TGTCTTTTAATAAGTGAGGTGCGTTAATA |
| 24 | blaCTX-M_5 | Beta Lactam | GCGATAACGTGGCGATGAAT | GTCGAGACGGAACGTTTCGT |
| 25 | penA | Beta Lactam | AGACGGTAACGTATAACTTTTTGAAAGA | GCGTGTAGCCGGCAATG |
| 26 | blaCTX-M_8 | Beta Lactam | CGTCACGCTGTTGTTAGGAA | CGCTCATCAGCACGATAAAG |
| 27 | blaNDM | Beta Lactam | GGCCACACCAGTGACAATATCA | CAGGCAGCCACCAAAAGC |
| 28 | blaACC | Beta Lactam | CACACAGCTGATGGCTTATCTAAAA | AATAAACGCGATGGGTTCCA |
| 29 | bla1 | Beta Lactam | GCAAGTTGAAGCGAAAGAAAAGA | TACCAGTATCAATCGCATATACACCTAA |
| 30 | blaCMY_2 | Beta Lactam | AAAGCCTCAT GGGTGCATAAA | ATAGCTTTTGTTTGCCAGCATCA |
| 31 | blaCMY_3 | Beta Lactam | CTGGCGCATACCTGGATTAC | GCCAGTTCAGCATCTCCCA |
| 32 | ampC_cefa | Beta Lactam | CAGGATCTGATGTGGGAGAACTA | TCGGGAACCATTTGTTGGC |
| 33 | blaSME | Beta Lactam | GAGGAAGACTTTGATGGGAGGATTG | CGCTATATTGCAATGCAGCAGAAG |

| 34 | blaCTX-M | Beta Lactam | CGTACCGAGCCGACGTTAA | CAACCCAGGAAGCAGGCA |
|---|---|---|---|---|
| 35 | blaFOX | Beta Lactam | CCTACGGCTATTCGAAGGAAGATAAG | CCGGATTGGCCTGGAAGC |
| 36 | blaIMIR | Beta Lactam | AGCCGGACTAGAGCTTCATG | GGCAGAACTCATCATCTGCAAA |
| 37 | blaOXA51 | Beta Lactam | CGACCGAGTATGTACCTGCTTC | TCAAGTCCAATACGACGAGCTA |
| 38 | blaOXY1 | Beta Lactam | AAAGGTGACCGCATTCGC | CCAGCGTCAGCTTGCG |
| 39 | blaPER | Beta Lactam | GCAAATGAAGCGCAGATGC | GACCACAGTACCAGCTGGTA |
| 40 | blaSHV11 | Beta Lactam | TTGACCGCTGGGAAACGG | TCCGGTCTTATCGGCGATAAAC |
| 41 | blaTEM | Beta Lactam | CGCCGCATACACTATTCTCAG | GCTTCATTCAGCTCCGGTTC |
| 42 | blaKPC | Beta Lactam | GCCGCCAATTTGTTGCTGAA | GCCGGTCGTGTTTCCCTTT |
| 43 | beta_ccra | Beta Lactam | CACTGGCACGGCGATTGTA | CGGCAGCCAAACCACGATA |
| 44 | bl1acc | Beta Lactam | TGTTATCCGTGATTACCTGTCTGG | CTCAGCGAGCCAACTTCAAATA |
| 45 | beta_B2 | Beta Lactam | GTAACGCCTACTGGAAGTCCA | CAGCTTCTCCTTGAGAATGCAG |
| 46 | blaACT | Beta Lactam | AAGCCGCTCAAGCTGGA | GCCATATCCTGCACGTTGG |
| 47 | blaB | Beta Lactam | CGTGCCGGAGGTCTTGAATA | GGGATAGTAAACCTGAAACTCGGA |
| 48 | blaCARB | Beta Lactam | TGATTTGAGGGATACGACAACTCC | CTGTAATACTCCGAGCACCAA |
| 49 | blaGOB | Beta Lactam | CTTGGGCTTGAATGCTCAGGTA | TGTATGGTCGTAGTGAGCCTGA |
| 50 | blaHERA | Beta Lactam | GGGCAACCGCATTCTGAC | GCATCTCCCACTTTATCGTCAC |
| 51 | blaIMI | Beta Lactam | ACATCTACACCTGCAGCAGTAG | AATCGCTTGGTACGCTAGCA |
| 52 | blaIND | Beta Lactam | CGCCTGTTAAACCCAACCTGTA | CGCTCTGTCATCATGAGAGTGG |
| 53 | blaLEN | Beta Lactam | TGTTCGCCTGTGTGTTATCTCC | GCAGCACTTTAAAGGTGCTCAC |
| 54 | blaMIR | Beta Lactam | CGGTCTGCCGTTACAGGTG | AAAGACCCGCGTCGTCATG |
| 55 | blaBEL-nonmobile | Beta Lactam | ATGTCCATGGCACAGACTGTG | CCTGTCTTGTCACCCGTTACC |
| 56 | blaADC-nonmobile | Beta Lactam | GGTATGGCTGTGGGTGTTATTCA | AGGCAAGGTTACCACTTGTATACG |
| 57 | ampC_1 | Beta Lactam | AACAAAAGATCCCCGGTATGG | ACGCCCGTAAATGTTTTGCT |
| 58 | blaCMY_1 | Beta Lactam | CCGCGGCGAAATTAAGC | GCCACTGTTTGCCTGTCAGTT |
| 59 | ampC_2 | Beta Lactam | TCCGGTGACGCGACAGA | CAGCACGCCGGTGAAAGT |
| 60 | blaSHV_1 | Beta Lactam | TCCCATGATGAGCACCTTTAAA | TTCGTCACCGGCATCCA |
| 61 | blaOKP | Beta Lactam | GCCGCCATCACCATGAG | GGTGACGTTGTCACCGATCTG |
| 62 | blaOXA10_1 | Beta Lactam | CGCAATTATCGGCCTAGAAACT | TTGGCTTTCCGTCCCATTT |
| 63 | cphA_2 | Beta Lactam | GTGCTGATGGCGAGTTTCTG | GGTGTGGTAGTTGGTGTTGATCAC |
| 64 | blaCMY2 | Beta Lactam | GCGAGCAGCCTGAAGCA | CGGATGGGCTTGTCCTCTT |
| 65 | fox5 | Beta Lactam | GGTTTGCCGCTGCAGTTC | GCGGCCAGGTGACCAA |
| 66 | ampC_3 | Beta Lactam | CCGCCCAGAGCAAGGACTA | GCTCGACTTCACGCCGTAAG |
| 67 | blaOXA1/blaOXA30 | Beta Lactam | CGGATGGTTTGAAGGGTTTATTAT | TCTTGGCTTTTATGCTTGATGTTAA |
| 68 | blaCTX-M_1 | Beta Lactam | GGAGGCGTGACGGCTTTT | TTCAGTGCGATCCAGACGAA |
| 69 | blaCTX-M_2 | Beta Lactam | GCCGCGGTGCTGAAGA | ATCGGATTATAGTTAACCAGGTCAGATTT |
| 70 | blaCTX-M_3 | Beta Lactam | CGATACCACCACGCCGTTA | GCATTGCCCAACGTCAGATT |
| 71 | blaCTX-M_4 | Beta Lactam | CTTGGCGTTGCGCTGAT | CGTTCATCGGCACGGTAGA |

