INVESTIGATING CONTROL STRATEGIES TO LIMIT BIOFILM FORMATION AND/OR QUORUM SENSING BY *Aeromonas* spp. ISOLATES

BY

TONDANI MBONENI

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor, I have approved this dissertation for submission.

Signed: _____ **Name:** _____ **Date:** _____

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from April 2011 to December 2012, under the supervision of Dr. H.Y. Chenia. These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1 – PLAGIARISM

I, ……..……....., declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

……

ACKNOWLEDGEMENTS

The author records his appreciation to:

Dr. H.Y Chenia for her supervision of this project and support and guidance during the course of this project;

The National Research Foundation for financial support; and

Staff and post-graduate students of the Department of Microbiology, University of KwaZulu-Natal (Westville campus) for their motivation and moral support.

ABSTRACT

Aeromonas spp. are important biofilm-forming fish pathogens causing great economic loss in aquaculture. Bacterial cells within biofilms communicate with each other *via* the production of quorum sensing (QS) signalling molecules called acyl-homoserine lactones (AHLs), which influence biofilm development and production of virulence factors. QS together with efflux pumps, extracellular polymeric substances (EPS) and eDNA are associated with resistance of bacteria to antimicrobial agents. These mechanisms provide a target for different control strategies. The objectives of this study were to: (i) determine effective antimicrobial agents and exposure concentrations against aeromonad biofilms; (ii) ascertain whether *Aeromonas* spp. produce QS molecules or display efflux pump phenotypes, and (iii) investigate the effect of antimicrobial agents, lytic enzymes, efflux pump inhibitors and QS inhibitors on biofilm formation by *Aeromonas* spp. isolates.signalling MICs of azithromycin, ciprofloxacin, ceftazidime, and tetracycline ranged between 0.064-64 μg/ml. Gentamicin had the lowest MICs which ranged between 0.0048-32 μg/ml.The highest MBIC at which antimicrobial agents exhibited inhibition was 4096 μg/ml. Majority of the isolates displayed MIC levels ranging from 2-32 μg/ml, and thus a ≥ 128-fold increase was observed for MBICs. Of the sub-MIC, MIC and supra-MIC exposures tested, MIC exposure of biofilms was the most effective. Gentamicin MIC exposures inhibited initial attachment of 100% (28/28) of isolates tested, while azithromycin MIC exposure detached 82.1% (23/28) of isolates. Carbonyl cyanide 3-chlorophenylhydrazone completely inhibited efflux of cefpodoximeby 14.8% of isolates. However, 1-(1-naphthylmethyl)-piperazinewas more effective, decreasing adherence of 98.1% (53/54) of isolates and increasing detachment of 100% (54/54) of isolates. DNase I was more effective against the mature biofilm,where it increased biofilm detachment of 64.8% of isolates. Of the 48 *Aeromonas* spp. and six *Plesiomonas* spp. isolates used, only a single isolate induced the production of violacein by the *C. violaceum* CV026 biosensor, while all isolates induced the utilization of X-gal to produce a visible blue colour with the *A.tumefaciens* A136 biosensor. Based on the reaction to the two biosensors, aeromonads appeared to produce long-chain acylhomoserine lactones. By blocking QS, S-adenosyl homoserinewas more effective in inhibiting both initial attachment (72.2% of isolates) and pre-formed biofilms (detached 74.1% of isolates). The investigated strategies are promising for *Aeromonas* spp. biofilm inhibition. Thesecould be explored aspotential therapeutic measures in aquaculture systems to limit aeromonad pathogenicity and overcome antimicrobial resistance.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AHL	Acyl-homoserine lactones
AI-1	Auto-inducer 1
AI-2	Auto-inducer 2
AK	Amikacin
AMP	Ampicillin
AZM	Azithromycin
C	Chloramphenicol
C-4 AHL	N-butyryl homoserine lactone
C-6 AHL	N-hexanoyl homoserine lactone
C-8 AHL	N-octanoylhomoserine lactone
C-10 AHL	N-decanoylhomoserine lactone
C-12 AHL	N-dodecanoylhomoserine lactone
CAZ	Ceftazidime
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CIP	Ciprofloxacin
CN	Gentamicin
CPD	Cefpodoxime
E	Erythromycin
eDNA	Extracellular DNA
ENR	Enrofloxacin
EPI	Efflux pump inhibitors
EPS	Extracellular polymeric substances
GN	Gentamicin
HPLC	High performance liquid chromatography
MATE	Multidrug and toxic compound extrusion
MBIC	Minimum biofilm inhibitory concentrations
M-H	Mueller-Hinton
MIC	Minimum inhibitory concentrations
NA	Nalidixic acid
NMP	1-(1-naphthylmethyl)-piperazine
NOR	Norfloxacin
OD	Optical density
OFX	Ofloxacin
PaβN	Phenylalanine arginine β-naphthylamide
QS	Quorum sensing
QSI	Quorum sensing inhibitors
RL	Sulphamethoxazole
RND	Resistance-nodulation-division
S	Streptomycin
SAHC	*S*-adenosyl homoserine
SMR	Small multidrug resistance
TE	Tetracycline
TLC	Thin layer chromatography
W	Trimethoprim

TABLE OF CONTENTS

CHAPTER 1

Introduction and Literature Review

1. Introduction

Many bacteria reside in aquatic settlement and the majority of these are suggested to be pathogenic (Declerk *et al*., 2009). About 95% of the biomass is found in distribution water systems with 5% occurring only in the water phase (Declerk *et al*., 2009). In aquatic settings such as surface water or man-made treatment systems, bacteria survive and grow in limited amounts of nutrients (Vital *et al*., 2010). Within these aquatic settings water pathogens have been shown to exist as both planktonic cells and biofilms. This aids pathogens to survive in water since the biofilm is protected by the exopolysaccharide substance, which concentrates nutrients, prevents access of antimicrobial agents and prevents desiccation (Chmielewski and Frank, 2003).

Aeromonas spp. havea high tendency to form biofilmsand are suggested to be associated with the first stage of biofilm formation in aquatic environments (Dogruoz *et al*., 2009). Aeromonad biofilms are considered as the major food and water-borne pathogens (Igbinosa *et al*., 2012). The source of infection for *Aeromonas* spp. isolates is contaminated fish and water, animal faeces and food handlers (Elhariry, 2011). *Aeromonas*spp. cause different kinds of diseases in humans but more importantly they havebeen found to be associated with diarrhea in children, elderly people and immune-compromised patients (Igbinosa *et al*. 2012). In addition aeromonads cause diseases such as cellulitis, septicaemia and wound infections in fish and other animals (Farmer *et al*., 2006). Therefore, control strategies that can eradicate biofilms formed by members of this species are required in order to reduce their infections in both humans and animals.

1.1. Characterization of *Aeromonas* spp.

Aeromonas spp. isolates are aquatic bacteria that are often associated with diseases in fish and other animals (Janda and Abbott, 2010). Fish industries often face great economic lossesassociated with *Aeromonas* spp.(Janda and Abbott, 2010), and these bacteria are alsoassociated with opportunistic infections in humans (Corral *et al*., 1990). *Aeromonas* spp. are Gram-negative, rod-shaped, non-spore forming facultative anaerobes, which normally possess a single polar flagellum (Farmer *et al*., 2006). They are oxidase-, catalase- and decarboxylase- positive and produce a brown pigment when grown on a media that contains tyrosine.

Members of this genus can be divided into the motile group that grow well at 35-37 °C (cause diseases in humans) and the non-motile group that grow well at 22-25 °C (cause diseases in fish) (Janda and Abbott, 2010). Different *Aeromonas* species that are currently known include: *Aeromonas hydrophila, A. hydrophila* subsp. *dhakensis, A. hydrophila subsp. ranae, A. hydrophila*-like, *A. salmonicida, A. salmonicida* subsp. *achromogenes, A. salmonicida* subsp. *salmonicida, A. salmonicida* subsp. *masoucida, A.sobria, A. caviae A. bestiarum, A. media, A.eucrenophila, A. veronii* biovar *veronii, A. veronii* biovar *sobria, A. schubertii, A. trota, A. tecta, A. aquariorum, A. bivalvium, A. sharmana, A. allosaccharophila, A. encheleia, A. papoffi, A. culcicola, A. simiae, A. jandae* and *A. molluscorum* (Igbinosa*et al*., 2012).

1.2. Environmental and clinical importance of *Aeromonas* species

Aeromonads are ubiquitous in aquatic environments (Nishikawa *et al*., 1994), which serve as their primary habitat (Farmer *et al*., 2006) and their presence in aquatic environments is now considered a threat to public health (Senderovich *et al*., 2008). They are found in high numbers in polluted flowing water (Farmer *et al*., 2006), raw sewage, treated sewage, activated sludge, and mud sinks. Water drainage systems and swimming pools also provide a suitable environment for the growth of *Aeromonas* spp. (Farmer *et al*., 2006). *Aeromonas* spp. cause diseases in different animals with *A. hydrophila* and *A. salmonicida* being the major etiological agents. *A. salmonicida* causes furunculosis in salmon, trout, cutthroat trout, rocky mountain white fish, and brown trout. *A. hydrophila* causes red leg and other diseases in fish, red sore diseases of bass, ulcer diseases of carp, cod, channel cat fish, centrachid fish and other diseases in other animals (Farmer *et al*., 2006).

