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Impact of Industrial Biocides on Bacterial Isolates from Hydraulic Fracturing Produced Water

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IMPACT OF INDUSTRIAL BIOCIDES ON BACTERIAL ISOLATES FROM HYDRAULIC FRACTURING PRODUCED WATER

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To my

Country “Kingdom of Saudi Arabia”

Father and Mother

Sister and Brothers

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Preface

We would like to acknowledge the work of Andrew Baldwin in the isolation of the bacteria used in this study. These isolates were obtained by Andrew during his undergraduate research project in the lab. Whole genome sequencing of these isolates was performed by Dr. Techtmann prior to the start of this work.

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List of abbreviations

DBNPA	2,2-dibromo-3-nitrilopropionamide
MIC	Minimum inhibitory concentration
HF	Hydraulic fracturing
WHO	World Health Organization
PBS	Phosphate-buffered saline
Ppm	Parts per billion
µl	A microliter
MB	Marine Broth
OD600	Abbreviation indicating the absorbance
MiSeq	System offers the first DNA-to-data sequencing
DNA	The hereditary material in humans and almost all other organisms
Rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid

Abstract

Biocides are antimicrobial compounds that are designed to kill diverse groups of microbes in an untargeted fashion. Glutaraldehyde and DBNPA are commonly used to manage microbial growth in different industries. It is possible that biocide use may result in the development of bacterial resistance. However, resistance to DBNPA is believed to be limited under normal use conditions. We isolated a number of bacteria from produced water from a hydraulically fractured site in West Texas. We then tested the ability of these isolates to resist Glutaraldehyde and DBNPA. In this study, we found that these isolates have varying resistance to these biocides. Importantly we show that some of these isolates are resistant to DBNPA. Isolates had higher minimum inhibitory concentrations (MICs) for glutaraldehyde after twenty-four hour incubation than after one hour. The opposite was seen with DBNPA, where cells that survived one hour of treatment were killed after the twenty-four hour incubation. Previous work has shown that many bacteria that are resistant to biocides can also be resistant to antibiotics. We determined the minimum inhibitory concentration (MIC) of Tetracycline, Ampicillin and Streptomycin. We found that the resistance profiles for these isolates varied. There was little relationship between the bacteria resistant to biocide and antibiotics. To determine the genetic mechanism for biocide and antibiotic resistance we sequenced the genomes of several of these isolates. Genomes were sequenced using Illumina sequencing. Raw reads were Quality filtered, assembled using SPAdes, and annotated using RAST. Antibiotic Resistance genes (ARGs) were identified for each of these isolates using the RGI from the CARD database. The number of ARGs varied from eight in some organisms to zero in others. There was no

relationship between the number of genes and high antibiotic resistance. Our results suggest a complex relationship between the presence of antibiotic resistance genes and the antimicrobial resistance profiles. More work is needed to understand the prevalence and genetic basis for biocide resistance.

1.1 Introduction:

Biocides are chemical compounds designed to kill organisms. Their efficiency varies with concentration and duration of exposure (Siddiqui, Pinel et al. 2017). Biocides are extensively used in industries for disinfectants, including wastewater treatment, domestic water treatment, ship ballast water treatment, and as antifouling substances that prevent the accumulation of animals such as mollusks (Siddiqui, Pinel et al. 2017). Biocides can be produced as concentrates, ready-to-use mixtures or in powders.

While biocides are often able to inhibit the growth of microorganisms some strains of microorganisms can develop a resistance to their effects. Generally speaking, organisms are considered resistant to biocides if their strains are not inhibited or killed by a concentration of biocide typically used to do so (Siddiqui, Pinel et al. 2017). In principle, bacteria develop tolerance as they exhibit properties that impair the action of biocides. In some cases, higher concentrations of biocides are required to overcome the tolerance developed by resistant bacteria. Many bacteria have developed resistance to antimicrobials including biocides and antibiotics. One mechanism by which antimicrobial resistance can be spread is through transfer of genetic material such as transposons and plasmids that contain genes for resistance to other bacteria (Tenover 2006; Siddiqui, Pinel et al. 2017). This may result in cross-resistant bacteria further insusceptible to antimicrobials with every generation.

Glutaraldehyde is a common biocide used to inhibit microbial growth in various industries (Wen, Zhao et al. 2009). Studies have illustrated that bacteria can develop resistance glutaraldehyde (Chapman. 2003) and other antimicrobials. On the other hand,

2,2-dibromo-3-nitrilopropionamide (DBNPA) is a powerful biocide with limited reports of resistant microbes (Donofrio. 1988; de La Fuente-Núñez, Reffuveille et al. 2013). Thus, it can be hypothesized that there is potential for DBNPA resistance to be developed in the future.

The menace of antibiotic resistance is escalating as more antimicrobial resistant organism are identified. Humans and animals infected with antibiotic resistant microorganisms have a higher likelihood of death since bacteria resist antibiotics used to treat infections. Therefore, there is need to reduce the presence of antibiotic resistant microorganisms in the environment by countering the mechanisms of resistance to antimicrobials. Linkages between antibiotic resistance and biocide resistance have previously been shown (de La Fuente-Núñez, Reffuveille et al. 2013). Therefore, it is possible that exposure to biocides could increase the rate of development of antibiotic resistance. However, the study of antibiotics and other antimicrobial agents has tended to progress separately and focus on pathogenic strains (Sütterlin, Alexy et al. 2008). This study seeks to combine an analysis of biocide and antibiotic resistance for the same set of environmental isolates to better understand this linkage.

In addition to studying the biocide resistance profiles of environmental isolates, the activities of three antibiotics (ampicillin, tetracycline and streptomycin) against environmental isolates were examined in this study for the further investigation of bacterial resistance. Ampicillin is a beta-lactam antibiotic that inhibits the synthesis of peptidoglycan in the bacterial cell wall (Gutmann, Kitzis et al. 1986). What is more, ampicillin does not influence the growth and metabolism of eukaryotes. Streptomycin is

aminoglycoside antibiotic and inhibits the actions of protein synthesis. Tetracycline inhibits the activity of gram-negative and gram-positive bacteria, protozoan parasites and chlamydiae to mention a few through inhibiting protein synthesis (Azam, Ahmed et al. 2012). However, some bacteria have acquired resistance against these drugs. One of our hypotheses is that bacteria resistant to biocides would display a higher resistance to antibiotics as observed in a higher minimum inhibitory concentration.

1.2 Importance of Studying Biocides and Biocide Resistance:

Bacterial resistance to biocides is not necessarily a new problem, in fact reports appeared various times ago in which environmental and laboratory isolates were shown to exhibit resistances to biocides (Chaplin 1951). Despite the previous studies, new biocides are routinely developed for various different industrial applications and are used in new and expanding industries. One such industrial application is in the oil and gas industry. Biocides are key components of hydraulic fracturing fluids used during gas and oil extraction (Karharlis. 2014). Relatively few studies have explored the impact of biocides on bacterial isolates from the produced water from hydraulic fracturing sites. Thus, this study aims at bridging this gap by providing evidence of how industrial biocides impact bacterial isolates from wastewater generated during hydraulic fracturing.

1.3 Background on Hydraulic Fracturing:

Hydraulic fracturing (HF) is a technique used in the production of gas and oil. It involves the injection of fluids under high pressure into an oil or gas-bearing shale. In most cases, the components of hydraulic fluids include iron-control chemicals, friction reducers,

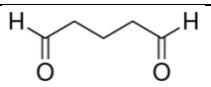
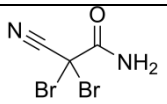
surfactants, pH adjusters, cross-linkers, foaming, gelling agents, clay stabilizers, corrosion inhibitors and biocides (Stringfellow, Camarillo et al. 2017). HF is informally referred to as fracking and is used after the drilling of a horizontal well into a shale. During hydraulic fracturing water, along with sand and other chemicals are injected into a shale to induce cracks which release oil and gas. The largest component of the HF injection fluid is water, which amounts to 25 million liters per well (Fink. 2013). The second largest component of HF fluid is sand or other proppants that are used to hold open the cracks in the shale to release the oil and gas. After injection, the injected water begins to return to the surface. The initial water that returns is known as flowback water. As the process continues this water changes to reflect more of the properties of the geological formation and becomes what is known as produced water. A hallmark of produced and flowback water is the high salinities with flowback water being saline or hyper-saline with total dissolved solids between 20,000 and 250,000 mg/L and high concentrations of cations. These conditions make flowback and produced water a harsh environment.

1.4 The Use of Biocides in Oil and Gas Industry:

The use of biocides in the oil and gas industry has increased with industry development. In hydraulic fracturing, biocides are key components of the fracturing fluid, with glutaraldehyde, DBNPA and quaternary ammonium compounds being the most commonly used biocides (de La Fuente-Núñez, Reffuveille et al. 2013; Stringfellow, Camarillo et al. 2017). The addition of biocides helps prevent gas souring, bio-clogging of equipment and pipes, and corrosion brought about by acid-producing and sulfate-reducing bacteria (Stringfellow, Camarillo et al. 2017). The frequency of Glutaraldehyde use in

hydraulic fractures is higher (27%) than DBNPA use (24%) (Kahrilas.2014). As shown in table 1, Glutaraldehyde ($C_5H_8O_2$) and DBNPA ($C_3H_8Br_2N_2O$) both undergo degradation through hydrolysis, photolysis and biodegradation. However, glutaraldehyde (the most commonly used biocide in hydraulic fracturing) biodegrades in both aerobic and anaerobic conditions (Campa, Techtmann et al. 2018.).