| 72 | blaIMP_1 | Beta Lactam | AACACGGTTTGGTGGTTCTTGTA | GCGCTCCACAAACCAATTG |
|---|---|---|---|---|
| 73 | blaSHV_2 | Beta Lactam | CTTTCCCATGATGAGCACCTTT | TCCTGCTGGCGATAGTGGAT |
| 74 | blaTEM_1 | Beta Lactam | AGCATCTTACGGATGGCATGA | TCCTCCGATCGTTGTCAGAAGT |
| 75 | blaCTX-M_6 | Beta Lactam | CACAGTTGGTGACGTGGCTTAA | CTCCGCTGCCGGTTTTATC |
| 76 | pbp2x | Beta Lactam | TTTCATAAGTATCTGGACATGGAAGAA | CCAAAGGAAACTTGCTTGAGATTAG |
| 77 | blaPER_1 | Beta Lactam | TGCTGGTTGCTGTTTTTGTGA | CCTGCGCAATGATAGCTTCAT |
| 78 | blaIMP_2 | Beta Lactam | AAGGCAGCATTTCCTCTCATTTT | GGATAGATCGAGAATTAAGCCACTCT |
| 79 | ampC_4 | Beta Lactam | GCAGCGAAGCGTCAGTCA | AGATCCGTGGCCGCATAA |
| 80 | ampC_5 | Beta Lactam | CAGCCGCTGATGAAAAAATATG | CAGCGAGCCCACTTCGA |
| 81 | blaOXA58 | Beta Lactam | GCAATTGCCTTTTAAACCTGA | CTGCCTTTTCAACAAAACCC |
| 82 | blaKPC_3 | Beta Lactam | CAGCTCATTCAAGGGCTTTC | GGCGGCGTTATCACTGTATT |
| 83 | blaSHV_3 | Beta Lactam | GCGTTATTTTCGCCTGTGTA | AGGTGCTCATCATGGGAAAG |
| 84 | blaCTX-M_7 | Beta Lactam | CGATGTGCAGTACCAGTAA | GCAATGGGATTGTAGTTAA |
| 85 | blaIMP_3 | Beta Lactam | GGAATAGAGTGGCTTAATTC | GGTTTAACAAAACAACCACC |
| 86 | pbp2b | Beta Lactam | AGACGGTAACGTATAACTTTTTGAAAGA | GCGTGTAGCCGGCAATG |
| 87 | blaKPC_2 | Beta Lactam | GCCGCCGTGCAATACAGT | GCCGCCCAACTCCTTCA |
| 88 | bla-SME | Beta Lactam | AACGGCTTCATTTTTGTTTAG | GCTTCCGCAATAGTTTTATCA |
| 89 | ampC_6 | Beta Lactam | GCAGCACGCCCCGTAA | TGTACCCATGATGCGCGTACT |
| 90 | blaACT_1 | Beta Lactam | CTGTTCGAGCTGGGTTCTATAAGTAAA | CAGTATCTGGTCACCGGATCGT |
| 91 | blaACT_2 | Beta Lactam | CCGCTCAAGCTGGACCATAC | CCATATCCTGCACGTTGGTTT |
| 92 | imiR | Beta Lactam | CCGGACTAGAGCTTCATGTAAGC | CCCACGCGGTACTCTTGTAAA |
| 93 | blaOXA48 | Beta Lactam | TGTTTTTGGTGGCATCGAT | GTAAMRATGCTTGGTTCGC |
| 94 | blaSFC | Beta Lactam | GGCTTACTGAACCAGCGAATTC | GGTCCAGACGAAATACATTGTCAC |
| 95 | blaGIM | Beta Lactam | GACGACGAATTCACACTGGGAA | GAACTTCCAACTTTGCCATGCC |
| 96 | blaKHM | Beta Lactam | GACCTACGCATCGACCCA | GCCGAGATTGCCCAAGC |
| 97 | blaSIM | Beta Lactam | TCCAGGCCCAGGACACA | GAGTTTCAATAGTGATGCGTCTCC |
| 98 | blaSPM-45 | Beta Lactam | ATGAAGCCGAAGAAAGTAGTAGCC | CAGGATGGGAACTCAGAATCCTTC |
| 99 | blaTMB | Beta Lactam | AAGTCATACAGCCGGGTGGA | GCTTCCCGCCAGCTCATAC |
| 100 | blaSFH | Beta Lactam | GGTATCTTTGTGTACTTCCCAGCA | GCAATGATCGAGTCGACTTTAAGC |
| 101 | blaAIM | Beta Lactam | GAGATCGCCACATGAAACGTC | GCGGATGTTGGCCAGGA |
| 102 | blaOXA23 | Beta Lactam | GCCGCGCAAATACAGAATATG | GCTTCATGGCTTCTCCTAGTG |
| 103 | blaOXA24 | Beta Lactam | GGCATTGTCAGCAGTTCCAGTATA | AGGTAATCGGTTATGTGCAAGGTC |
| 104 | blaOXA48_2 | Beta Lactam | AATAGCTTGATCGCCCTCGA | TTTGGCGGGCAAATTCTTGA |
| 105 | blaOXA51_2 | Beta Lactam | AATGATCTTGCTCGTGCTTCGA | CATAGCATCGCCTAGGGTCATG |
| 106 | blaOXA54 | Beta Lactam | GTGATAGTGCTTTGGAACGAGAAC | GCCGCGATATCCCGAGTC |
| 107 | blaOXA55 | Beta Lactam | GCCTTATTGCGTTGGAAACCG | GCCAACTGCTGATATACAGGCA |
| 108 | blaOXA58_2 | Beta Lactam | GTCGTATTGGTCCAAGCTTAATGC | TCTGCGCTCTACATACAACATCTC |
| 109 | intI1_2 | Integrons | CGAAGTCGAGGCATTTCTGTC | GCCTTCCAGAAAACCGAGGA |