Diseases that are caused by *Aeromonas* spp.in humans are extraintestinal infections, meningitis, bacteremia, wound infections (Farmer *et al*., 2006), cellulitis, peritonitis, and myonecrosis (Janda and Abbott, 2010). *A. schubertii* in humans is associated with blood infections, while *A. sobria* in humans is the most invasive in tissues. *Aeromonas* spp.utilizes adhesins, hemolysins and cytotonic enterotoxins as virulence factors to cause diseases in humans(Senderovich *et al*., 2008). The presence of extracellularenzymes such as proteases, lipases, and elastases, production of amonabactin, enterobactin, siderophores, α- and β-haemolysins, thermo-stableand thermo-labile enterotoxins, invasins and adhesins, also plays a major role in the pathogenicity of *Aeromonas* spp.*Aeromonas* spp.also display biofilm

formation, which may be associated with their ability to persist and cause disease in diverse hosts (Parker and Shaw, 2011).

1.3 Biofilm formation

Bacteria transitionfrom planktonic cells to sessile cellswhere they live as a population of cells within a biofilm (Landini *et al*., 2010).A biofilm is a community of cells living together within the extracellular polymeric substances (EPS) attached to the surface (del Pozo and Patel, 2007). The EPS is composed of proteins, lipids, polysaccharides, extracellular DNA, phospholipids and humeric substances (Simoes *et al*., 2010). The matrix provides protection for the biofilm against harsh conditions prevents antimicrobial agents from penetrating within and is also responsible for attachment of the biofilm (Simoes *et al*., 2010). There are four stages involved in biofilm formation (Fig. 1.1), i.e., attachment, colonization, maturation and detachment (Behlau and Gilmore, 2008).

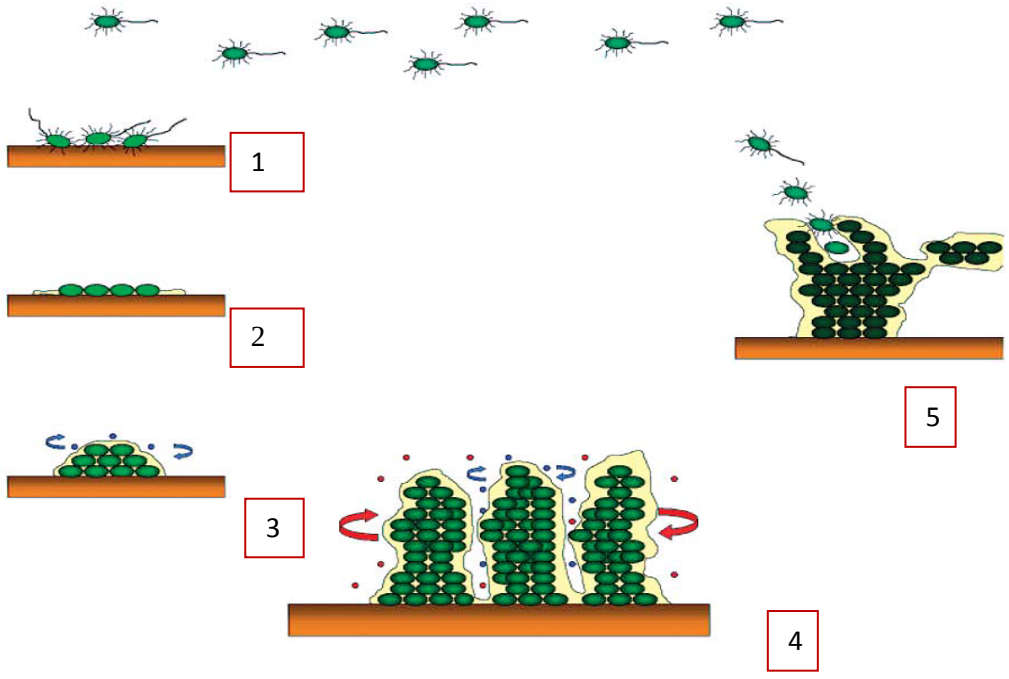

Figure 1.1: Stages involved in biofilm formation.Firstly, the planktonic cells that are dispersed from the biofilm or cells from the environment attach to the surface. The cells then form micro-colonies, which is then followed by formation of the developing biofilm. Before the cells are dispersed the fully matured biofilm is formed (Behlau and Gilmore, 2008).

The attachment stage occurs on rough and hydrophobic surfaces (Simoes *et al*., 2010). Initial attachment of cells to the surface is reversible and it involves weak interactions such as

hydrophobic, Van der Waals and electrostatic forces (Kaplan, 2010). Irreversible interaction which follows reversible interaction, involves strong attachment of appendages (flagella, pili and fimbrae) to the surface area (Simoes *et al*., 2010). Cells that are attached to the surface multiply to form micro-colonies to which the secondary colonizers will attach (Simoes *et al*., 2010).As the cell density increases, cells produce signalling molecules during a process called quorum sensing (QS). This process has been suggested to influence biofilm development (Cataldi *et al*., 2007). The last step of biofilm formation is dispersal of cells which occurseither by erosion, sloughing and seeding (Fig.1.1) (Kaplan, 2010).

1.3.1. Evidence of biofilm formation by *Aeromonas* spp.

Biofilm formation by *Aeromonas* spp. is a characteristic which they share with *Vibrio* and *Yersinia* species (Basson *et al*., 2008). *Aeromonas* spp. isolates have been shown to form biofilmson both synthetic and natural objects (Declerk *et al*., 2009). Aeromonads were also identified as the bacteria responsible for biofilm formation in potable and recycled water systems (Bomo *et al*., 2004). *Aeromonas* species are found in water where they infect fish and if outside their host form biofilms to survive. Aeromonads have been shown to survive in conditions where nutrients are limited, however, nutrients increase the biomass and rate of biofilm development (Bomo *et al*., 2004). *A. caviae* was shown to form biofilms on the surface of glass flasks (Bechet and Blondeau, 2003), while *A. hydrophila* was shown to form biofilms *in vitro* when cultured on a polystyrene surface (Elhariry, 2011). *Aeromonas* spp. were shown to form both single and mixed biofilms (with *Flavobacterium* spp. isolates) within 24 and 48 hours (Basson *et al*., 2008). Since *Aeromonas* spp. are mostly associated with surface colonization and biofilm formation in water distribution systems, food processing and the gastrointestinal tract for clinical strains, attachment is one of the most important aspects of pathogenicity (Santos *et al.*, 2010).

Flagella are useful for movement of cells towards the surface area and sufficient flow rates of water and nutrient concentrations enhance attachment of cells to surfaces (Simoes *et al*., 2010). Flagella are involved in the first step of biofilm formation (Kirov *et al*., 2004). Thedetailed mechanism of the involvement of flagella in biofilm formation is not well understood, however, they are important in colonization which is followed by biofilm formation (Wilhems *et al*., 2009). Bacteria can either have polar or lateral flagella, however, Gavin *et al*. (2002) suggested that the number of bacteria having both is increasing. Polar flagella are responsible for swimming activity of bacteria and lateral flagella are responsible

for swarming activity (Gavin *et al*. 2002). While polar flagella are produced on all culture conditions, lateral flagella are produced on solid media. *Aeromonas* spp. were observed to possess both types of flagella which are involved in biofilm formation (Gavin *et al*., 2002). Canals *et al*. (2007) observed that polar flagella are more important than lateral flagella in *Aeromonas* biofilm formation. Their study suggested that *Aeromonas* spp. isolates that were polar flagella-positive but lateral flagella negative had 62% reduction in biofilm formation. However, an *A.hydrophila* lateral flagella mutant could not form a biofilm until lateral flagella genes were inserted. Santos *et al.* (2010) observed that *A. caviae* possess both polar and lateral flagella which are involved with biofilm formation. Aeromonads are suggested to have type IV pili which are associated with autoaggregation of these bacterial cells. In addition to its involvement in biofilm formation, QS in *Aeromonas* spp. like other different species have been shown to mediate communication (Lynch *et al*., 2002).