Table 1.1 Biocides consider

Biocide	Glutaraldehyde	DBNPA
Chemical Formula	$C_5H_8O_2$	$C_3H_2Br_2N_2O$
Chemical Structure		
Frequency of use in HF	27%	24%
Degradation pathways	Hydrolysis Photolysis Biodegradation	Hydrolysis Photolysis Biodegradation

1.5 Background on Industrial Biocides:

In addition to the oil and gas industry, many industries such as hospitals, food industry, animal husbandry and industrial water systems routinely use biocides. Biocides are also widely used in many consumer goods such as paints, detergents and cosmetics. (Deborah K. 1988 ; Kähkönen , Nordström. 2008). The energy sector has the fastest growing biocide demand in part due to the increased use of biocides in HF operations (Bolger, A. M., et al 2014). Biocides used in hydraulic fracturing have different modes of

action in controlling bacteria. They are divided into two classes: oxidizing and non-oxidizing compounds. Non-oxidizing biocides are more commonly used and they are grouped into electrophilic and lytic (Campa, Techtmann et al. 2018). The functioning of lytic biocides is based on the disruption of bacterial cell walls. Electrophilic biocides contain electron-accepting functional groups that react with electron-rich chemical classes in membrane proteins. However, glutaraldehyde (the commonly used biocide in HF) biodegrades in both aerobic and anaerobic conditions (Campa, Techtmann et al. 2018). Biocides are considered one of as one of the prime chemicals of concern in hydraulic fracturing based on their toxicity and possible impact to the environment (Stringfellow, Camarillo et al. 2017).

1.6 Development of Biocide Resistance:

Biocides are widely used to control pathogenic microbes. In the food industry, for example, they are used to protect both the food being processed along with the processing equipment from bacteria. Other uses of biocides include clinical applications, wastewater treatment and in the oil and gas industry (Carey and McNamara. 2015). These uses, however, are being impeded with the continued increase in development of biocide resistance by bacteria. A number of studies have shown that bacteria can develop resistance to several types of biocides (Forbes, Dobson et al. 2014).

The mechanism for the development of resistance by bacteria are diverse and some studies have been carried out in an attempt to explain this (Azam, Ahmed et al. 2012). Mechanisms of resistance can often be broken down into genetic changes that alter

sensitivity or phenotypic resistance, which is resistance that is not linked to a genetic change (Chapman. 2003). One such study investigating biocide resistance was carried out to determine the cause of the resistance of *Salmonella enteric* to chlorhexidine (Condell, Power et al. 2014; Randall, Cooles et al. 2004). This study concluded that *Salmonella enteric* is resistant to the biocide due to the complex cell wall and membrane it possesses which enables it to develop phenotypic resistance. The idea that phenotypic resistance is prevalent among most bacteria seems common, although this cannot be confirmed without an in-depth look into the mechanisms of biocide resistance and further research (Henly, Dowling et al. 2019). Though there is no defined period of time for a microbe to develop resistance to biocides, frequent exposure makes development of resistance more likely (Edwards and Holt 2013).

It has also been shown that the resistance of some bacteria to biocides involves genetic changes (Gupta, Bhatia et al. 2018), involving extensive cell alteration (Felden and Cattoir 2018). In another study to find out mechanism for the development of resistance to glutaraldehyde by *Pseudomonas* bacteria found that efflux pumps were an important genetic component of glutaraldehyde resistance (Vikram *et al*. 2015). Though there is no defined period for a microbe to develop resistance to biocides, frequent exposure makes it more likely (Edwards and Holt 2013).

The mechanisms by which bacteria exhibit biocide resistance can either be classified as natural or acquired (Soumet, Méheust et al. 2016). The natural or naturally occurring mechanisms include the development of biofilms and change in cell permeability barrier (Vikram, Lipus et al. 2014). Those resistance mechanisms that are acquired include

increase in efflux pump expression, gene acquisition and changes in cell impermeability (Vikram, Lipus et al. 2014), alteration of enzymes, alteration of target sites, and other mutations (Lipus, Vikram et al. 2017). Since the mechanism of biocides is not typically targeted at a particular enzyme or protein, efflux pumps and increased cell impermeability are more likely mechanisms for biocide resistance than alterations of target sites. The continuous use of biocides in industries may have resulted in microbes developing resistance through both phenotypic and genetic adaptations (Sharma, Sharma et al. 2016).

1.7 Link between Biocide Resistance and Antibiotic Resistance:

A recent World Health Organization (WHO) report suggests that antibiotics may soon become ineffective based on the prevalence of antibiotic resistance (Daniel and Patrick Joseph. 2015). In addition to improper use of antibiotics, one additional reason for the surge in this resistance may be the common use of biocides, as recent studies have shown (Jessica, Mark et al. 2014). In an attempt to study the effects of biocides on the spread of antibiotic resistance, one such study deduced that pathogenic bacteria for example *Staphylococcus epidermidis* showed reduced susceptibility to both triclosan a biocide and antibiotics (Sissel, Lene Nørby et al. 2013). Exposure to a low concentration of biocide can result in changes in the genetic composition of the bacteria, resulting in its resistance to unrelated compounds (Sissel, Lene Nørby et al. 2013). Increased resistance to biocides by bacteria has also been found to impact the degradation potential in stream water after it was found that the bacteria were resistant to glutaraldehyde (Campa, Techtmann et al. 2018).

In summary, studies have shown that biocide resistance may be, a major contributing factor to antibiotic resistance in bacteria (Pal, Bengtsson-Palme et al. 2015). Further research is still needed on this subject, since existing research has focused on pathogens and commonly used household or medical biocides. In this study we are interested in the resistance of environmental isolates to understudied industrial biocides.

1.8 Goals of the Study and Hypotheses

While biocides are used to control microbial growth in HF operations, a number of studies have shown the presence of viable microbes in produced and flowback waters (Struchtemeyer and Elshahed 2012). To understand the efficiency of biocides on microbes present in produced and flowback waters, we isolated a number of strains of bacteria from flowback water on saline media. These isolates were then used to determine the sensitivity to biocides and antibiotics, and seek to explain these observed resistance profiles with genome analysis of these strains. In this study we sought to address the following hypotheses.

1. Bacteria can develop resistance to DBNPA.
2. The resistance in DBNPA would be different from that of Glutaraldehyde.
3. Bacterial resistance to biocide increases the antibiotic minimum inhibitory concentration within the aquatic environment.
4. Organisms that show higher resistance to antibiotics and biocides will have more genes associated with antibiotic resistance.

2-Methods:

2.1 Isolation of bacteria from flowback and produced water

Isolations were previously performed by an undergraduate student in the lab (Andrew Baldwin). Flowback and produced water were obtained from a HF site in Western Texas in the Permian Basin. To obtain biocide resistant isolates the HF wastewater was exposed to either glutaraldehyde, DBNPA or the combination of the two biocides. The water was treated with 100 ppm of the biocides and incubated at room temperature for one hour. After incubation the water was plated onto marine agar and incubated at 30 °C until observable colonies were identified. Additionally, a control was performed where raw HF wastewater was plated on to marine agar and incubated at 30 °C. Colonies were picked and streaked three times on to marine agar and incubated at 30 °C to obtain isolates. The identity of these isolates was determined by 16S rRNA gene sequencing using the 27F and 1492R primers. The near-full length sequence was obtained by sequencing this fragment in the forward and reverse direction and assembling the two reads. Taxonomy of these strains was determined through BLAST of the 16S rRNA gene against the non-redundant database.

2.2 Determination of Minimum Inhibitory Concentration for Biocides:

Minimum inhibitory concentrations (MIC) were determined according to protocols previously described in (Vikram et al 2014). MICs were determined for glutaraldehyde and DBNPA. Glutaraldehyde reacts with free amine groups (Peng, Glattauer et al. 2017), which

are common in most bacterial media. Therefore, MICs were determined by treating the cell with the biocide in marine PBS. Biocide MIC protocol involved the use of the marine PBS solution, (NaCl 22.79 g/L, KCl 0.2 g/L, Na_2HPO_4 1.42 g/L, and KH_2PO_4 0.24 g/L). Cultures of each strain were grown in Marine Broth at 20 °C until a dense culture was obtained. From the overnight grown culture, 1 ml of the culture was added to a tube and centrifuged at 10,000 x g for a minute. The spent media was removed from the cell pellet.

A 1000 ppm solution of either glutaraldehyde or DBNPA was made in marine PBS just before each experiment. This 1000 ppm solution was diluted in marine PBS to make the following concentrations of biocides (0 ppm, 25 ppm, 37.5 ppm, 50 ppm, 62.5 ppm, 75 ppm, 100 ppm and 125 ppm). The pellet was then suspended again in 0.5 ml of PBS with biocide followed by incubation for one hour. From these tubes, 10 µl of the cell solution was spotted on Marine agar. The spots were allowed to dry for 1 hour. These plates were then incubated for 24 hours at 26 °C. The biocide treated tubes were allowed to incubate for 24 hours and the plating was again performed to determine the impact of longer-term biocide incubations on survivability. All conditions were set up in triplicate. Growth was observed on the plates and scored according to the following system: No growth (-), observable growth, but substantially less growth than the 0 ppm control (+), marginally less growth than the 0 ppm control (++) and similar growth to the 0 ppm control (+++).