| 110 | intI1_1 | Integrons | CGAACGAGTGGCGGAGGGTG | TACCCGAGAGCTTGGCACCCA |
|---|---|---|---|---|
| 111 | intl3 | Integrons | CAGGTGCTGGGCATGGA | CCTGGGCAGCATCACCA |
| 112 | intI1_3 | Integrons | GCCTTGATGTTACCCGAGAG | GATCGGTCGAATGCGTGT |
| 113 | intI3_1 | Integrons | GCCACCACTTGTTTGAGGA | GGATGTCTGTGCCTGCTTG |
| 114 | intI2_2 | Integrons | TGCTTTTCCCACCCTTACC | GACGGCTACCCTCTGTTATCTC |
| 115 | intI3_2 | Integrons | GCCACCACTTGTTTGAGGA | GGATGTCTGTGCCTGCTTG |
| 116 | intI1_4 | Integrons | CTGGATTTCGATCACGGCACG | ACATGCGTGTAAATCATCGTCG |
| 117 | pmrA | MDR | TTTGCAGGTTTTGTTCCTAATGC | GCAGAGCCTGATTTCTCCTTTG |
| 118 | acrB_1 | MDR | AGTCGGTGTTCGCCGTTAAC | CAAGGAAACGAACGCAATACC |
| 119 | acrF | MDR | GCGGCCAGGCACAAAA | TACGCTCTTCCCACGGTTTC |
| 120 | adeA | MDR | CAGTTCGAGCGCCTATTTCTG | CGCCCTGACCGACCAAT |
| 121 | cmr | MDR | CGGCATCGTCAGTGGAATT | CGGTTCCGAAAAAGATGGAA |
| 122 | acrA_1 | MDR | GGTCTATCACCCTACGCGCTATC | GCGCGCACGAACATACC |
| 123 | emrD_1 | MDR | CTCAGCAGTATGGTGGTAAGCATT | ACCAGGCGCCGAAGAAC |
| 124 | mdtE | MDR | CGTCGGCGCACTCGTT | TCCAGACGTTGTACGGTAACCA |
| 125 | mexA | MDR | AGGACAACGCTATGCAACGAA | CCGGAAAGGGCCGAAAT |
| 126 | emrB/qacA_1 | MDR | CTTTTCTCTAACCGTACATTATCTACGATAAA | AGAACGTAGCGACTGATAAAATGCT |
| 127 | mtrE | MDR | CGATGTGTCGTTTTGGAAGGT | CCTGCACCATGATTCCTCAATA |
| 128 | oprD | MDR | ATGAAGTGGAGCGCCATTG | GGCCACGGCGAACTGA |
| 129 | ttgA | MDR | ACGCCAATGCCAAACGATT | GTCACGGCGCAGCTTGA |
| 130 | mepA | MDR | ATCGGTCGCTCTTCGTTCAC | ATAAATAGGATCGAGCTGCTGGAT |
| 131 | mexE | MDR | GGTCAGCACCGACAAGGTCTAC | AGCTCGACGTACTTGAGGAACAC |
| 132 | cfr | MDR | GCAAAATTCAGAGCAAGTTACGAA | AAAATGACTCCCAACCTGCTTTAT |
| 133 | mexB | MDR | CTGGAGATCGACGACGAGAAG | GAAATCGTTGACGTAGCTGGAA |
| 134 | mdsA | MDR | CGGAGTCCATCGACCATTTG | ATCGTCGGCAAGGAGAATCA |
| 135 | tolC_2 | MDR | CAGGCAGAGAACCTGATGCA | CGCAATTCCGGGTTGCT |
| 136 | acrR_1 | MDR | GCGCTGGAGACACGACAAC | GCCTTGCTGCGAGAACAAA |
| 137 | marR_3 | MDR | GCTGTTGATGACATTGCTCACA | CGGCGTACTGGTGAAGCTAAC |
| 138 | oqxA | MDR | GAGTCAACCTACCTCCACTATCA | GCTGCGAGTTATCCAGCAG |
| 139 | adeI | MDR | CAGTCTGGTTTGCAGTAACCA | CACTCCTACAACAACAGGCAA |
| 140 | bexA/norM | MDR | TCGGGCATCCCGTTTATGATC | GTAGGCTGCGCATAATACCCA |
| 141 | mdtA | MDR | ACAAGCCCAGGGCCAAC | CCTTAATGGTGCCTTCGGTTTC |
| 142 | mdtH | MDR | ATGCTGGCTGTACAAGTGATG | CACTCCAGCGGGCGATA |
| 143 | cefa_qacelta | MDR | TAGTTGGCGAAGTAATCGCAAC | TGCGATGCCATAACCGATTATG |
| 144 | qacA/B | MDR | AAGGGCCACTGCATTAGCTG | CCAGTCCAATCATGCCTGCA |
| 145 | qacF/H | MDR | CTGAAGTCTAGCCATGGATTCACTAG | CAAGCAATAGCTGCCACAAGC |
| 146 | arsA | MDR | CAGGTCAGCCGCATCAACC | GCCTGAAACACGGCAATTTCTTC |
| 147 | cadC | MDR | CGCTCTGTGTCAGGATGAAGAG | CTTTCTTATGTGCTAGGGCGATCA |