1.4. Quorum sensing

Quorum sensing (QS) is a mechanism employed by cells of either the same or different species to communicate with each other *via* production of signalling molecules. The produced signal molecules induce expression of the target genes, which then allows the bacteria to achieve different important functions (Cataldi *et al*., 2007). There are different signalling molecules produced by different bacterial species. The produced signalling molecules include: acyl homoserine lactones (AHLs), auto-inducer 2 (AI-2), 4-quinolones, 3-hydroxypalmitic acid methyl ester, *cis*-11-methyl-2-dodecanoic acid and butyrolactone (Tarighi and Taheri, 2011). AHLs are QSsignalling molecules that are produced by Gram-negative bacteria and are responsible for mediating communication between these bacteria. Gram-negative and Gram-positive bacteria also share a universal auto-inducer called auto-inducer 2 (AI-2), which is a type of signalling molecule that functions as a common language between interspecies bacteria (Kozlova *et al*., 2008).The 4-quinolones are involved with controlling expression of virulence factors, biofilm development, iron transport system and C4-AHL production. A molecule called 3-hydroxypalmitic acid methyl ester, which is produced by converting fatty acid to methyl ester by methyl transferase, has also been shown to mediate cell-density dependent signals between Gram-negative bacteria (Tarighi and Taheri, 2011).Diffusible signal factor molecules, *cis*-11-methyl-2-dodecanoic acid and butyrolactone have been shown be involved in a cell-dependent signalling mechanism (Gudesblat *et al*., 2009).

The two types of signal molecules involved in QS which have been most thoroughly studied are AHLs and AI-2, and for the purpose of the current study the focus will be on AHLs. The production of both AHLs and AI-2 is dependent on bacterial cell density. As the cell density of the bacteria increases (Fig. 1.2), the amount of signalling molecules also increases (Pan and Ren, 2009).The major function of AHLs is suggested to be inducing biofilm formation in different bacterial species (Lynch *et al*., 2002; Cataldi *et al*., 2007; Kozlova *et al*., 2008). AHLs with different lengths within a biofilm were suggested to be responsible for bio-fouling (Ponnusamy *et al*., 2009). AHL production is also associated with bioluminescence, antibiotic production, swarming motility and production of virulence factors in other bacterial species(Ponnusamy *et al*., 2009).

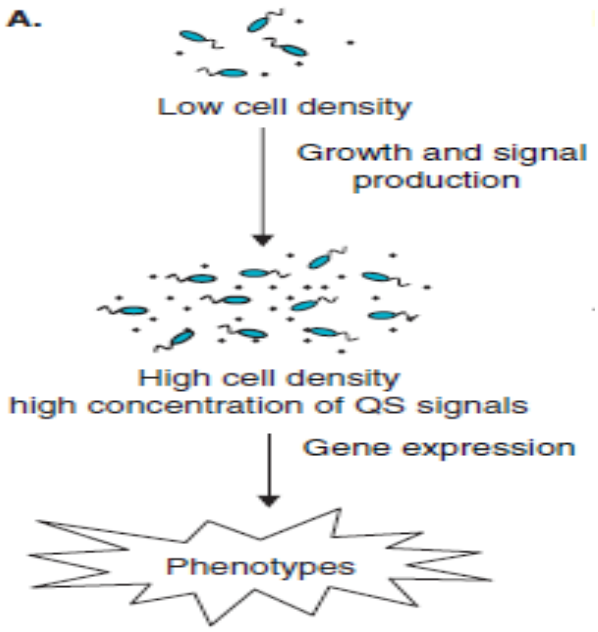

Figure 1.2: The mechanism of quorum sensing.When the cell density of the population is low, the amount of signal produced is low and *vice versa*. The signals produced induce the expression of target genes which then result in different phenotypes of the bacteria (Pan and Ren, 2009).

1.4.1. Strategies used to extract, identify and characterize AHLs

Different strategies have been used to identify AHL production. These include the use of biosensors, thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC) (Wang *et al.*, 2010). The biosensors that are commonly used are *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136. The former

detects short and medium AHLs (C-6, C-6-3-oxo, C-8, C8-3-oxo, C-4) and the latter detects a broad range of AHLs (all 3-oxo, C-6, C-8, C-10, C-12, C-14, C-6-3-hydroxy, C-8-3-hydroxy and C-10-3-hydroxy) (Steindler and Venturi, 2007).

Detection of AHLs by *C. violaceum* CV026 is indicated by the production of a purple violacein pigment, while in *A. tumefaciens* A136 identification is indicated by the presence of blue color which appears after this bacterium utilizes 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Steindler and Venturi, 2007). The *C. violaceum* CV026 reporter strain was constructed by inserting a transposon in the *cviI* AHL synthase gene (responsible for production of AHL) and the putative violacein repressor locus, so that this strain can only produce violacein against exogenous AHL. The *A. tumefaciens* A136 strain was constructed by introducing a mutation in the *traI* gene (responsible for the production of AHL), and the construct contains two plasmids, *viz*, pCF218 inserted with *traR* expressed from *tetR* vector promoter and pCF372, which is transcriptionally linked to *lacZ*. As a result, the reporter can utilize X-gal and produce a detectable blue color (Steindler and Venturi, 2007).

Biosensor assays do not give information on the exact structure of AHLs. TLC is commonly used to determine the type of AHLs produced by a certain bacterial species. Control AHLs with known migration characteristics are used for comparison with the unknown (Shaw *et al*., 1997). TLC chromatograms can also be overlaid with agar-containing biosensors which makes it easy to locate the migrating AHLs (Steindler and Venturi, 2007). Using TLC, *Yersinia enterocolitica* was shown to produce 3-oxo-hexanoyl homoserine lactone and hexanoyl homoserine lactone (Medina-Martínez *et al*., 2006). However, TLC cannot give structural information of the AHLs and hence HPLC is used (Wang *et al*, 2010). HPLC can also be used to purify AHLs before analysis (Steindler and Venturi, 2007).

1.4.2. Acyl homoserine lactones

Gram-negative bacteria produce AHLs as their major signal molecules (Estrela *et al*., 2009). Signals are specific so that QS occurs between Gram-negative bacteria of the same species, due to differences in the lengths and side chains of AHLs produced by different species (Cataldi *et al*., 2007). The side chains of AHLs that are produced by different species have N-acyl chains with carbons that range from 4-14, and this resultsin production of diverse AHLs (Fig. 1.3). The signal diversity also results from the C-3 position on the side chain of AHLs which can either be substituted by 3-oxo, 3 hydroxyl, fully methylene group or have unsaturated bonds(Cataldi *et al*., 2007). Taga and Bassler (2003) have suggested that

differences in AHLs of different species enables bacteria of the same species to communicate without confusion in a community where different bacterial species are found. Therefore,bacteria of the same species will only produce signals that are recognized by the same species.

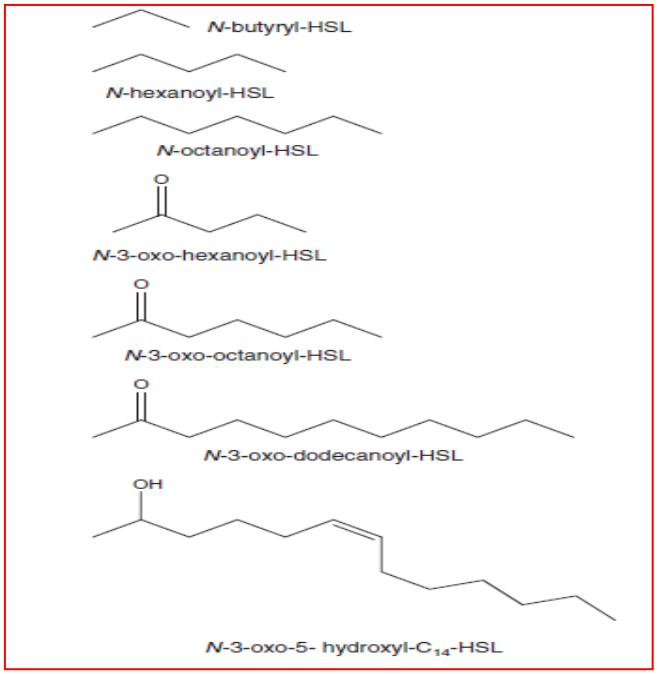

Figure 1.3: Different types of AHLs that are produced by different bacterial species. The AHL structures represented are produced by *Pseudomonas aeruginosa, Aeromonas salmonicida, Burkholderia cepacia, Vibrio fischeri, Agrobacterium tumefaciens, Rhizobium leguminosarum* and *Rhodopseudomonas palustris*, respectively (Pan and Ren, 2009).

AHLs produced by Gram-negative bacteria involved in QS aresuggested to be homologues of LuxI and LuxR of *Vibrio fischeri* (Taga and Bassler, 2003). LuxI-type proteins are involved in catalyzing the production of AHL. The produced AHLs diffuse out of the cells and accumulate around the biofilm until a sufficient number of cells is reached. Stimulated by the high densities of cell populations, concentrated AHLs diffuse into the cells where they bind to the LuxR-type proteins. Thereafter, the complex binds to *lux* boxes where they induce expression of specific genes (Fig. 1.4) (Steindler and Venturi, 2007). The AHLs bind to LuxR at the N-terminus of the transcriptional activator, and its C-terminus binds to *lux* boxes, which contain the *lux* gene (Wang *et al*., 2010).