2.3 Determination of Minimum Inhibitory Concentration for Antibiotics:

The minimum inhibitory concentration for ampicillin, tetracycline and streptomycin were determined for each strain. Marine Broth with various concentrations of antibiotics was made. A stock solution of antibiotic in medium was made and filter sterilized. This stock solution was added at different volumes to autoclaved Marine Broth to reach a final volume of 50 ml of medium and then filter sterilized. The concentrations used were 0 ppm, 5 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm. Triplicate cultures were set up with 9 ml of the medium with appropriate concentrations of antibiotic and 1 ml of an overnight culture. One tube of the medium was left un-inoculated. Before incubation, the OD600 was measured for each tube using a spectrophotometer set at 600 nm. Triplicate cultures were set up for each strain and each concentration of antibiotics. Cultures were incubated at in a shaker set a 165 rpm and 26 °C for 24h. Growth was measure by measuring the OD600 of each tube. Marine Broth has precipitate in the medium naturally. To correct for any absorbance due to this precipitate, the OD600 of the tube prior to incubation was subtracted from the OD600 after incubation to determine the amount growth. Minimum inhibitory concentrations were determined as the concentration of antibiotic at which there was no observable growth.

2.4 Genome Sequencing:

Whole genome sequencing was previously performed by Dr. Techtmann and Andrew Baldwin (an undergraduate researcher in the lab). Genome sequencing of each

strain was performed using the Illumina MiSeq. Overnight cultures were grown, and 1.8 ml of culture were centrifuged and the supernatants were removed. DNA was extracted from cells using the Zymo Quick-DNA fungal/bacteria mini-prep kit (Zymo Research, Irvine CA). DNA concentration was determined using Qubit (Invitrogen). The DNA was diluted and sequencing libraries were prepared using Nextera-XT library preparation kit (Illumina, San Diego CA). The libraries were pooled and sequenced on the Illumina MiSeq using v3 2x300 cycle kit. Libraries were demultiplexed on the MiSeq.

2.5 Genome Assembly:

Genome quality filtering, assembly and annotation were performed using the DOE KBase website (Arkin, Cottingham et al. 2018). Raw data for 10 of the strains were uploaded in FASTQ format to KBase. Quality assessment and reporting of the raw reads was done using FastQC (Ewels, Krueger et al. 2016). Read trimming to remove low quality regions and adaptor removal was performed using Trimmomatic (Bolger, Lohse et al. 2014). Quality filtered reads were then assembled into contigs using the SPAdes assembler implemented at KBase (Tesler 2012). Quality of assemblies (number of contigs, N50, G+C content) was assessed using Quast (Gurevich, Saveliev et al. 2013), as implemented in Kbase. The genomes were further analyzed to identify completeness and contamination using the Check-M program (Parks, Imelfort et al. 2015) as implemented in KBase.

2.6 Genome Annotation:

To identify and functionally classify the genes in these genomes annotation was performed. Bacterial genome annotation is readily carried out by uploading a genome assembly to an automated web-based tool such as RAST (Rapid Annotations using Subsystems Technology; Gurevich, A.,et al 2013). Annotation of structural and functional features of prokaryotic genomes was performed on all isolates using RAST. RAST will identify rRNA, tRNAs, repeats, CRISPRs and annotate protein-encoding genes.

2.7 Genome Analysis:

Potential antibiotic resistance genes were identified using Use the Resistance Gene Identifier(RGI) to predict resistomes from genome data based on homology models as part of The Comprehensive Antibiotic Resistance Database (CARD) database (Jia et al., 2016). Only perfect and strict hits were considered to potential genes. The genes from each of these genomes were identified.

3 Result:

3.1 Identification of the isolates from produced water:

A number of microbes were isolated from produced water. 16S rRNA sequencing indicated that a number of these isolates are related to isolates from the marine environment. Isolates such as *Marinobacter*, *Idiomarina*, *Halomonas*, and *Planomicrobium*. Additionally, a number of these isolates are Firmicutes such as *Bacillus* spp. and *Planomicrobium.*

Table 3.2 List of isolates used, with their closest relatives.

Strains ID	Closest Relative
N3	*Planomicrobium okeanokoites*
N7	
N4	*Idiornarina loihiensis*
G11	*Marinobacter vinifirmus*
G15	*Halomonas alimentaria*
G16	*Bacillus subtillis*
D23	
DG33	
D19	*Bacillus sp.*
D24	*Halomnonas xianhensis*
DG30	*Bacillus thuringiensis*

3.2 Differential Resistance to Biocides

MICs of glutaraldehyde and DBNPA were determined to test the hypotheses that bacteria can develop a resistance to DBNPA and that the species that are resistant to DBNPA would be different from that of Glutaraldehyde. The bacteria isolated from produced water from a hydraulically fractured site were tested. There are eleven strains, as shown in table 2, with the strain IDs being N3, N4, N7, G11, G15, G16, D19, D23, D24, DG30 and DG33. The objective of the experiment was to determine the MIC for these strains for glutaraldehyde and DBNPA.

Results indicated that the different isolates demonstrated varying levels of resistance to Glutaraldehyde and DBNPA and show that the resistance tends to increase with longer exposure. MICs of glutaraldehyde for these isolates ranged from 25 ppm to concentrations higher than 125 ppm. The MICs were generally lower after one hour of exposure to glutaraldehyde and the MICs increased substantially after 24 hours of exposure. Results for the 11 different strains, including N3, N4, N7, G11, G15, G16, D19, D23, D24, DG30 and DG33 are described in detail below.

3.3 Growth patterns analysis of the strains at different concentrations of glutaraldehyde

MICs of glutaraldehyde were determined for each strain in triplicate. Strain N3 showed a high MIC after both 1 hour and 24 hours with the MIC not being able to be determined as high growth was seen in all concentrations. This was also the case for N7 and G15. The growth pattern of N4 after 1 hour declined with increase in concentration

though scanty growth still occurred at the highest concentration of biocide. In the 24 hours set, N4 showed constant high growth for all tested concentration. The growth pattern for G11 for 1 hour showed a slight decline in growth with increase in concentration. There was moderate growth at the highest concentration. This was also the case for G16 for 1 hour and 24 hours and DG33 for 1 hour set. In the 24 hours set, G11 showed constant high growth with increase in concentration while DG33 showed decline in the growth until 100 ppm. Further increase in concentration caused growth inhibition. The MIC of this strain was 125 ppm. Growth of D19, D23 and DG30 at the 1 hour set occurred in the absence of glutaraldehyde. The lowest concentration of 25 ppm inhibited growth. The MIC of glutaraldehyde for D19, D23 and DG30 after 1 hour treatment was 25 ppm. In the 24 hour set, the MIC on D19 was 62.5 ppm and 50 ppm for D23. DG30 had an MIC of 25 ppm in the 24 hour set as well. D24 on 1 hour set showed decline in the growth to scanty growth at the highest concentration. In 24 hour set, constant high growth pattern was observed with an increase in concentration. Therefore the MIC for D24 was not able to be determined.

Table 3.3 (A) Growth pattern of the 11 strains of Bacteria after 1 hour of treatment at different Concentrations of the MICs biocide glutaraldehyde. +++: indicates high growth of isolate, ++: moderate growth, +: scanty or single bacterial colony and, -: indicates no bacterial growth. A, B and C are replicates for each treatment.

Strain		0 ppm	25 ppm	37.5 ppm	50 ppm	62.5 ppm	75ppm	100ppm	125ppm	MIC
N3	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
N4	A	+++	+++	++	+	+	+	+	+	Res
	B	+++	+++	++	+	+	+	+	+	Res
	C	+++	+++	+	+	+	+	+	+	Res
N7	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
G11	A	+++	++	++	++	++	++	++	++	Res
	B	+++	++	++	++	++	++	++	++	Res
	C	+++	++	++	++	++	++	++	++	Res
G15	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
G16	A	+++	++	++	++	++	++	++	++	Res
	B	+++	++	++	++	++	++	++	++	Res
	C	+++	++	++	++	++	++	++	++	Res
D19	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D23	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D24	A	+++	+	+	+	+	+	+	+	Res
	B	+++	+	+	+	+	+	+	+	Res
	C	+++	+	+	+	+	+	+	+	Res
DG30	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
DG33	A	+++	++	++	++	++	++	++	++	Res
	B	+++	++	++	++	++	++	++	++	Res
	C	+++	++	++	++	++	++	++	++	Res

Table 3.3 (B): Growth pattern of the 11 strains of Bacteria after 24 hours at different concentrations of the MICs biocide glutaraldehyde. *+++:* indicates high growth of isolate, ++: moderate growth, +: scanty or single bacterial colony and, -: indicates no bacterial growth. A, B and C are replicates for each condition.