| 148 | copA | MDR | TGCACCTGACVGGSCAYAT | GVACTTCRCGGAACATRCC |
|---|---|---|---|---|
| 149 | czcA | MDR | GCCTTGTTCATCGGCGAAC | GGCAATGTCGCCTTCGTTC |
| 150 | pbrT | MDR | GATGCGCACTGGGCTTG | TCGGAATATGCGGAAATGCG |
| 151 | pcoA | MDR | TGGCGTATGGAGTTTCAATGC | GAATAATGCCGTGCCAGTGAA |
| 152 | sugE | MDR | CTTAGTTATTGCTGGTCTGCTGGA | GCATCGGGTTAGCGGACTC |
| 153 | tcrB | MDR | GTGCCGGAACTCAAGTAGCA | GCACCGACTGCTGGACTTAA |
| 154 | terW | MDR | TCAAAGAGCTACGCGAGTCATA | CCTTCCCTGTGGACTCACC |
| 155 | acrB_2 | MDR | TGGTAGTGGGCGTCATTAACAC | GGCAACGTAATCCGAAATATCC |
| 156 | bexA | MDR | GCGGATCTCTGGTCAGCAA | TGATTGATGGTTCCCCGTACA |
| 157 | cmeA | MDR | GCAGCAAAGAAGAAGCACCAA | AGCAGGGTAAGTAAAACTAAGTGGTAAATCT |
| 158 | mdtA_1 | MDR | CCTAACGGGCGTGACTTCA | TTCACCTGTTTCAAGGGTCAAA |
| 159 | mdetl1 | MDR | ATACAGCAGTGGATATTGGTTTAATTGT | TGCATAAGGTGAATGTTCCATGA |
| 160 | mdtG_1 | MDR | TGGCACAAAATATCTGGCAGTT | TTGTGTGGCGATAAGAGCATTAG |
| 161 | yceL/mdtH | MDR | TCGGGATGGTGGGCAAT | CGATAACCGAGCCGATGTAGA |
| 162 | mdtH_1 | MDR | CTGCCGTTAAATGGATGTATGC | ACTCCAGCGGGCGATAGG |
| 163 | mexD | MDR | TTGCCACTGGCTTTCATGAG | CACTGCGGAGAACTGTCTGTAGA |
| 164 | qacH | MDR | CATCGTGCTTGTGGCAGCTA | TGAACGCCCAGAAGTCTAGTTTT |
| 165 | sdeB_1 | MDR | CACTACCGCTTCCGCACTTAA | TGAAAAAACGGGAAAAGTCCAT |
| 166 | mtrD_1 | MDR | CCGCCAAGCCGATATAGACA | GGCCGGGTTGCCAAA |
| 167 | oprJ | MDR | ACGAGAGTGGCGTCGACAA | AAGGCGATCTCGTTGAGGAA |
| 168 | mexF | MDR | CCGCGAGAAGGCCAAGA | TTGAGTTCGGCGGTGATGA |
| 169 | mtrC | MDR | GGACGGGAAGATGGTCCAA | CGTAGCGTTCCGGTTCGAT |
| 170 | acrA_2 | MDR | TACTTTGCGCGCCATCTTC | CGTGCGCGAACGAACAT |
| 171 | emrD_2 | MDR | TTTAGGCAGCCTCGCTTCA | CCGAATCCAAATAAAACCCAATAA |
| 172 | qacA/qacB | MDR | TGGCAATAGGAGCTATGGTGTTT | AAGGTAACACTATTTTCGGTCCAAATC |
| 173 | tolC_1 | MDR | GCCAGGCAGAGAACCTGATG | CGCAATTCCGGGTTGCT |
| 174 | emrB/qacA_2 | MDR | GCAGTAGAAGGAACGATTGTTAGTACAG | TGCGTAAACCCAGCTAACAAGTT |
| 175 | qac | MDR | GGAGATTTAGCTCATGTAGCTGAAGAA | AAGCTGTTTTATCCCCGTAGCTTTA |
| 176 | mdtF | MDR | CCACCATCGGGCTTTCC | CCCTTCTTTCTGCATCATCTCA |
| 177 | acrA_3 | MDR | CAACGATCGGACGGGTTTC | TGGCGATGCCACCGTACT |
| 178 | mdtG_2 | MDR | TTATCTGTTTTCTGCTCACCTTCTTTT | GCGTGGTGACAAACAGGCTTA |
| 179 | mdtH_2 | MDR | CGCGTGAAACCTTAAGTGCTT | AGACGGCTAAACCCCATATAGCT |
| 180 | mtrD_2 | MDR | TGCGCGTAGTCGTTCATCTC | CGTTCCAATTTCCTGATGATTG |
| 181 | mtrD_3 | MDR | GGTCGGCACGCTCTTGTC | TGAAGAATTTGCGCACCACTAC |
| 182 | acrA_4 | MDR | CAGACCCGCATCGCATATT | CGACAATTTCGCGCTCATG |
| 183 | acrA_5 | MDR | CGTGCGCGAACGAACA | ACTTTGCGCGCCATCTTC |
| 184 | qacG | MDR | CAATAATAACCGAAATAATAGGGACAAGTT | AATAAGTGTTCCTAGTGTTGGCCATAG |
| 185 | sdeB_2 | MDR | GGCATGCAGAAAGTGTTTATGC | TTAAGTGCGGAAGCGGTAGTG |