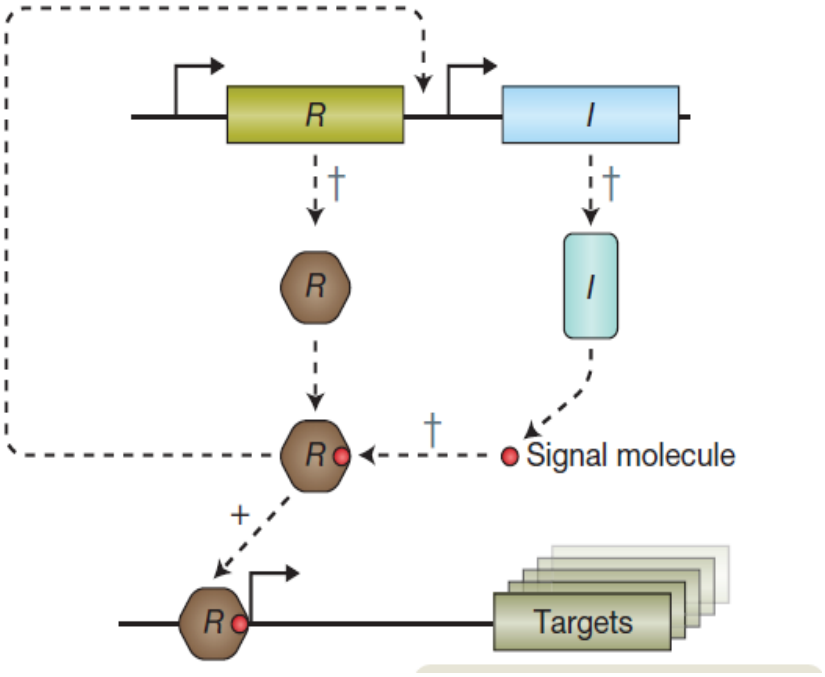

Figure 1.4: The LuxR and LuxI system mechanism. R proteins which are AHL cognate proteins are produced by *luxR* and *luxI* produces AHL synthetase, which catalyze the production of AHLs. AHLs then bind to their cognate genes *via* the N-terminus and induce expression of target genes after binding these genes on their C-terminus (Bjarnsholt *et al*., 2010).

LuxI-type proteins and the LuxR-type proteins are very specific in their activation (Taga and Bassler, 2003) and this specificity is important in preventing gene expression of other species (Taga and Bassler, 2003). The LuxI-type protein is specific when binding to its substrate, which is the acyl-acyl carrier protein on the homocysteine moiety of *S*-adenosylmethionine and LuxR-type only binds to the AHL molecule that it recognizes as its cognate molecule.

1.4.3. Auto-inducer 2

AI-2is the signalling molecule responsible for cell-to-cell communication in both Gram-negative and Gram-positive bacteria (Kozlova *et al*., 2008). The major function of these auto-inducers was first described in *Vibrio harveyi*, where it is responsible for the production of light. In other bacterial species such as *E. coli, V. cholerae*, *Clostridium perfringens*, and *Streptococcus pyogenes* this molecule is suggested to be responsible for the production of

virulence factors (Taga and Bassler, 2003).The structure of AI-2 is similar to the structure of the furanosyl-borate di-ester moleculeand this molecule, like AHLs,isderived from*S*-adenosyl-methionine.The LuxS protein converts*S*-adenosyl-methionine to dihydroxy-2, 3-pentanedione, which then undergoes cyclization to produce 2,4-dihydroxy-2-methylhydro-3-furanone, which forms a diesterboric acid to form AI-2. Boyen *et al*. (2009) demonstrated that in *V.harveyi*, AI-2 binds to the LuxP protein after which the complex binds to LuxQ which possess both the sensor kinase domains and a response regulator domain. They suggested that in low concentration the repressor protein blocking the transcription of luciferase is activated after LuxQ phosphorylates LuxO, and this reaction is aided by LuxU (intermediary protein) (Fig. 1.5).

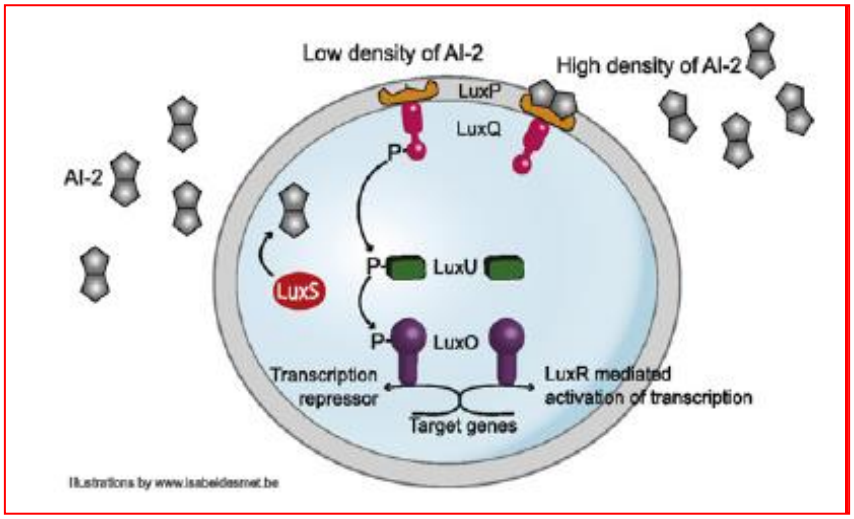

Figure 1.5: AI-2 mediated signalling in *V. harveyi*. Transcription of target genes is achieved in high density and *vice versa* (Boyen *et al*., 2009).

At high concentration LuxO is inactivated after AI-2 induces the phosphatase activity of LuxQ. The reaction then induces transcription of the luciferase operon in return (Fig. 1.5). The major function of AI-2 is mediating inter-species communication, and this is because bacterial species that cannot produce this molecule can, however, respond to its signal (Ryan and Dow, 2008). Ryan and Dow (2008) have suggested that *P. aeruginosa* does not produce AI-2 but it can detect it and express virulence genes. A *luxS Caenorhabditis elegans* mutant was observed to have attenuated virulence, while a *luxS* mutant *Vibrio vulnificus* showed a delayed time required for it to kill mice when compared to the wild type (Kozlova *et al*., 2008). In addition to mediating communication and production of virulence factors between

different species, AI-2 is also associated with the activated methyl cycle (Tarighi and Taheri, 2011).

1.4.4. Quorum sensing in *Aeromonas* spp. isolates

In *Aeromonas* spp. the genes responsible for QS are *ahyRI* and *asaRI* (Swift *et al.*, 1997). The AhyRI and AsaRI QS system of *Aeromonas* spp. functions in a similar manner to the LuxI and LuxR systems. The gene that is responsible for production of AI-2 in *A. hydrophila* and *Vibrio* spp. is *luxS.* In the latter species, the receptors of the LuxS protein are recognized as LuxP and in the former species the receptors have not being identified (Kozlova *et al*., 2008). AHLs are suggested to be the major molecules responsible for signalling by *Aeromonas* spp. (Khajanchi *et al.*, 2010). Swift *et al*. (1997) observed that *A. hydrophila* and *A. salmonicida* produce diffusible AHLs in which N-butyryl homoserine lactone (C-4 AHL) was the main signalling molecule. *A. hydrophila* isolates produce N-octanoylhomoserine lactone (C-8 AHL), N-dodecanoylhomoserine lactone (C-12 AHL) and N-tetradecanoylhomoserine lactone (C-14 AHL), while *A. salmonicida* producesC-8 AHL, dodecanoylhomoserine lactone (C-12 AHL), N-tetradecanoylhomoserine lactone (C-14 AHL) and N-decanoylhomoserine lactone (C-10 AHL) (Cataldi *et al.*, 2007). *Aeromonas* spp. isolates obtained from patients with malaria were shown to produce C-4 AHL and N-hexanoylhomoserine lactone C-6 AHL as the two major types of AHLs. *A. hydrophila* isolates were shown to produce both C-4 AHL and C-6 AHL, while *A. sobria* isolates only produced C-4AHL (Chan *et al*., 2011). *A. hydrophila* was shown to produce C-4 AHL, as the major AHL and *A. caviae* was shown to produce 3-oxo-C-6AHL (Medina-Martínez *et al*., 2006). *Aeromonas* spp. isolates isolated from municipal activated sludge also produced C-4 AHL and C-6 AHL (Morgan-Sagastume *et al*., 2005).

Aeromonas QS has been implicated in the production of virulence factors. C-4 AHL produced during QS by *A. hydrophila* was shown to be responsible for the production of extracellular protease (Kirke *et al*., 2004). Chan *et al*. (2011) also suggested that QS in *Aeromonas* spp. is associated with the production of virulence factors. The production of virulence factors such as hemolysins, cytotonic and cytotoxic enterotoxins, proteases, lipases, leucocidins, endotoxin, adhesions, and an S layer in *Aeromonas* spp. is associated with high cell density, showing that it is QS-mediated (Khajanchi *et al*., 2010). In addition to production of virulence factors, QS is involved in the development of a biofilm (Lynch *et al*., 2002). Kirke *et al*. (2004) observed that a mutant strain of *Aeromonas* spp. Without *ahyI* did

not form a mature biofilm when compared to the parent strain. Ponnusamy *et al.* (2010) observed that the production of auto-inducers is responsible for the formation of the three-dimensional structure of a biofilm. Lynch *et al.* (2002) observed that C-4AHL or C-6AHL produced by a mutant strain of *A. hydrophila* was important in biofilm formation and for its development when compared with its wild type that is incapable of producing AHLs. In a study conducted by Khajanchi *et al*. (2009), similar results were obtained. Labbate *et al*. (2004) suggested that AHL production by aeromonads is important in the formation of microcolonies. AI-2 is also suggested to be responsible for production of virulence factors and biofilm formation in *A. hydrophila* (Khajanchi *et al*., 2010). AI-2 was shown to be responsible for the formation of well-defined biofilm structures of *A. hydrophila*, when compared with an AI-2 mutant strain that formed an altered biofilm (Kozlova *et al*., 2008).