Strain		0 ppm	25 ppm	37.5ppm	50 ppm	62.5 ppm	75ppm	100ppm	125ppm	MIC
N3	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
N4	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
N7	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
G11	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
G15	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
G16	A	+++	++	++	++	++	++	++	++	Res
	B	+++	++	++	++	++	++	++	++	Res
	C	+++	++	++	++	++	++	++	++	Res
D19	A	+++	+++	+++	+++	---	---	---	---	250 ppm
	B	+++	+++	+++	+++	---	---	---	---	250 ppm
	C	+++	+++	+++	+++	---	---	---	---	250 ppm
D23	A	+++	+++	+++	---	---	---	---	---	200 ppm
	B	+++	+++	+++	---	---	---	---	---	200 ppm
	C	+++	+++	+++	---	---	---	---	---	200 ppm
D24	A	+++	+++	+++	+++	+++	+++	+++	+++	Res
	B	+++	+++	+++	+++	+++	+++	+++	+++	Res
	C	+++	+++	+++	+++	+++	+++	+++	+++	Res
DG30	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
DG33	A	+++	++	++	++	++	++	++	---	500 ppm
	B	+++	++	++	++	++	++	++	---	500 ppm
	C	+++	++	++	++	++	++	++	---	500 ppm

3.4 Growth pattern analysis of the strains at different concentrations of DBNPA:

MICs for DBNPA were determined for each strain. In the 1 hour set, all the strains except D19 and DG30 showed no growth in the presence of DPNPA at concentrations between 100 ppm to 500 ppm. Increase in concentration on D19 showed a decline in the growth as shown in table 4, but measurable growth was observed even at 500 ppm. Increase in concentration on DG30 showed a decline in the growth until 200 ppm. No growth was observed for DG30 at 250 ppm or above. Therefore, the MIC of DBNPA for DG30 was determined to be 250 ppm. Further increase in concentration inhibited growth of DG30.

In the 24 hour set, all the strains except for D19 showed no growth in the presence of DPNPA from 100 ppm to 500 ppm. The growth pattern for D19 declined with increase in concentration until 200 ppm and no growth was seen at 250 ppm. The MIC of DBNPA for D19 was at 250 ppm. With the analysis above, we can conclude that lower concentrations of DPNPA than glutaraldehyde were required for inhibition of these bacterial strains and that DBNPA is more efficient at controlling microbial growth than glutaraldehyde. Furthermore, strains that were resistant to glutaraldehyde were not resistant to DBNPA. However, our results indicate that after one hour of incubation two strains were resistant to DBNPA. This indicates that it is possible for bacteria to exhibit resistance to DBNPA. Interestingly, DG30 was sensitive to glutaraldehyde but resistant to DBNPA at one hour. This suggests a potential difference in mechanism for DBNPA resistance.

Table 3.4 (A): Growth pattern of the 11 strains of Bacteria after 1 hour at different Concentrations of the MICs biocide DBNPA. +++: indicates high growth of isolate, ++: moderate growth, +: scanty or single bacterial colony and, -: indicates no bacterial growth. A, B and C are replicates for each treatment.

Strain		0 ppm	100 ppm	150 ppm	200 ppm	250 ppm	300 ppm	400 ppm	500 ppm	MIC
N3	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
N4	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
N7	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G11	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G15	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G16	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D19	A	+++	++	++	++	++	+	+	+	Res
	B	+++	++	++	++	++	+	+	+	Res
	C	+++	++	++	++	++	+	+	+	Res
D23	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D24	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
DG30	A	+++	+	+	+	---	---	---	---	250 ppm
	B	+++	+	+	+	---	---	---	---	250 ppm
	C	+++	+	+	+	---	---	---	---	250 ppm
DG33	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm

Table 3.4 (B): Growth pattern of the 11 strains of Bacteria after 24 hours at different Concentrations of the MICs biocide DBNPA. +++: indicates high growth of isolate, ++: moderate growth, +: scanty or single bacterial colony and, -: indicates no bacterial growth. A, B and C are replicates for each treatment.

Strain		0 ppm	100 ppm	150 ppm	200 ppm	250 ppm	300 ppm	400 ppm	500 ppm	MIC
N3	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
N4	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
N7	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G11	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G15	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
G16	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D19	A	+++	++	+	+	---	---	---	---	250 ppm
	B	+++	++	+	+	---	---	---	---	250 ppm
	C	+++	++	+	+	---	---	---	---	250 ppm
D23	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
D24	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
DG30	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm
DG33	A	+++	---	---	---	---	---	---	---	100 ppm
	B	+++	---	---	---	---	---	---	---	100 ppm
	C	+++	---	---	---	---	---	---	---	100 ppm

3.5 Minimum inhibitory concentrations of antibiotics:

Antibiotics are used to cure as well as prevent bacterial infections or diseases. With time and exposure, some bacteria become resistant to these drugs. On the other hand, biocides are used to manage or destroy organisms that cause harm to human beings. Previous work has shown that many bacteria that acquire resistance to biocides are also resistant to antibiotics (A.D. Russell. 2002). To test the hypothesis that bacteria that are resistant to biocides will also be resistant to antibiotics, we determined the MIC for three antibiotics for the same strains tested above. The data obtained from an experiment to test the resistance of strains to biocides and antibiotics was recorded and graphs drawn for each strain. The minimum inhibitory concentrations (MICs) as obtained from the graphs are tabulated below: and the results of the analysis is shown in Figure 1 to Figure 11.