| 186 | acrR_2 | MDR | GATGATACCCCCTGCTGTGAGA | ACCAAACAAGAAGCGCAAGAA |
|---|---|---|---|---|
| 187 | marR_1 | MDR | GCGGCGTACTGGTGAAGCTA | TGCCCTGGTCGTTGATGA |
| 188 | marR_2 | MDR | TCTGGCGTTAGCTTCACCAGTAC | GTGCAAAGGCTGGATCGAA |
| 189 | acrR_3 | MDR | TGCAACACGCGCTTTCTC | ACGATTGCGGGCAGGTT |
| 190 | Tp614 | MGE | GGAAATCAACGGCATCCAGTT | CATCCATGCGCTTTTGTCTCT |
| 191 | IS613 | MGE | AGGTTCGGACTCAATGCAACA | TTCAGCACATACCGCCTTGAT |
| 192 | tnpA_1 | MGE | GCCGCACTGTCGATTTTTATC | GCGGGATCTGCCACTTCTT |
| 193 | tnpA_2 | MGE | CCGATCACGGAAAGCTCAAG | GGCTCGCATGACTTCGAATC |
| 194 | tnpA_3 | MGE | GGGCGGGTCGATTGAAA | GTGGGCGGGATCTGCTT |
| 195 | tnpA_4 | MGE | CATCATCGGACGGACAGAATT | GTCGGAGATGTGGGTGTAGAAAGT |
| 196 | tnpA_5 | MGE | GAAACCGATGCTACAATATCCAATTT | CAGCACCGTTTGCAGTGTAAG |
| 197 | tnpA_6 | MGE | TGCAGATGGTTTAACCTTGGATATTT | TCGGTTCATCAAACTGCTTCAC |
| 198 | tnpA_7 | MGE | AATTGATGCGGACGGCTTAA | TCACCAAACTGTTTATGGAGTCGTT |
| 199 | trfA | MGE | ACGAAGAAATGGTTGTCCTGTTC | CGTCAGCTTGCGGTACTTCTC |
| 200 | orf37-IS26 | MGE | GCCGGGTTGTGCAAATAGAC | TGGCAATCTGTCGCTGCTG |
| 201 | ISPps | MGE | CACACTGCAAAAACGCATCCT | TGTCTTTGGCGTCACAGTTCTC |
| 202 | IS1247_2 | MGE | TGGATCGACCGGTTCCAT | GCTGACCGAGCTGTCCATGT |
| 203 | ISAba3 | MGE | TCAGAGGCAGCGGTATACGA | GGTTGATTCAGTTAAAGTACGTAAAAC TTT |
| 204 | ISEfm1 | MGE | AGGTGTCCATGACGTGAAAGTG | TCCTTTGTCCCCTAGGATATTGG |
| 205 | IS1111 | MGE | GTCTTAAGGTGGGCTGCGTG | CCCCGAATCTCATTGATCAGC |
| 206 | IS1133 | MGE | GCAGCGTCGGGTTGGA | ACGCGTTCGAACAACTGTAATG |
| 207 | Tn5 | MGE | TCAGAGGCAGCGGTATACGA | GGTTGATTCAGTTAAAGTACGTAAAAC TTT |
| 208 | IncN_rep | MGE | AGTTCACCACCTACTCGCTCCG | CAAGTTCTTCTGTTGGGATTCCG |
| 209 | IncN_oriT | MGE | TTGGGCTTCATAGTACCC | GTGTGATAGCGTGATTTATGC |
| 210 | IncP_oriT | MGE | CAGCCTCGCAGAGCAGGAT | CAGCCGGGCAGGATAGGTGAAGT |
| 211 | IncQ_oriT | MGE | TTCGCGCTCGTTGTTCTTCGAGC | GCCGTTAGGCCAGTTTCTCG |
| 212 | IncW_trwAB | MGE | AGCGTATGAAGCCCGTGAAGGG | AAAGATAAGCGGCAGGACAATAACG |
| 213 | pAMBL | MGE | CAGGCTCTTAATGTGATA | TTATGCTCAATACTCGTG |
| 214 | pAKD1 | MGE | GGTAAGATTACCGATAAACT | GTTCGTGAAGAAGATGTA |
| 215 | cro | MGE | AGATGTTATCGACCACTTCGGA | CCGCTTGGCGATAAGCG |
| 216 | EAE_05855 | MGE | CCCATCACCGCTGAACTGG | TGGGCGCTGCCATCTAAAC |
| 217 | IncHI2-smr0018 | MGE | ATAATGATTCACCGGGGTAG | CTTCAGGCTATCGTTTCG |
| 218 | IncI1_repI1 | MGE | CGAAAGCCGGACGGCAGAA | TCGTCGTTCCGCCAAGTTCGT |
| 219 | IncN_korA | MGE | GGAACGTTTGTAYCTTGTATTG | ACTCACTATCTTCTGTTGATTG |
| 220 | IS1247_1 | MGE | CGGCCGTCACTGACCAA | TCGGCAGGTTGGTGACG |
| 221 | IS26_2 | MGE | CAATACCTTTGATGGTGGCGTAAG | CTTACGCCACCATCAAAGGTATTG |
| 222 | IS200_1 | MGE | CCAAATACCGAAGACAAGCGTTC | CCAAACTGCTCGTAAAGCATCAG |
| 223 | IS200_2 | MGE | GCACACCCGATGGAACTGTAAA | TCGGCGGGATCTCCAGAAG |