1.4.5. Association of auto-inducers with biofilm formation

Auto-inducers that are produced by bacteria induce expression of target genes only when high cell density is reached and these molecules often have an effect on biofilm formation (Khajanchi *et al*., 2010). The mechanism by which AHL contributes to biofilm formation is not clear, however, interfering with their signals during QS results in reduction in biofilm formation (Morohoshi *et al*., 2008). This shows that AHLs are indeed associated with biofilm formation. Addition of exogenous AHLs to a bacterial species which is incapable of producing AHLs often also results in biofilm formation (McClean *et al*., 1997). AHLs are produced by diverse Gram-negative bacteria such as *Serratia marcescens* (Rice and Koh, 2005), *P. aeruginosa* (Davies *et al*., 1998), *Hafnia alvei* (Viana *et al*., 2009) and *Vibrio anguillarum* (Morohoshi*et al*., 2008), which have been shown to form biofilm mediated by AHLs during QS.Nadell *et al*.(2008) suggested that even though QS is responsible for biofilm development, it is also responsible for the production of exopolymeric substance (EPS). QS controls when the polymers that makes up the EPS should be produced and when they should be repressed. This then influences biofilm formation when the cells are deprived of nutrients (Nadell *et al*., 2008). The EPS protects cells within the biofilm and also is responsible for attachment of cells to the substrate which initiates biofilm formation (Behlau and Gilmore, 2008). AHLs in biofilm formation of Gram-negative bacteria have been suggested to affect heterogeneity, architecture, stress resistance, maintenance and sloughing (Viana *et al*., 2009). While the influence of AHLs on biofilm formation is still a mystery, their production plays a crucial role.

1.5. Biofilm resistance

Biofilms cause problems in the paper, food, cosmetic and pharmaceutical industries (de Carvalho, 2007) as well as being linked to health-care associated infections (del Pozo and Patel, 2007). Biofilmcells are difficult to kill because of their increased resistance to antimicrobial agents(del Pozo and Patel, 2007). Biofilm formation by bacteria is suggested to be a major strategy to achieve pathogenicity and to develop resistance to antimicrobial agents (Landini *et al*., 2010). Biofilms are 10-1000 times more resistant to antimicrobial agents when compared to their planktonic counterparts (Mah and O'Toole, 2001). Biofilms adapt easily to environmental stress due to their existence as a population (Declerk *et al*., 2009). Severalmechanisms have been proposed (Fig. 1.6) that may contribute to biofilmcells being resistant to a wide range of antimicrobials (del Pozo and Patel, 2007). The antimicrobial agents may be prevented from entering beyond the surface layer of the biofilm, and these may be due to changes in the environment within the biofilm; growth inside the biofilm maychange in favor of the biofilm rendering the antimicrobial agents inactive; enzymes within the matrix of the biofilm may destroy the incoming antimicrobial agentsand biofilms also expressspecific genes associated withefflux pumps (del Pozo and Patel, 2007). Gene transfer also plays an important role in providing resistance, since a planktonic cell that is resistant to a specific antimicrobial agent may transfer resistance to other cells within a biofilm.QS is now also considered one of the major mechanisms associated with biofilm resistance to different antimicrobial agents (Molin and Tolker-Nielsen, 2003).

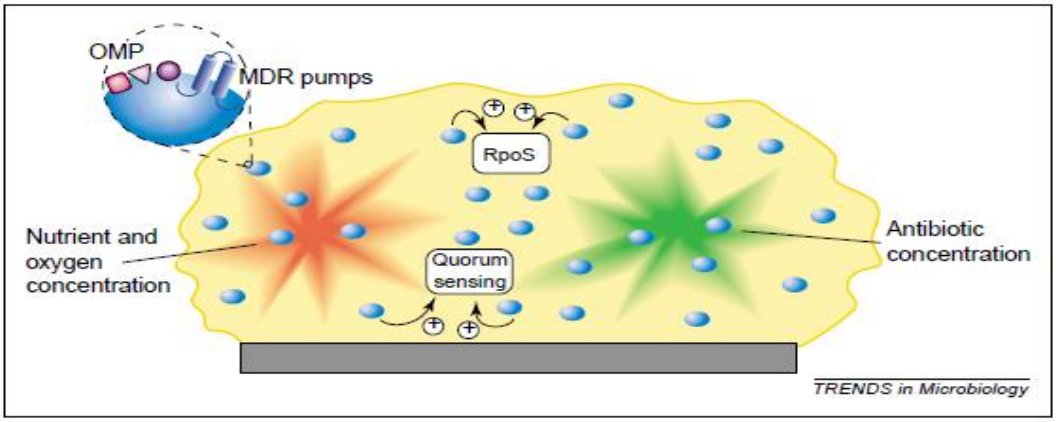

Figure 1.6: Different aspects associated with biofilm resistance. The EPS is represented by yellow and the bacterial cells by blue circles. Quorum sensing, nutrient and oxygen concentration, induction of general stress response, change in profiles of outer membrane proteins and efflux pumps are responsible for resistance of a biofilm (Mah and O'Toole, 2001).

Efflux pump activation is one of the major mechanisms employed by bacteria to confer resistance to different antimicrobial agents (Poole, 2001). Efflux pumps are proteins that are utilized by bacteria to pump out antimicrobial agents, and may either occur as a single or multi-component system (Kvist *et al*., 2008). A typical bacterial cell may have five or more of the different classes of efflux pumps(Fig. 1.7), i.e., the major facilitator (MF) super-family, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family (Poole,2001). The RND class is suggested to be unique for Gram-negative bacteria. Pumping out of the drugs from the bacterial cell by the efflux pump can be drug-specific or class-specific (Poole and Lomovskaya, 2006).

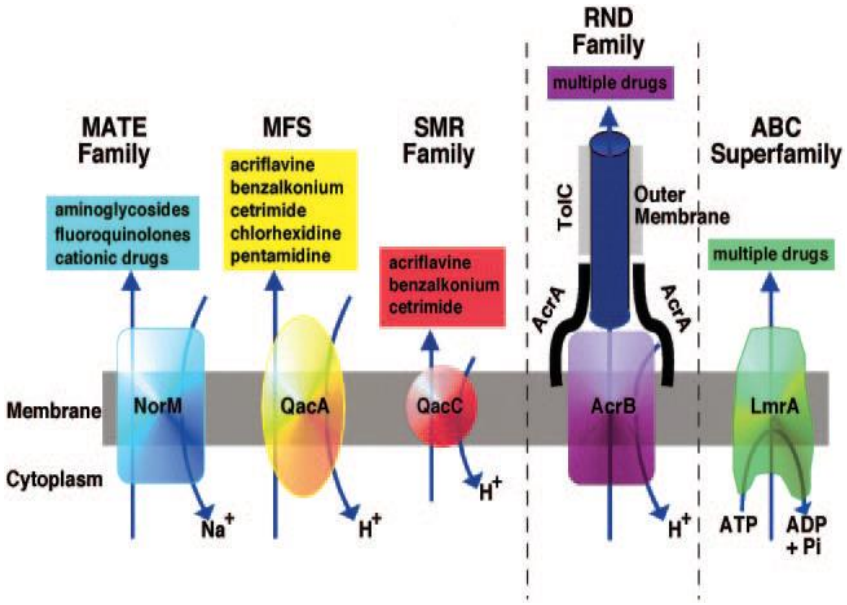

Figure 1.7: Five different efflux pump classes of Gram-negative bacteria:the efflux pump multidrug and toxic compound extrusion (MATE) family, the major facilitator (MF) super-family, the small multidrug resistance (SMR) family, resistance-nodulation-division (RND) family and the ATP-binding cassette (ABC) family (Piddock, 2006).

QSin a biofilm is also important in resistance because signal molecules cause transcription of genes that allows the cells to survive longer in the presence of antimicrobial agents. Butler *et al*. (2010) observed that the high cell density alone of cells within a biofilm is enough to confer resistance at high concentrations of antimicrobial agents. This might be because in biofilm populations, cells run out of nutrients and oxygen which results in

development of resistance. Also when the cell density is high, cells communicate *via*QS confering resistance to the neighbor cell.

Extracellular DNA (eDNA) is one of the major components of the EPS and this matrix inhibits the ability of antimicrobial agents to penetrate within the biofilm. eDNA is suggested to be involved in biofilm formation (Allesen-Holm *et al*., 2006), and it is more effective when it is intact with the EPS than when it is freely released by planktonic cells (Böckelmann*et al*., 2006). Even though biofilms are resistant to a wide variety of antimicrobial agents, many control strategies are being developed.