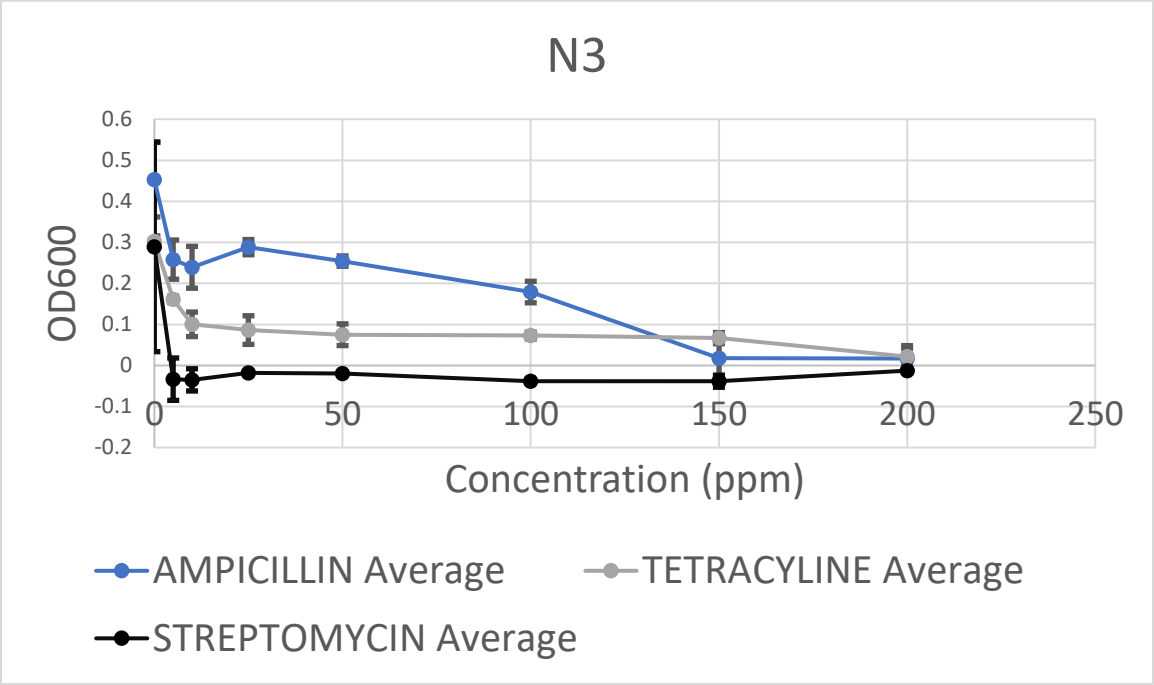


Figure 1: N3 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

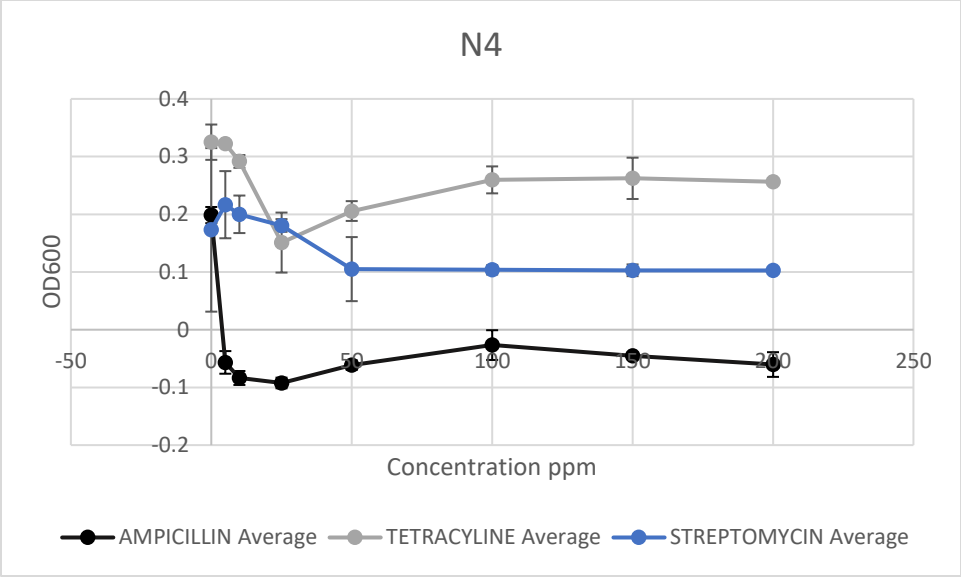


Figure 2: N4 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

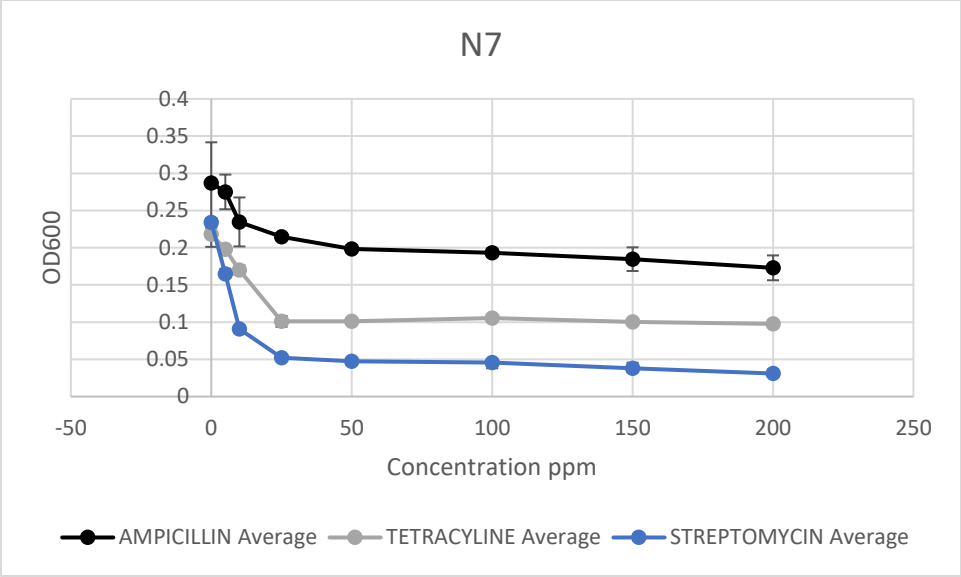


Figure 3: N7 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

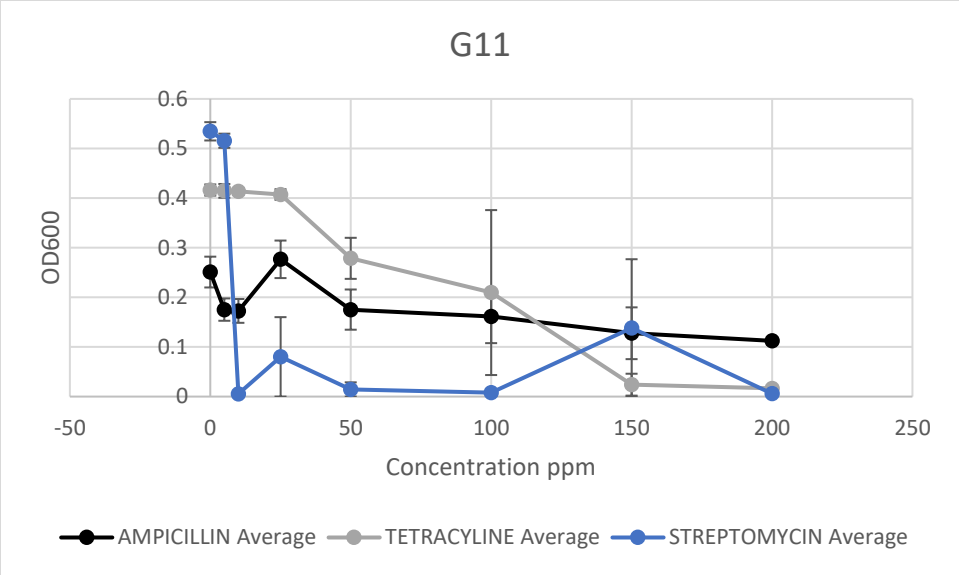


Figure 4: G11 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

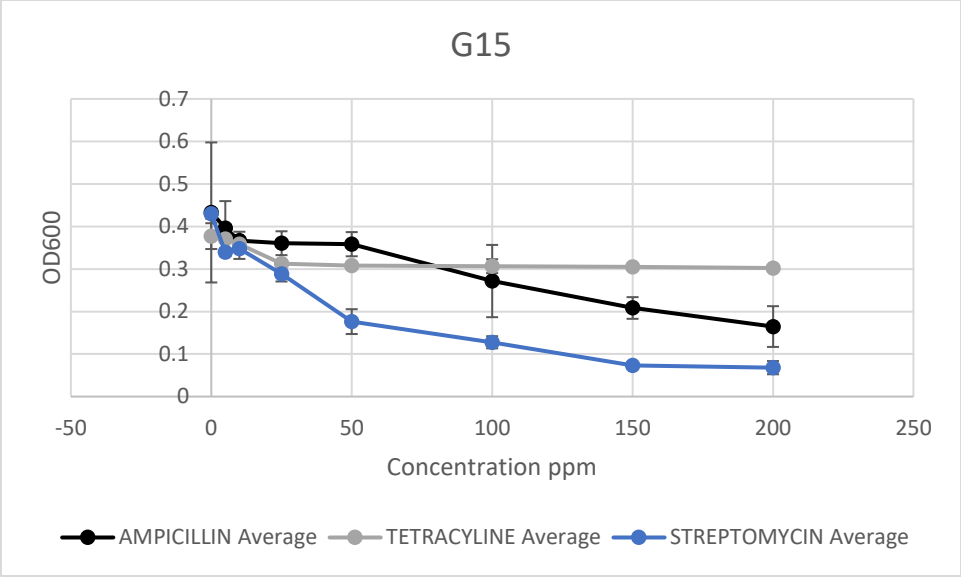


Figure 5: G15 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

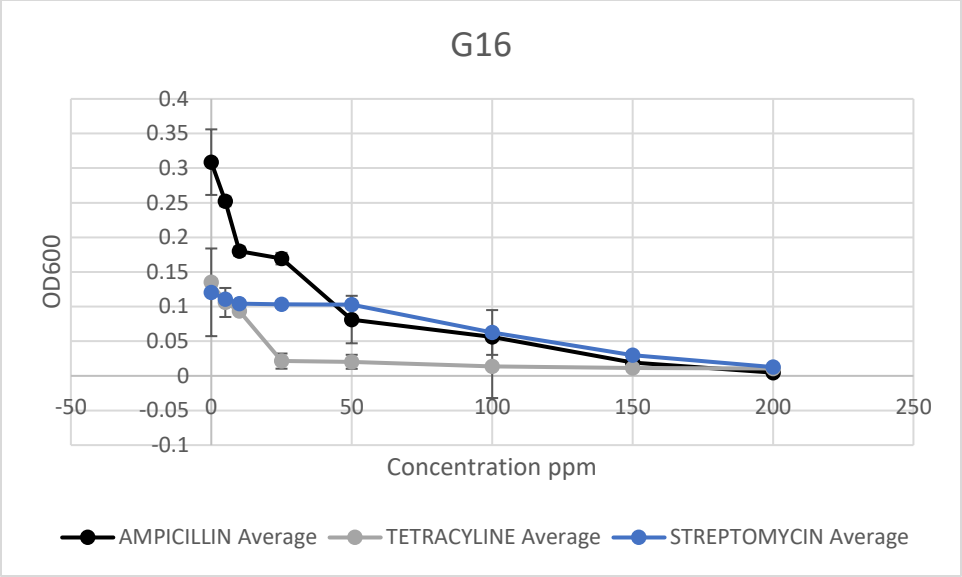


Figure 6: G16 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

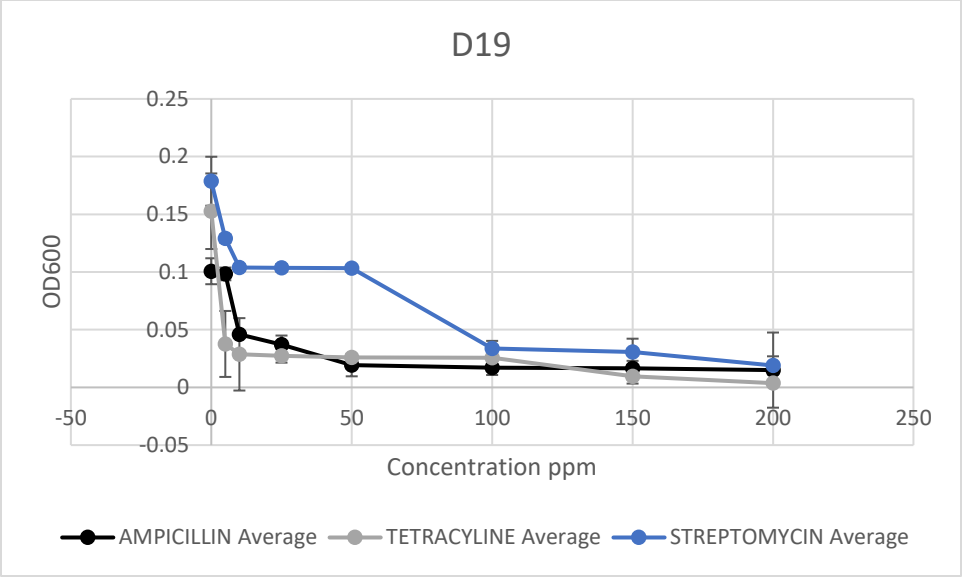


Figure 7: D19 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

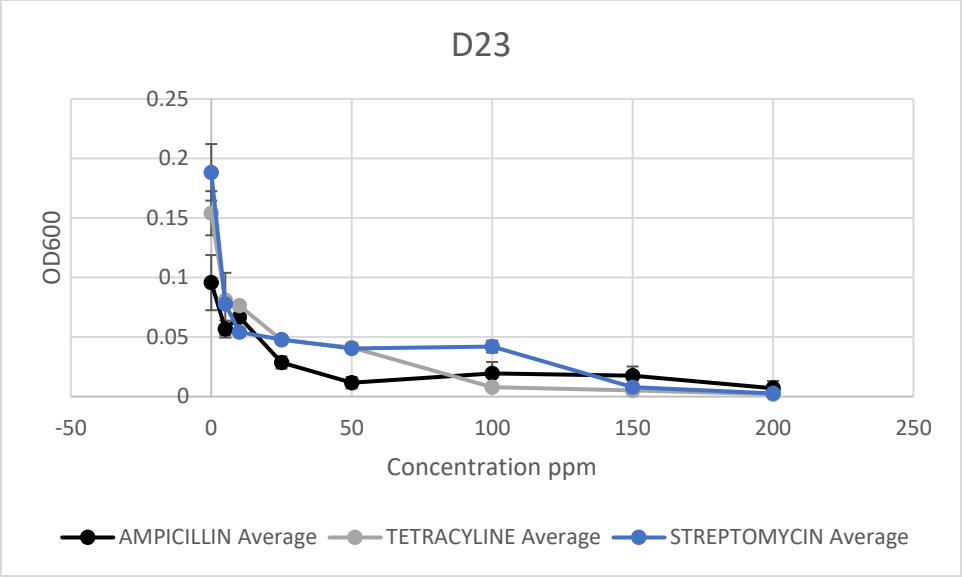


Figure 8: D23 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

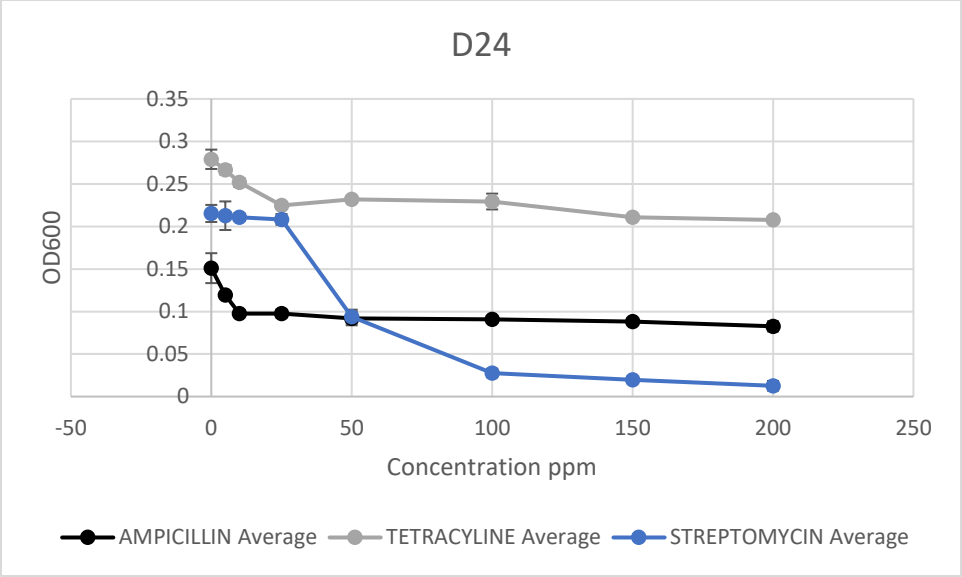


Figure 9: D24 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

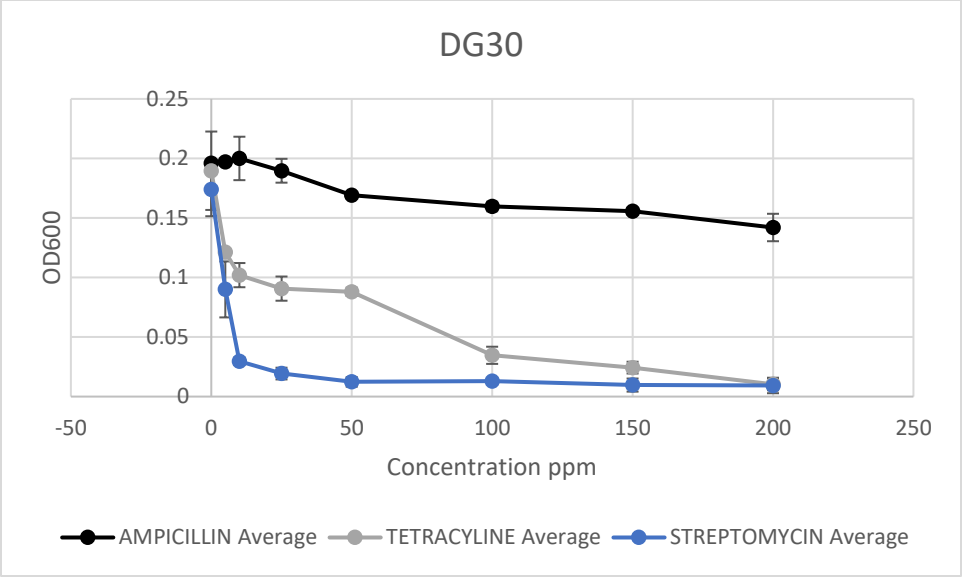


Figure 10: DG30 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

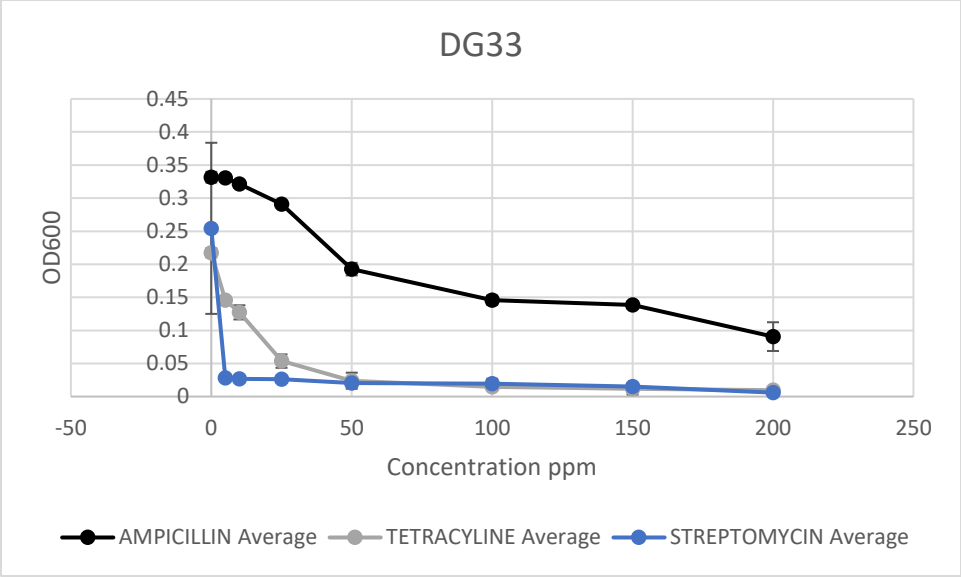


Figure 11: DG33 Resistance Patterns against Ampicillin (black), Tetracycline (gray) and Streptomycin (dark blue).