| | | | | |
|---|---|---|---|---|
| 224 | IS21-ISAs29 | MGE | GGTCCGTCAGGCACAAGTC | GGGATCGTATCGGCAAGCC |
| 225 | IS256 | MGE | CTTGCGCATCATTGGATGATGG | AAGAACGGCTCCAATTAAGCGA |
| 226 | IS26_1 | MGE | ATGGATGAAACCTACGTGAAGGTC | CGGTACTTAATCTGTCGGTGTTCA |
| 227 | IS3 | MGE | CGGTCTGAGCTTCGGGAA | AGAACTGTCACTCCGGTCTG |
| 228 | IS5/IS1182 | MGE | TTCTCGAAGAATCGCCATGGC | GCTTTGGATCGCTCCAATCGA |
| 229 | IS6/257 | MGE | ATATCGTGCCATTGATGCAGAG | ACCATTGCTACCTTCGTTGAAG |
| 230 | IS6100 | MGE | CGCACCGGCTTGATCAGTA | CTGCCACGCTCAATACCGA |
| 231 | IS630 | MGE | CCGCCACCAGTGTGATGG | TTGGCGCTGACTGGATGC |
| 232 | IS91 | MGE | GGATGCCACTGCTGGTCA | ACAGTGGATACAGTATCTGCTGAG |
| 233 | ISCR1 | MGE | ATGGTTTCATGCGGGTT | CTGAGGGTGTGAGCGAG |
| 234 | ISEcp1 | MGE | CATGCTCTGCGGTCACTTC | GACGCACCTTCTTGATGACC |
| 235 | lncF_FIC | MGE | GTGAACTGGCAGATGAGGAAGG | TTCTCCTCGTCGCCAAACTAGAT |
| 236 | mobA | MGE | GCTTCCCGTAACGAGGTAGT | CCTTGAACGGTATCAGCACG |
| 237 | Tn3 | MGE | GCTGAGGTGTTCAGCTACATCC | GCTGAGGTAGTCACAGGCATTC |
| 238 | Tn5403 | MGE | AAGCGAATGGCGCGAAC | CGCGCAGGGTAAACTGC |
| 239 | traN | MGE | GCTTGGCGGTCAGCAATT | TTAGGAATAACAATCGCTACACCTTTA |
| 240 | trbC | MGE | CGGYATWCCGSCSACRCTGCG | GCCACCTGYSBGCAGTCMCC |
| 241 | repA | MGE | CCCCCAGGACTTGCGAGCG | GAGGCATGCACGCCGACCA |
| 242 | pNI105 | MGE | CGCTAAGGATGTTTACAC | CTCAACCGTTCTAGGATT |
| 243 | tetA_2 | Tetracycline | CTCACCAGCCTGACCTCGAT | CACGTTGTTATAGAAGCCGCATAG |
| 244 | tetA/B_1 | Tetracycline | AGTGCGCTTTGGATGCTGTA | AGCCCCAGTAGCTCCTGTGA |
| 245 | tetD | Tetracycline | AATTGCACTGCCTGCATTGC | GACAGATTGCCAGCAGCAGA |
| 246 | tetA_1 | Tetracycline | GCTGTTTGTTCTGCCGGAAA | GGTTAAGTTCCTTGAACGCAAACT |
| 247 | tetD_1 | Tetracycline | TGCCGCGTTTGATTACACA | CACCAGTGATCCCGGAGATAA |
| 248 | tetA/B_2 | Tetracycline | GCCCAGTGCTGTTGTTGTCAT | TGAAAGCAAACGGCCTAAATACA |