1.6. Biofilm control strategies

A number of strategies are being pursued in order to eradicate or prevent biofilm formation by diverse microorganisms. Use of enzymes, phages, antimicrobial molecules from microbial origin (Simoes *et al*., 2010), persister cell-destroying substances, phosphorylation inhibitors (benzalkonium chloride, chlorhexidine), electrical current, radio-frequency,electromagnetic fields, ultrasound in combination with antimicrobial agents (del Pozo and Patel, 2007), hydrophilic coatings and a combination of drugs (Francolini and Donelli, 2010) are the commonly used strategies to control biofilms. These agents can be used singly or in combination to inhibit biofilm formation depending on the biofilm under investigation.

1.6.1. Use of antimicrobial agents

The use of antimicrobial agents to treat bacterial pathogens has been the most commonly used method of controlling infections, including biofilm-associated ones. However, the major development of resistance to antimicrobial agents by bacteria is limiting their application in clinical, agricultural andindustrial fields (Francolini and Donelli, 2010). Biofilms are more resistant to antimicrobial agent than planktonic cells due to the presence of different resistance mechanisms within a biofilm (Høiby*et al*., 2010).

Antimicrobial agentscan sometimes be effective when used alone, but they are more effective when used in combination as they provide a synergistic effect (Francolini and Donelli, 2010). Rifampicin killed strains of *Staphylococcus aureus* in a biofilm when used in combination with linezolid (Raad *et al*., 2007), while tobramycin and silver inhibited the growth of micro-organisms (Kim *et al*., 2009). Curtin and Cormican (2003) suggested that the resistance of bacteria to antimicrobial agents does not always mean that the agent has become ineffective, rather that the activity is reduced. They also stated that determining the

lowest effective concentration, i.e, minimum inhibitory concentrations (MIC), is then required. MICs are the lowest concentrations of antimicrobial agents that will inhibit the bacterial growth after overnight incubation (Andrews, 2001). Minimum bactericidal concentrations (MBCs) are used to determine the ability of antibiotics to inhibit the growth of bacteria within a specific time (Pankey and Sabath, 2004). MBCs are defined as the lowest concentration that inhibit growth of the bacteria in the initial inoculumof the subculture. When using antimicrobial agents, MIC and MBC valuesare used to determine the required concentration to control microbial growth. Tobramycin, ticarcillin-clavulanate, and ceftazidime at their MICs, were shown to be effective in inhibiting biofilm formed by *P.aeruginosa* (Francolini and Donelli, 2010). Clindamycin, daptomycin, linezolid, tigecycline and vancomycin were shown to be more effective against *S. aureus* biofilm than against planktonic cells (Perez-Giraldo *et al*., 2003).

In addition to the use of both MIC and MBC, the use of half the amount of the MIC is suggested to be effective. The effective concentration of antimicrobial agents should be above the MIC as suggested by Pompilio *et al*. (2010). However they further suggested that after a certain period of applying these antimicrobial agents, the concentration within a cell becomes lower than the MIC and is called sub-MIC. Sub-MICs do not kill the micro-organisms, however, they change the chemical and physical cell-surface characteristics which affects the functionality and expression of some virulence factors, adhesion, biofilm formation, hydrophobicity and motility (Pompilio*et al*., 2010). Sub-MIC levels of roxithromycin and sansanmycin were observed to inhibit the generation of *P. aeruginosa* biofilms and proliferation of bacteria (Li*et al*., 2009). Moxifloxacin at its sub-MIC caused reduction in biofilm formation of *Stenotrophomonas maltophilia* (Pompilio *et al*., 2010). The sub-MIC of gentamicin was shown to be effective in inhibiting the growth of *Salmonella typhimurium*, when compared to ciprofloxacin and cefotaxime (Landini *et al*., 2010). Even though the sub-MICs are aimed at inhibiting biofilm formation, these concentrations have also been shown to enhance biofilm formation. Haddadin *et al*. (2009) observed that sub-MICs of ciprofloxacin and roxithromycin inhibited *S. aureus* biofilm formation, while sub-MICs of cefalexin increased biofilm formation. They suggested that since cefalexin is a cell wall synthesis inhibitor, its sub-MIC could have affected the cell surface of the bacteria which increased hydrophobicity leading to increased adherence.While the sub-MIC of vancomycin was observed to increase cell density of *S. epidermidis*, the MIC of this antimicrobial agent reduced the cell density (Cargill and Upton, 2009). The authors suggested

that this might be due to the thick staphylococcal walls in response to antimicrobial agents or it maybe because low concentrations might have affected expression of genes involved in biofilm formation.

1.6.2. Use of efflux pump inhibitors

Efflux pump inhibitors (EPIs) are substances that block the activity of the efflux pumps (Kvist *et al*., 2008). Efflux pump inhibitorsblock and deactivate the efflux pumps and when used in combination with antimicrobial agents they increase their activity since they will prevent antimicrobial agents from being pumped out (Kvist *et al.,* 2008).In order for the EPIs to be effective, several factors should be taken into consideration such as: whether the resistance mediated by efflux pump is dominant, EPIs occur in multiples, and also that efflux might work together with other mechanisms responsible for resistance in the bacteria.Effective EPIs are the ones that will make the resistant bacteria susceptible, make the bacteria that acquired resistance from other bacteria susceptible and inhibit the strain that is transferring resistance to other strains (Lomovskaya and Watkins, 2001). EPIs can be used to restore the activity of the antimicrobial agents and to block biofilm formation (Kvist *et al*., 2008).

There are different types of EPIs that can be used to inhibit biofilm formation (Kvist *et al.,* 2008), however,the current study only focused on phenylalanine arginine β-naphthylamide (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP). PAβN and NMP target the resistance-nodulation-cell division (RND) super-family, and CCCP targets the proton motive force. PAβN and NMP affect biofilms directly by binding directly to the target sites, while CCCP affects the energy level of the bacterial membrane. Three efflux pump inhibitors, *viz*: thioridazine, 1-(1-naphthylmethyl) piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) were used by Kvist *et al.* (2008) and inhibition of *E. coli* and *Klebsiella* spp. biofilms wereobtained.They also obtained inhibition of species not belonging to Enterobacteriaceaesuch as *S. aureus* and *P. putida.*PAβN and NMP are competitive inhibitors, which target the RND efflux pumps of Gram-negative bacteria (Kvist *et al.*, 2008). PAβN and NMP were shown toinhibit the biofilm formed by *V.cholerae* (Kvist *et al.*, 2008). Ikonomidis *et al*. (2008) demonstrated that CCCP inhibited biofilm formation by *P. aeruginosa.*

1.6.3. Quorum sensing inhibitors

QS induces expression of virulence factors, pathogenesis (Hentzer *et al.,* 2003) and biofilm formation (Asahi *et al*., 2010) in Gram-negative bacteria. It is thus likely that disruption of this process will inhibit production of virulence factors and biofilm development (Asahi *et al*., 2010). One strategy involves enzymes that degrade QS molecules by a process termed quorum quenching (Tarighi and Taheri, 2011).These enzymes include AHL-lactonases produced by *Bacillus,Variovorax paradoxus, Pseudomonas* spp., *Comamonas* spp.,*Rhodococcus* spp., and AHL-acyclase produced by *Ralstonia* spp. The other strategy includes the use of synthetic compounds and natural products from plants fungi, plants and algae (Kociolek, 2009). Quorum sensing inhibitors (QSIs) are compounds that inhibit cell-to-cell communication within the bacteria population. These compounds can be used to control bacterial species that are infectious without affecting their growth (Hentzer *et al.,* 2003). The QSI molecule that is considered the best candidate must have a low molecular mass and prevent expression of the genes that are controlled by QS. The compounds must be very specific for the QS regulator and should not have toxic effects to the bacteria and also the eukaryotic host (Rasmussen and Givskov, 2006).

The three important target sites for QS inhibition are the signal generator (LuxI homologue), the signal molecule (AHL) and the receptor of the signal (Rasmussen and Givskov, 2006). Different compounds have different mechanisms and different efficaciesin inhibiting biofilm formation (Tarighi and Taheri, 2011). Phytochemicals are now recognized as one of the best QSI candidates (Hentzer *et al.,* 2003). Rio red and Marsh white which are two types of compounds found in grapes were observed to inhibit AI-1 and AI-2 receptor systems in *V.harveyi* (Kociolek, 2009). Cinnamaldehyde inhibited the bioluminescence of *V. harveyi* by blocking AI-1 and also it inhibited AI-2 (Niu and Gilbert, 2004). Cinnamaldehyde was shown to inhibit the growth of different *Pseudomonas*species and *E. coli* (Niu and Gilbert, 2004). The mechanism of inhibition by cinnamaldehyde is not fully understood, however Niu and Gilbert (2004) hypothesized that to inhibit the growth of *E. coli*, cinnamaldehyde might have prevented these bacteria from reaching their substratum. Different concentrations of trans-cinnamaldehyde were used in the study by Amalaradjou *et al*. (2010), who obtained inhibition of uro-pathogenic *E.coli* with all concentrations when compared to the untreated isolates. Kociolek (2009) stated that cinnamaldehyde affects the mass of the biofilm and not the number of viable cells, and thus this inhibitor inhibits biofilm formation in *V. anguillarum* and *V. vulnificus*.