Table 3.5 Determination of Minimum Inhibitory Concentration for Antibiotics. *R= resistance, S= sensitive

Isolates	AMPICILLIN		TETRACYCLINE		STREPTOMYCIN	
N3	150 ppm	R	150ppm	R	5ppm	S
N4	200 ppm	R	200ppm	R	200ppm	R
N7	50ppm	R	25ppm	R	10ppm	R
G11	100 ppm	R	25ppm	R	10ppm	R
G15	50ppm	R	25ppm	R	50ppm	R
G16	25ppm	R	10 ppm	R	50ppm	R
D19	5ppm	S	5ppm	S	50ppm	R
D23	10 ppm	S	10 ppm	R	100 ppm	R
D24	200ppm	R	200ppm	R	25ppm	R
DG30	200 ppm	R	50 ppm	R	10 ppm	R
DG33	200ppm	R	50 ppm	R	5ppm	S

The European Food Safety Administration defines resistance as and MIC above 8 ppm for Ampicillin, above 16 ppm for Streptomycin, and above 8 ppm Tetracycline (EFSAs). As shown in table 3.5, for N3, tetracycline had the highest MIC of 150 ppm followed by ampicillin (100 ppm) and then streptomycin with an MIC of 5 ppm. For N4, tetracycline and streptomycin had the highest MICs of 200 ppm, followed by ampicillin (200 ppm). For N7, ampicillin had the highest MIC of 50 ppm, followed by tetracycline with MICs of 10 ppm and streptomycin 25 ppm. For G11, ampicillin had the highest MIC of 100 ppm, followed by tetracycline (25 ppm) and then streptomycin (10 ppm). For G15, streptomycin and tetracycline both had the highest MIC of 50 ppm, followed by ampicillin

(25 ppm). For G16, streptomycin had the highest MIC of 50 ppm, followed by ampicillin (25 ppm) and then tetracycline (10 ppm).