# Appendix B – Gene Targets Used in Experiment

| | Gene | Target antibiotics (major) | Forward Primer | Reverse Primer |
|---|---|---|---|---|
| 1 | 16S rRNA | 16S rRNA | GGGTTGCGCTCGTTGC | ATGGYTGTCGTCAGCTCGTG |
| 2 | penA | Beta Lactam | AGACGGTAACGTATAACTTTTTGAAAGA | GCGTGTAGCCGGCAATG |
| 3 | fox5 | Beta Lactam | GGTTTGCCGCTGCAGTTC | GCGGCCAGGTGACCAA |
| 4 | cfxA | Beta Lactam | TCATTCCTCGTTCAAGTTTTCAGA | TGCAGCACCAAGAGGAGATGT |
| 5 | blaTMB | Beta Lactam | AAGTCATACAGCCGGGTGGA | GCTTCCCGCCAGCTCATAC |
| 6 | blaSHV_1 | Beta Lactam | TCCCATGATGAGCACCTTTAAA | TTCGTCACCGGCATCCA |
| 7 | blaSFO | Beta Lactam | CCGCCGCCATCCAGTA | GGGCCGCCAAGATGCT |
| 8 | blaOXA48_2 | Beta Lactam | AATAGCTTGATCGCCCTCGA | TTTGGCGGGCAAATTCTTGA |
| 9 | intI1_2 | Integrons | CGAAGTCGAGGCATTTCTGTC | GCCTTCCAGAAAACCGAGGA |
| 10 | intI1_1 | Integrons | CGAACGAGTGGCGGAGGGTG | TACCCGAGAGCTTGGCACCCA |
| 11 | intl3 | Integrons | CAGGTGCTGGGCATGGA | CCTGGGCAGCATCACCA |
| 12 | acrA_5 | MDR | CGTGCGCGAACGAACA | ACTTTGCGCGCCATCTTC |
| 13 | acrR_3 | MDR | TGCAACACGCGCTTTCTC | ACGATTGCGGGCAGGTT |
| 14 | arsA | MDR | CAGGTCAGCCGCATCAACC | GCCTGAAACACGGCAATTTCTTC |
| 15 | IS1111 | MGE | GTCTTAAGGTGGGCTGCGTG | CCCCGAATCTCATTGATCAGC |
| 16 | IS1247_1 | MGE | CGGCCGTCACTGACCAA | TCGGCAGGTTGGTGACG |
| 17 | IS6100 | MGE | CGCACCGGCTTGATCAGTA | CTGCCACGCTCAATACCGA |
| 18 | IS630 | MGE | CCGCCACCAGTGTGATGG | TTGGCGCTGACTGGATGC |
| 19 | ISEcp1 | MGE | CATGCTCTGCGGTCACTTC | GACGCACCTTCTTGATGACC |
| 20 | orf37-IS26 | MGE | GCCGGGTTGTGCAAATAGAC | TGGCAATCTGTCGCTGCTG |
| 21 | repA | MGE | CCCCCAGGACTTGCGAGCG | GAGGCATGCACGCCGACCA |
| 22 | Tn5403 | MGE | AAGCGAATGGCGCGAAC | CGCGCAGGGTAAACTGC |
| 23 | tnpA_2 | MGE | CCGATCACGGAAAGCTCAAG | GGCTCGCATGACTTCGAATC |
| 24 | tnpA_3 | MGE | GGGCGGGTCGATTGAAA | GTGGGCGGGATCTGCTT |
| 25 | tnpA_5 | MGE | GAAACCGATGCTACAATATCCAATTT | CAGCACCGTTTGCAGTGTAAG |
| 26 | tetQ | Tetracycline | CGCCTCAGAAGTAAGTTCATACACTAAG | TCGTTCATGCGGATATTATCAGAAT |
| 27 | blaMIR | Beta Lactam | CGGTCTGCCGTTACAGGTG | AAAGACCCGCGTCGTCATG |
| 28 | blaAIM | Beta Lactam | GAGATCGCCACATGAAACGTC | GCGGATGTTGGCCAGGA |
| 29 | mepA | MDR | ATCGGTCGCTCTTCGTTCAC | ATAAATAGGATCGAGCTGCTGGAT |
| 30 | mexF | MDR | CCGCGAGAAGGCCAAGA | TTGAGTTCGGCGGTGATGA |
| 31 | oprD | MDR | ATGAAGTGGAGCGCCATTG | GGCCACGGCGAACTGA |
| 32 | oprJ | MDR | ACGAGAGTGGCGTCGACAA | AAGGCGATCTCGTTGAGGAA |
| 33 | tolC_1 | MDR | GCCAGGCAGAGAACCTGATG | CGCAATTCCGGGTTGCT |
| 34 | trbC | MGE | CGGYATWCCGSCSACRCTGCG | GCCACCTGYSBGCAGTCMCC |
| 35 | 16S rRNA2 | 16S rRNA | CCTACGGGAGGCAGCAG | ATTACCGCGGCTGCTGGC |
| 36 | blaACT | Beta Lactam | AAGCCGCTCAAGCTGGA | GCCATATCCTGCACGTTGG |

# Appendix C – Statistical Analysis of Methanogenesis and Amoxicillin

ANOVA – Rate ~ Feed type + Amoxicillin

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

| | | Df | Sum Sq | Mean Sq | f value | Pr(>F) | |
|---|---|---|---|---|---|---|---|
| Run 1 | Feed | 7 | 673659 | 96237 | 101.808 | <0.001 | *** |
| | Amox | 1 | 2536 | 2536 | 2.682 | 0.104 | |
| Run 2 | Feed | 7 | 1848152 | 264022 | 70.012 | <0.001 | *** |
| | Amox | 1 | 35363 | 35363 | 9.377 | 0.00276 | ** |

Aov (Rate ~ Amoxicillin) – With amoxicillin as a continuous variable

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

| Rate | | | | | | | | 0.5 ppm | | 1 ppm | | 2.5 ppm | | 5 ppm | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feed | Df | Sum Sq | Mean Sq | f value | Pr(>F) | | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* |
| Run 1 | Acetate | 1 | 517 | 517 | 4.83 | 0.0467 | * | 77% | 0.09 | 55% | 0.09 | 64% | 0.04 | 59% | 0.16 |
| | Ethanol | 1 | 1314 | 1314.3 | 2.84 | 0.116 | | 123% | 0.38 | 96% | 0.72 | 221% | 1.16 | 193% | 1.00 |
| | Butyrate | 1 | 83.66 | 83.66 | 3.617 | 0.0796 | . | 181% | 0.36 | 134% | 0.15 | 144% | 0.52 | 194% | 0.16 |
| | Propionate | 1 | 212.3 | 212.3 | 3.784 | 0.0737 | . | 50% | 0.06 | 72% | 0.50 | 51% | 0.19 | 38% | 0.14 |
| | Glucose | 1 | 867 | 867.1 | 2.316 | 0.152 | | 117% | 0.31 | 129% | 0.28 | 116% | 0.05 | 78% | 0.15 |
| | Broth | 1 | 7904 | 7904 | 8.137 | 0.0136 | * | 122% | 0.25 | 136% | 0.08 | 95% | 0.04 | 75% | 0.08 |
| | H2/CO2 | 1 | 975 | 975 | 0.182 | 0.677 | | 78% | 0.02 | 53% | 0.37 | 74% | 0.02 | 77% | 0.04 |