Vanillin, which is a compound from vanilla beans and is used mostly in food industries as a flavoring agent, was shown to inhibit both short and long chain AHLs in *A. hydrophila* (Ponnusamy *et al*., 2009). Vanillin is suspected to interact with AHL receptors and interfere with binding of AHLs to their cognate receptors (Ponnusamy *et al*., 2009).

Synthetic compounds mimic the QS signalling, however, unlike AI-1 and AI-2 these compounds block the signals rather than promoting it (Hentzer *et al.*, 2003). Asahi *et al*. (2010) demonstrated that ten out of 17 AHL analogues that were made by replacing the AHL moiety with different amines and alcohols inhibited biofilm formation of *Porphyromonas gingivalis.* The analogs of *S*-adenosyl methionine are *S*-adenosylhomocysteine, *S*-adenosylcysteine, and sinefungin, and these analogs inhibit synthesis of AHL, thus disrupting QS at its early stages (Hentzer and Givskov, 2003). Hentzer and Givskov(2003) observed that the use of *S*-adenosylmethionine analogs, which are compounds that act as amino group donors during formation of the homoserine lactone ring, were found to have inhibitory activity against *P. aeruginosa. S*-adenosylhomocysteine, sinefungin and butyryl-*S*-adenosyl methionine are suggested to have the ability to inhibit the production of AHLs *in vitro* but not *in vivo*.

Halogenated furanones and usnic acid are the most commonly used inhibitors of Gram-negative bacteria (Francolini and Donelli, 2010). Hentzer *et al.* (2003) observed that halogenated furanones inhibited QS in *P. aeruginosa*, production of virulence factors and biofilm formation without interfering with its growth. Halogenated furanones act as competitive inhibitors by binding to regulatory protein and preventing AHLs from binding to the regulatory protein and thus disrupting QS (Landini *et al*., 2010). 4-hydroxy-2,5-dimethyl-3(2H) furanonewas shown to inhibit the growth of *A. hydrophila* (Ponnusamy *et al*, 2010) and *Hafnia alvei*which is an opportunistic pathogen associated with noscomial infections and typically isolated from fish and meat (Viana *et al.*, 2009). Raina *et al*. (2009) observed that (5*Z*)-4-bromo-5-bromomethylene-3-butylfuran-2(5*H*)-one inhibited swarming motility and biofilm formation in *E. coli* by interfering with AI-2.

1.6.4. Use of matrix-degrading enzymes

EPS of a biofilm is composed of proteins, polysaccharides, lipids and eDNA and these can be used as targets of degrading enzymes. When a biofilm forms, the cells first attach to the surface by weak interactions, followed by strong interactions which are followed by production of the matrix. The matrix also plays an important role in inhibiting the penetration

of the antimicrobial agents intothe biofilm (del Pozo and Patel, 2007). The need for enzymes that degrade the matrix and makes the cells within the biofilm accessible is necessary.

Enzymes such as dispersin B (del Pozo and Patel, 2007; Francolini and Donelli, 2010; Simoes *et al*., 2010) and some other proteases and polysaccharides-hydrolyzing enzymes (Simoes *et al*., 2010) are used in controlling biofilms due to their ability to digest the extracellular matrix.Chaignon *et al.*(2007) observed that dispersin B degraded poly-N-acetylglucosamine and reduced the biomass of *S.epidermidis*. Proteinase K and trypsin were also shown to reduce the biomass of *S. epidermidis* by degrading the peptide bonds.When serine protease, α-amylase and polysaccharidase were used to treat 16 different food bacterial species, serine protease was shown to be the mosteffective in removing biofilm formed by those species, followed by α-amylase (Lequette *et al*., 2010).

The presence of eDNA has been suggested to be responsible for biofilm formation in other bacterial species, however, for *Aeromonas*information is still limited. Evidence of the presence of eDNA was provided by Tetz and Tetz (2010) who observed a 30 kb eDNA in *S. aureus* and Böckelmann *et al*. (2006) who observed a 29 kb eDNA in an unspecified F8 isolate (suspected to be Gammaproteobacterium or *Rheinheimera baltica*). eDNA interconnects the matrix component of cells within a biofilm (Allesen-Holm*et al*., 2006). The exact mechanism by which eDNA influences biofilm formation is not well understood. However, Tetz and Tetz (2010) observed that the shape of the biofilms treated with DNase I was different from the untreated biofilm. They also observed that biofilm cells that received DNase I treatment had formed a mesh-like structure containing increased area of cell free zones. Biofilms of *P. aeruginosa* were observed to be affected after the addition of DNase I (Allesen-Holm *et al*., 2006). Cleavage of extracellular DNA by DNase I through its exonuclease activity is the mechanism employed to reduce the biomass of a biofilm (Tetz *et al*., 2009).

1.7. Rationale for the study

It is estimated that approximately 99% of bacteria form biofilms to survive (de Carvalho, 2007). *Aeromonas* spp. isolates are one of the major biofilm forming species in aquatic environments and are often associated with fish diseases and human (food and water-borne) infections. These bacteria have been identified in medical and industrial biofilms, resulting in their association with a wide variety of medical and industrial problems. Biofilm formation is not only an important stage in the pathogenicity of organism but it limits the effectiveness of

antimicrobial therapy, protects against host defence mechanisms and also facilitates bacterial communication QS leading to the expression of virulence determinants. Understanding the effect of different biofilm inhibitors such as antimicrobial agents, lytic enzymes, phytochemicals and EPIs on biofilm formation by *Aeromonas* spp. isolates is critical as it could facilitate removal of these biofilms either clinically or in an aquaculture environment. These would then be solutions to limit infections caused by aeromonad biofilms in man or in fish.

It is hypothesized that biofilm formation by *Aeromonas* spp. may be limited or completely eradicated with the use of antimicrobial agents, lytic enzymes, EPIsor QSIs. It is further hypothesized that *Aeromonas* spp. isolates from different sources communicate with each other by producing AHL signalling molecules, which may display diversity from other known *Aeromonas* spp.

1.8. Objectives

The following objectives have been established:

1.8.1. To investigate strategies to inhibit *Aeromonas* spp. biofilm formation and QS; and

1.8.2. To identify the ability of *Aeromonas* spp.isolates to communicate by producing signalling molecules.

1.9.Aims

The following aims will be pursued:

1.9.1. To determine the MIC of azithromycin, ciprofloxacin, ceftazidime, gentamicin, and tetracyclineagainst *Aeromonas*spp;

1.9.2. To identify the prevalence and diversity of efflux pumps in *Aeromonas* spp.isolates using the disk diffusion assay on Mueller-Hinton (MH) agar containing EPIs;

1.9.3. To investigate the effect of EPIs [carbonyl cyanide 3-chlorophenylhydrazone, phenylalanine arginine β-naphthylamide or 1-(1-naphthylmethyl)-piperazine]on initial attachment and detachment using microtiter plate assays;

1.9.4.To determine the inhibition of adhesion or detachment from pre-formed biofilms using microtiter plate assays, in the presence of;

1.9.4.1. Antimicrobial agents (tetracycline, azithromycin, ciprofloxacin, ceftazidime, gentamicin);

1.9.4.2. Lytic enzymes (DNase I);

1.9.4.3. QS inhibitors [4-hydroxy-2,5-dimethyl-3(2H) furanone and *S*-adenosylhomocysteine], and

1.9.4.4. Phytochemicals (vanillin and cinnamaldehyde);

1.9.5. To identify the expression of QS signalling molecules by *Aeromonas* spp. isolates using biosensors:

1.9.5.1. *C.violaceum* CV026; and

1.9.5.2. *A.tumefaciens* A136.

1.10. Questions to be answered

A number of specific questions are relevant to this topic:

1.10.1. Does exposure to varying concentrations of antimicrobial agents significantly reduce biofilm formation?

1.10.2. Which antimicrobial agents are effective against aeromonad biofilms?

1.10.3. Do *Aeromonas* spp. isolates demonstrate the efflux phenotype?

1.10.4. Do antimicrobial agents, lytic enzymes, EPIs and QSIs inhibit or increase bacterial adhesion and/or detachment from biofilms?