For D19, streptomycin had the highest MICs of 50 ppm, followed by tetracycline and ampicillin with MICs of 5 ppm. For D23, streptomycin had the highest MIC of 100 ppm, followed by ampicillin and tetracycline with MICs of 10 ppm each. D24 had ampicillin and tetracycline with the highest MIC of 200 ppm, followed by streptomycin (25 ppm). DG30 had ampicillin with the highest MIC of 200 ppm followed by tetracycline (50 ppm) and then streptomycin (10 ppm). Lastly, DG33 had ampicillin with the highest MIC of 200 ppm followed by tetracycline (25 ppm) and then streptomycin at 5 ppm. These results demonstrate that there is large variation in sensitivity and resistance to these antibiotics. Even within members of the same species such as G16, D23 and DG33, there are large differences in the resistance profiles to these antibiotics. Our results show that most of these isolates are resistant to all three of the tested antibiotics based on the MIC cutoffs from the EFSA.

Finally, for D19 was sensitive for both ampicillin and tetracycline. For the D23 that sensitive just to ampicillin. And for streptomycin only N3 and DG33 that are sensitive.

Table 3.6 the effect of biocides and antibiotic on the bacteria 11 strains **Glutaraldehyde=G, 2,2-dibromo-3-nitrilopropionamide=DBNPA, AMPICILLIN=A, TETRACYLIN=T And STREPTOMYCIN=S

Isolates	Biocide				Antibiotic		
	G- 1	G-24	DBNPA-1	DBNPA-24	A	T	S
N3	+	+	-	-	+	+	-
N4	+	+	-	-	+	+	+
N7	+	+	-	-	+	+	+
G11	+	+	-	-	+	+	+
G15	+	+	-	-	+	+	+
G16	+	+	-	-	+	+	+
D19	-	+	+	+	-	-	+
D23	-	+	-	-	-	+	+
D24	+	+	-	-	+	+	+
DG30	-	-	+	-	+	+	+
DG33	+	+	-	-	+	+	-

From the table 3.6, it can be concluded that these bacterial strains are highly resistant to glutaraldehyde but for the most part sensitive to DBNPA. However, most of the strains are able to tolerate at least some antibiotic. There also does not appear to be a trend in terms of resistance or sensitivity to biocides being a predictor of antibiotic resistance. For example, DG30 is sensitivity to glutaraldehyde, but yet is able to tolerate high concentrations of some of the antibiotics. Additionally, D19 is resistant to both Glutaraldehyde and DBNPA, but is sensitive to two of the three antibiotics.

3.6 Genomic analysis of the isolates

To better understand the molecular basis for the observed MICs calculated above, we sequenced and analyzed the genomes of ten of the studied isolates. These genomes were assembled into draft assemblies. The number of contigs can be used to determine how good an assembly is because most bacterial genomes have one or very few chromosomes and therefore, the fewer the number of contigs, the better. Typically, more than 200 contigs is

not a good assembly. Another measure of how good a genome assembly is N50, which is the length of the smallest contig needed to cover 50% of the genome. Another measure is length of the longest contig is related to N50 and the longer the longest contig the better the assembly.

The length of the total assembly can provide information about the potential for contamination. The average bacterial genome is on the order of 5,000,000 bp. As shown table 3.7, N4, D19 and D24 all had very high number of contigs, relatively small N50s and short longest contigs. This indicates that these assemblies are not very good. However, all of the other assemblies are very good with most assemblies having less than 100 contigs and very large N50 values. Strain DG33 had the largest contig with longest contig having a length of 705,966 as well as a high N50 value of 284,189 and a total length of 4,236,180 bp. Although the largest contigs for strain N4 may not have been the largest among the ten strains, it nevertheless had the highest total length of 19,612,901 bp, which is surprisingly large for a bacterial genome. The strain with the shortest of the longest contig was D24 with the longest contig having a length of 49,124 as shown in Table 3.7.

Table 3.7: Demonstrates the difference between the Number of contigs, N50, the GC contigs, the largest contigs and the total of length for the 10 strains of Bacteria.

Strain	Number of contigs	N50	GC contigs	largest contigs	The total of length
N3	35	290,212	45,79 %	599, 071	3, 288,494 bp
N4	7396	6,867	52,02 %	289, 140	19,612,901 bp
G11	120	109,800	57,90 %	288, 494	4,040,906bp
G15	98	98,335	65,48 %	318, 971	3,809934 bp
G16	62	287,078	43,89 %	476, 815	4,259,424 bp
D19	1132	15,100	40,02 %	126 ,267	6,919,805 bp
D23	43	239,015	43.81 %	576, 084	4,338,296 bp
D24	2575	2,585	57.62 %	49 ,124	4,160,891 bp
DG30	111	379,380	34.93 %	437, 827	5,805,959 bp
DG33	48	284,189	43,94 %	705 ,966	4,236,180 bp

To determine the quality of genomes and attempt to explain the poor assemblies for N4, D19, and D24, we calculated contamination and completeness for the 10 strains of bacteria using Check-M (Table 3.8). Contamination is an indication of if the sequences were obtained from a single organism or if the DNA for the whole genome sequencing was from multiple organisms. Completeness is a measure of how much of the genome was obtained in these draft assemblies. All of the assemblies had high completeness. The D24 strain had the lowest level of completeness at 81.387 %. Nevertheless, three of the strains also had high levels of contamination. N4 had the highest level of contamination at 376.386 %. This means that there are estimated between three and four different strains in these genomes. D24 also had the second highest level of contamination at 9.436 %. D19 also showed minor levels of contamination (5.4 %) The strains with the lowest level of contamination (no record of contamination identified) were D23, DG30, and DG33. The contamination levels in these strains may explain the poor assemblies and the high number of contigs that were present in these genome assemblies.

Table 3.8: Demonstrates the difference between the completeness and the Contamination for the 10 strains of Bacteria.

Strain	Completeness	Contamination
N3	99.338	0.662
N4	1000.0	370.386
G11	100.0	0.086
G15	99.856	0.625
G16	99.17	0.041
D19	98.907	5.398
D23	99.17	0.00
D24	81.387	9.436
DG30	99.425	0.00
DG33	99.17	0.00

In order to help determine the mechanisms of resistance observed, we examined these genomes for antibiotic resistance genes. In this case, the antibiotic resistance genes were identified for the different isolates using the Comprehensive Antibiotic Database (CARD). Using the Resistance Gene Identifier (RGI) algorithm, potential antimicrobial resistance genes were identified. This was done to test the hypothesis that the number of resistance genes in a genome is related with the levels of biocide resistance for the organism.

Table 3.9, presents the number of identified antibiotic resistance genes in the isolates and their corresponding strict hits. The isolate N3 and G15 had zero strict hits. Strain D24 had one potential antibiotic resistance gene. The isolate G11 had 2 strict hits. The isolates N4 and D19 had 3 potential antibiotic resistance genes. Isolate DG30 contained five predicted antibiotic resistance genes. The isolates G16, D23 and DG30 had the most potential antibiotic resistance genes with 8 strict hits. This finding indicates that these isolates had a wide range in number of potential antibiotic resistance genes.

Interestingly, there is very little relationship between the number of antibiotic resistance genes and resistance to biocides. For example, all of the isolates with eight antibiotic resistance genes were sensitive to DBNPA. Additionally, G16, and D23 were only able to tolerate low levels of the tested antibiotics. On the other hand, the two isolates with zero predicted antibiotic resistance genes (N3 and G15) both were highly resistant to glutaraldehyde and to some of the tested antibiotics.

Table 3.9: Demonstrates the difference between Strict Hits for the 10 strains of Bacteria.

Isolates	Strict Hits
N3	0
N4	3
G11	2
G15	0
G16	8
D19	3
D23	8
D24	1
DG30	5
DG33	8

These antibiotic resistance genes were analyzed in more detail to better understand the diversity of antibiotic resistance genes in these genomes. A bit score is an indicator of the statistical value of the BLAST output. All of the bit scores for these potential antibiotic resistance genes are high indicating that they are good hits. Efflux pumps have been associated with both antibiotic and biocide resistance. Therefore, it is possible that increase biocide resistance could be associated with higher numbers of efflux pumps encoded in the genome. Five of the ten sequenced isolates encode at least one efflux pump. These efflux pumps are from the classes of “resistance-nodulation-cell division (RND) antibiotic efflux

pump" family, "the small multidrug resistance (SMR) antibiotic efflux pump" and "ATP-binding cassette (ABC) antibiotic efflux pump" families. Many of these isolates encode multiple efflux pumps. For example, N4, G11, G16, D23 and DG33 all encode two or more efflux pumps. In addition to efflux pumps many other potential antibiotic resistance mechanisms are encoded in these genomes. For example, many genes encode for antibiotic inactivation, antibiotic target protection or alteration. This suggests that these microbes contain diverse set of genes that are capable enabling resistance to diverse antimicrobial.