| | | | | | | | | 10 ppm | | 25 ppm | | 50 ppm | | 100 ppm | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feed | Df | Sum Sq | Mean Sq | f value | Pr(>F) | | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* |
| Run 2 | Acetate | 1 | 193 | 192.6 | 0.678 | 0.425 | | 66% | 0.49 | 32% | 0.05 | 44% | 0.17 | 52% | 0.18 |
| | Ethanol | 1 | 819 | 818.6 | 1.489 | 0.244 | | 73% | 0.41 | 54% | 0.12 | 174% | 1.56 | 137% | 1.02 |
| | Butyrate | 1 | 296.3 | 296.33 | 5.021 | 0.0431 | * | 150% | 0.84 | 129% | 0.13 | 198% | 0.94 | 58% | 0.13 |
| | Propionate | 1 | 7.6 | 7.585 | 0.3 | 0.593 | | 124% | 0.80 | 17% | 0.05 | 30% | 0.08 | 26% | 0.07 |
| | Glucose | 1 | 8672 | 8672 | 11.43 | 0.00493 | ** | 127% | 0.43 | 83% | 0.19 | 65% | 0.26 | 27% | 0.04 |
| | Broth | 1 | 12705 | 12705 | 14.1 | 0.00241 | ** | 103% | 0.28 | 88% | 0.06 | 48% | 0.06 | 20% | 0.05 |
| | H2/CO2 | 1 | 101508 | 101508 | 4.512 | 0.0534 | . | 92% | 0.17 | 80% | 0.11 | 56% | 0.42 | 57% | 0.09 |

# Appendix D – Statistical Analysis of Total Methane and Amoxicillin

ANOVA – Volume ~ Feed type + Amoxicillin

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

| | | Df | Sum Sq | Mean Sq | f value | Pr(>F) | |
|---|---|---|---|---|---|---|---|
| Run 1 | Feed | 7 | 2313.2 | 330.5 | 153.488 | <2e-16 | *** |
| | Amox | 1 | 1.5 | 1.5 | 0.683 | 0.41 | |
| | | | | | | | |
| Run 2 | Feed | 7 | 1639.4 | 234.19 | 70.67 | < 2e-16 | *** |
| | Amox | 1 | 43.6 | 43.6 | 13.16 | 0.00043 | *** |

Aov (Volume ~ Amoxicillin)

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

| | | | | | | | | 0.5 ppm | | 1 ppm | | 2.5 ppm | | 5 ppm | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feed | Df | Sum Sq | Mean Sq | f value | Pr(>F) | | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* |
| Run 1 | Acetate | 1 | 0.22 | 0.22 | 5.63 | 0.03 | * | 95% | *11%* | 87% | *17%* | 79% | *20%* | 58% | *17%* |
| | Ethanol | 1 | 0.58 | 0.58 | 2.79 | 0.12 | | 107% | *47%* | 74% | *16%* | 135% | *13%* | 136% | *20%* |
| | Butyrate | 1 | 0.04 | 0.04 | 1.50 | 0.24 | | 126% | *32%* | 120% | *16%* | 98% | *36%* | 146% | *9%* |
| | Propionate | 1 | 0.10 | 0.10 | 2.94 | 0.11 | | 110% | *18%* | 100% | *61%* | 49% | *10%* | 65% | *37%* |
| | Glucose | 1 | 0.05 | 0.05 | 0.79 | 0.39 | | 121% | *21%* | 118% | *14%* | 121% | *16%* | 93% | *23%* |
| | Broth | 1 | 0.01 | 0.01 | 0.07 | 0.80 | | 107% | *22%* | 108% | *16%* | 106% | *10%* | 106% | *6%* |
| | $H_2$/$CO_2$ | 1 | 8.96 | 8.96 | 0.52 | 0.48 | | 106% | *6%* | 70% | *51%* | 111% | *1%* | 119% | *7%* |

| | | | | | | | | 10 ppm | | 25 ppm | | 50 ppm | | 100 ppm | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feed | Df | Sum Sq | Mean Sq | f value | Pr(>F) | | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* | % of control | *STDEV* |
| Run 2 | Acetate | 1 | 0.06 | 0.06 | 2.37 | 0.15 | | 106% | *44%* | 47% | *14%* | 63% | *37%* | 56% | *33%* |
| | Ethanol | 1 | 0.11 | 0.11 | 0.22 | 0.65 | | 79% | *29%* | 49% | *13%* | 132% | *110%* | 99% | *57%* |
| | Butyrate | 1 | 0.18 | 0.18 | 7.53 | 0.02 | * | 100% | *20%* | 63% | *11%* | 74% | *33%* | 48% | *10%* |
| | Propionate | 1 | 0.03 | 0.03 | 5.65 | 0.03 | * | 125% | *15%* | 63% | *16%* | 95% | *9%* | 63% | *10%* |
| | Glucose | 1 | 1.57 | 1.57 | 7.23 | 0.02 | * | 87% | *40%* | 64% | *24%* | 56% | *16%* | 39% | *7%* |
| | Broth | 1 | 4.73 | 4.73 | 9.76 | 0.01 | ** | 163% | *7%* | 161% | *3%* | 89% | *28%* | 50% | *2%* |
| | $H_2$/$CO_2$ | 1 | 204.40 | 204.41 | 14.55 | 0.00 | ** | 92% | *4%* | 86% | *7%* | 64% | *35%* | 51% | *16%* |