Table 3.10: Demonstrates the difference between the Best_Hit_Bitscore, Resistance Mechanism AMR Gene Family for the 10 strains of Bacteria.

Isolates	Best_Hit_Bitscore	Resistance Mechanism	AMR Gene Family
N3	---------	----------	----------
N4	702.2	antibiotic target alteration	elfamycin resistant EF-Tu
	1189.1	antibiotic efflux	resistance-nodulation-cell division (RND) antibiotic efflux pump
	791.2	antibiotic efflux	resistance-nodulation-cell division (RND) antibiotic efflux pump
G11	773.9	antibiotic efflux	resistance-nodulation-cell division (RND) antibiotic efflux pump
	1260	antibiotic efflux	resistance-nodulation-cell division (RND) antibiotic efflux pump
G15		----------	----------
G16	984.6	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
	211.8	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
	1589.7	antibiotic target alteration	defensin resistant mprF
	706.4	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump
	199.9	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
	524.6	antibiotic inactivation	macrolide phosphotransferase (MPH)
	873.2	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
	747.7	antibiotic target alteration	daptomycin resistant pgsA
D19	526.2	antibiotic inactivation	macrolide phosphotransferase (MPH)
	793.9	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
	101.3	antibiotic target alteration	glycopeptide resistance gene cluster; vanR
D23	522.7	antibiotic inactivation	macrolide phosphotransferase (MPH)
	199.9	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
	874	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
	985.7	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
	1590.1	antibiotic target alteration	defensin resistant mprF
	705.7	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump
	748.4	antibiotic target alteration	daptomycin resistant pgsA
	211.8	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
D24	39.7	antibiotic target alteration	defensin resistant mprF
DG30	430.6	antibiotic target alteration	glycopeptide resistance gene cluster; vanR
	694.9	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
	484.2	antibiotic inactivation	subclass B1 Bacillus anthracis Bla beta-lactamase
	547	antibiotic inactivation	macrolide phosphotransferase (MPH)
	273.5	antibiotic inactivation	fosfomycin thiol transferase
DG33	211.8	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
	520.4	antibiotic inactivation	macrolide phosphotransferase (MPH)
	199.1	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump
	706.4	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump
	1591.6	antibiotic target alteration	defensin resistant mprF
	874	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
	989.6	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
	748.4	antibiotic target alteration	daptomycin resistant pgsA

4- Discussion:

According to WHO, the resistance of bacteria to antibiotics has escalated to extremely high levels all over the world. There are new mechanisms of resistance coming up and spreading thus threatening the ability to treat common infectious diseases (WHO). This study demonstrated that the number of these environmental bacterial isolates resistant to DBNPA was less compared to that of Glutaraldehyde. This confirms the concept that that DBNPA is a highly effective, non-oxidizing biocide. Therefore, DBNPA seems to be efficient at killing different bacteria and may provide economical control of microorganisms at low-use concentrations. The two isolates that were resistant to DBNPA (DG30 and D19) were both *Bacillus* spp. (*Bacillus* sp. and *Bacillus thuringiensis*). Many *Bacillus* spp. are known to forms endospores, which may provide protection against heat, cold, desiccation and disinfectants (Edwards, D. J. 2013) a characteristic that may enable these bacteria to thrive well under the action of DBNPA.

The degree of resistance of bacteria to DBNPA is completely different from that of Glutaraldehyde. Most of the isolated bacteria were resistant to Glutaraldehyde as compared to DBNPA. This variation can be associated with their different action mechanisms. Glutaraldehyde acts by alkylation of sulfhydryl, hydroxyl, carboxyl and amino groups in microorganisms. This alters the synthesis of protein, DNA and RNA. The biocide associates itself with the external parts of bacteria, specifically the unprotonated amines that represent the active sites. DBNPA however, has its cytotoxicity related to shifts in membrane fluidity. The biocide is electrophilic, hence it reacts with nucleophilic amino acids containing sulphur and amines in membrane proteins (Azam, Ahmed et al. 2012). DBNPA changes the protein structure completely hence a change in membrane fluidity.

From our results, it can be seen that some of the bacteria are resistant to biocides antibiotics like (N7, G11, G15, G16 and D24). For the biocide glutaraldehyde almost all of the tested bacteria are resistant and for DBNPA only some are resistant like D19. Also, most of the isolates showed some resistance to the tested antibiotics tetracycline, ampicillin and streptomycin. However, it is difficult to know why and how as there is no trend on resistance to both biocides. As much as mechanisms of resistance to antimicrobials vary from one agent to another, the basic steps involved are the modification of enzymes, limitation of drug accumulation and changing drug target in the bacterial cell (Ghannoum. 1999). Previous studies have shown that through increased efflux, bacterial cells are able to actively pump incoming antimicrobial agents through multi-drug transporters or specific transporters (Schwarz. 2017).

From the table 4.1 two isolates with zero antibiotic resistance genes and zero efflux (N3 and G15) both were highly resistant to glutaraldehyde, both were also sensitive to DBNPA and to some of the tested antibiotics. For isolates (G16, D23 and DG33) with eight antibiotic resistance genes and four efflux. All were resistant to glutaraldehyde and to all tested antibiotics except Streptomycin to DG33. All of the isolates with eight antibiotic resistance genes and four efflux were sensitive to DBNPA. However these were all Bacillus subtilis relatives.

Table 4.1The effect of biocides, antibiotic, ARG and efflux on the bacteria: **Glutaraldehyde=G, 2,2-dibromo-3-nitrilopropionamide=DBNPA, AMPICILLIN=A, TETRACYLIN=T, STREPTOMYCIN=S, ARG = Antibiotic Resistance Genes and E= Efflux.

Isolate	Biocide				Antibiotic			ARG	Efflux
	G- 1	G-24	DBNPA-1	DBNPA-24	A	T	S	ARGs	E
N3	+	+	-	-	+	+	-	0	0
N4	+	+	-	-	-	+	+	3	2
N7	+	+	-	-	+	+	+	-	-
G11	+	+	-	-	+	+	+	2	2
G15	+	+	-	-	+	+	+	0	0
G16	+	+	-	-	+	+	+	8	4
D19	-	+	+	+	-	-	+	3	0
D23	-	+	-	-	+	+	+	8	4
D24	+	+	-	-	+	+	+	1	0
DG30	-	-	+	-	+	+	+	5	0
DG33	+	+	-	-	+	+	-	8	4

5- Conclusion:

The accumulation of biocides any substance in the environment may lead to stages of uncontrolled effects. However, industries are currently using chemicals and organic compounds that hinder treatment of many prevalent diseases. As a result, the cure for diseases may be more difficult based on the substances used. This can be seen in terms of biocide resistance. When the resistance is formed, it can be very difficult to treat or control resistant microbes. In most cases, antibacterial resistance is owed to the many factors and the effects are realized upon exposure to a variety of biocides and antibiotics. Therefore, there is a significant role that biocides and antibiotics play in the enhancement of the antibacterial resistance. While previous studies have linked biocide and antibiotic resistance, in our study, we do not observe a no link between them. This implies that, the bacterial strains are not affected by the biocide concentration. The study shows that bacteria can be resistant to DBNPA (D19 and DG30) and that the biocide concentration affects bacterial strains. D19 is resistant to DBNPA and only sensitive to streptomycin. Alternatively, the bacteria that were resistant to DBNPA were not resistance to glutaraldehyde. This shows that many bacterial strains are highly resistant to glutaraldehyde while being sensitive to DBNPA. Our work also demonstrated that most of the tested strains were able to resist one or two antibiotics. We hypothesized that bacterial resistance to biocides and antibiotic would be linked to the number of antibiotic resistance genes. Therefore, we expected that the number of resistant genes might result in different resistant profiles (Wales. 2015). However, our results did not show that the higher the number of resistant genes resulted in higher resistance to antibiotics and biocides. Our

results also showed that there is a wide range of resistance mechanisms and the AMR gene families in these isolates. These bacteria have many genes related to antibiotic resistance genes as have shown in table 3.10. Yet the number and type of resistant genes doesn't necessarily explain the resistance profiles observed. As part of this work we have identified a number of isolates from produced water (Table 2). We could then use this information to study the effect of biocides and ARG gene concentration on relatives of these isolates, which can help identify the similarities and differences among these isolate. Further work on these isolates would involve comparing these produced water isolates with their close relatives to understand the impact of produced water on selecting for bacteria that are naturally resistant to biocides. Moreover, from our work we have shown it is possible for bacteria to be resistant to DBNPA like (D19 and DG30). We also have shown that Bacteria that are resistant to glutaraldehyde are not resistant to DBNPA and most bacteria are sensitive to DBNPA are resistant to glutaraldehyde. However strains D19 and DG30 are resistant to DBNPA as have shown table 3A\B (D19 and DG30). Both D19 and DG30 were sensitive to glutaraldehyde but resistant to DBNPA (Table 4). Our results show that there is little link between biocide and antibiotic resistance and the biocide resistance is a complex phenomenon. Previous data indicate that biocides can co-select for antibiotic resistance, but our data does not support this and shows little trend between biocide and antibiotic resistance. More work must be done to better understand the mechanism for resistance to DBNPA and in particular the role of changes in gene expression that may allow for DBNPA resistant. It is important to find alternative ways of managing antimicrobial resistance.

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