1.10.5. What effect do the phytochemicals have on *Aeromonas* spp. biofilm formation and/or QS?

CHAPTER 2

Characterization of biofilm-associated *Aeromonas* spp. resistance to antimicrobial agents and the effect of sub-MIC, MIC and supra-MIC antimicrobial agent exposures

2.1. Introduction

Biofilms are more resistant to antimicrobial agents compared to planktonic due to the activation of diverse resistance mechanisms that comes with cell density. They contain EPS, which inhibits penetration of antimicrobial agents within the biofilm. Resistance can also occur due to lack of oxygen and nutrients as well as accumulation of waste (Dhar and McKinney, 2007). Another major contributing mechanism is the presence of persister cells, which are a sub-population of cells that can withstand high doses of antimicrobial agents. Persister cells are responsible for the persistence of biofilms in the presence of certain types of antimicrobial agents (Gefen and Balaban, 2009). Keren *et al*. (2004) observed that *P. aeruginosa* which was tested for the formation of persister cells was not inhibited by ofloxacin throughout its growth phase. When the same test was perfomed with *S. aureus* it was observed that this species was not inhibited with ciprofloxacin and penicillin (Keren *et al*., 2004). Eventhough bacteria are resistant to antimicrobial agents, they remain the better candidates to treat infections due to ease of production and their affordability.

In order to determine the correct concentration of antimicrobial agents to use when treating infections, the MIC is used (Gould and MacKenzie, 2002). MIC helps to determine if the concentration of the antimicrobial agents should be reduced, increased or if it should remained unchanged. The correct MIC to use can vary between the types of drugs used or between bacterial species. The concentration below the MIC or the concentrations incapable of causing death but affecting the functionality of the cell are called sub-MICs and when these concentrations are doubled they are called supra-MIC. The sub-MIC is generally less effective when compared to the MIC and the supra-MIC.Supra-MIC is likely tobe more effective than sub-MIC and MICexposures. Sub-MIC affects different factors of bacteria such as morphology, virulence, ability to produce genetic variation (Couce and Blazquez, 2009), alteration of cell surface, inhibition of enzyme and toxin production and lastlysuppression of bacterial adhesion to host cells (Wojnicz and Jankowski, 2007). Høiby *et al*. (2010) suggested that sub-MICexposure of β-lactam induces biofilm formation of *P. aeruginosa* without affecting its growth. Landini *et al*. (2010) observed that MIC exposure of gentamicin was more effective in inhibiting the biofilm of *Salmonella typhimurium*. Takahashi *et al.*(2007)

observed that the biofilm of *Actinobacillus actinomycetemcomitans* was resistant to ofloxacin, tetracycline minocycline, ampicillin, erythromycin and cefalexin at their MICs. The same drugs were more effective against the biofilm of *A. actinomycetemcomitans* at higher concentration than their MICs (Takahashi *et al*., 2007). This,therefore,suggests that the use of Minimum biofilm inhibitory concentrations (MBICs) is required, which usually is investigated for cells within the biofilm that are protected by EPS which provide resistance together with other resistance mechanisms (Reiter *et al*., 2013). This might explain why MBICs are usually higher than MICs which are determined against planktonic cells (Garcia-Castillo *et al*., 2007).The following study aimed to compare the MICs of planktonic cells and MBICs of *Aeromonas* spp. biofilms.The study further determined the effect of of sub-MIC, MIC and supra-MIC exposures of five antimicrobial agents on initial attachment and biofilm detachment.

2.2. Material and Methods

2.2.1. Maintenance of bacterial cultures

Forty-eight *Aeromonas* spp. isolates and six *Plesiomonas shigelloides*isolates (Table 2.1) from catfish, koi-carp, tilapia and sea water were selected for study (Duma, 2012). The current study also included type strains (*A. caviae* ATCC 15468^{T} and *A. hydrophila* ATCC 7966^{T}). Isolates were maintained on tryptic soy agar (TSA) plates at 4 ºC and for long-term storage in TSB containing 20% glycerol at -70 ºC (Jacobs and Chenia, 2007).

Table 2.1: List of study isolates, their respective species designation and source of isolation

Isolate code	Species name	Source of isolates
M2	*A. hydrophila*	Catfish
M5	*A. hydrophila*	Catfish
M6	*A. hydrophila*	Catfish
M13	*A. hydrophila*	Catfish
M14	*A. hydrophila*	Tilapia
M17	*A. hydrophila*	Tilapia
M50	*A. hydrophila*	Catfish
M51	*A. hydrophila*	Catfish
M52	*A. hydrophila*	Tilapia
M53	*A. hydrophila*	Catfish
M60	*A. hydrophila*	Tilapia
M62	*A. hydrophila*	Tilapia
M64	*A. hydrophila*	Tilapia

M65	*A. hydrophila*	Tilapia
M86	*A. hydrophila*	Koi-carp
M94	*A. hydrophila*	Koi-carp
M95	*A. hydrophila*	Koi-carp
M22	*A. culicicola*	Sea water
M23	*A. culicicola*	Sea water
M25	*A. culicicola*	Sea water
M31	*A. culicicola*	Sea water
M32	*A. culicicola*	Sea water
M38	*A. culicicola*	Sea water
M39	*A. culicicola*	Sea water
M58	*A. culicicola*	Tilapia
M70	*A. bestiarum*	Koi-carp
M72	*A. bestiarum*	Koi-carp
M80	*A. bestiarum*	Koi-carp
M81	*A. bestiarum*	Koi-carp
M88	*A. bestiarum*	Koi-carp
M90	*A. bestiarum*	Koi-carp
M96	*A. bestiarum*	Koi-carp
M99	*A. bestiarum*	Koi-carp
M26	*Aeromonas* spp. 45	Sea water
M34	*Aeromonas* spp. 45	Sea water
M41	*Aeromonas* spp.	Sea water
M55	*A. veronii*	Tilapia
M57	*A. veronii*	Tilapia
M63	*A. veronii*	Tilapia
M18	*A. caviae*	Tilapia
M59	*A. caviae*	Tilapia
M68	*A. caviae*	Koi-carp
M76	*A. salmonicida*	Koi-carp
M77	*A. salmonicida*	Koi-carp
M8	*A. allosaccharophila*	Tilapia
M92	*A. allosaccharophila*	Koi-carp
M28	*A. jandaei*	Sea water
M49	*A. sobria*	Tilapia
M9	*Plesiomonas shigelloides*	Catfish
M45	*Plesiomonas shigelloides*	Tilapia
M46	*Plesiomonas shigelloides*	Tilapia
M47	*Plesiomonas shigelloides*	Tilapia
M66	*Plesiomonas shigelloides*	Tilapia
M67	*Plesiomonas shigelloides*	Tilapia
ATCC 15468^{T}	*A. caviae*	Type strain
ATCC 7966^{T}	*A. hydrophila*	Type strain

2.2.2. Determination of minimum inhibitory concentrations (MICs) using broth microdilution assays

Twenty-eight *Aeromonas* spp. isolates (Table 2.1) as well as the type strains were selected based on their biochemical and physiological characteristics for the determination of MICs of planktonic cells for five antimicrobial agents. Five antimicrobial agents [azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), and tetracycline (TET)] were tested against the isolates using thirteen concentrations: 0.008, 0.016, 0.064, 0.125, 0.5, 1, 2, 4, 16, 32, 64, 128 and 256 μg/ml. MICs of the various planktonic cultures for each of the selected antimicrobial agents were determined using the broth microdilution assay (Andrews, 2001). Two-fold serial dilutions of antimicrobial agents were prepared in Mueller-Hinton (M-H) broth. Cultures were grown overnight in TSB, washed three times with sterile distilled water and diluted until they were equivalent to a 0.5 MacFarland standard (Andrews, 2001). Microtiter plate wells, each containing 100 μl of M-H broth medium with the required antimicrobial agent concentration, were inoculated with 10 μl of cell suspension and incubated at 30 ºC for 24 h without shaking. The negative control wells contained M-H broth only and the positive control wells contained the respective cell suspensions with no antimicrobial agents added. This was done in triplicate, on two separate occasions (Andrews, 2001). TheMIC was the lowest concentration of antimicrobial agent, which inhibited visible growth of organism.

2.2.3. Determination ofMBICs of biofilm cells

Twenty-eight *Aeromonas* spp. isolates as well as the type strains were also used for the determination of MBICs of biofilm-forming isolatesfor five antimicrobial agents. Cultures were grown overnight in TSB, washed three times with sterile distilled water and diluted until they were equivalent to a 0.5 McFarland standard (Andrews, 2001).

MBICs of cells were determined using a modified microtiter plate assay. Biofilms were formed at 30ºCfor 24 husing M-H broth. Once the biofilms had formed, planktonic cells were washed off and the wells were air-dried. Serial dilutions of antimicrobial agents (azithromycin, ciprofloxacin, ceftazidime, gentamicin, and tetracycline) were added to 100 μl of fresh M-H broth at the required antimicrobial agent concentrations and transferred to wells to determine MBICs of the biofilm cells. Wells, in triplicate, contained 0.008, 0.5, 12, 32, 256, 1024, 2048, and 4096 μg/ml, respectively, of the antimicrobial agents to be tested. Plates were incubated for further 24 h at 30 ºC. The negative control wells contained M-H broth

onlyand the positive control wells contained the respective cell suspensions with no antimicrobial agents added.

Contents of each well wereaspirated, washed three times with 250 µl of sterile distilled water and the remaining cells were fixed with 200 µl of methanol for 15 min. After air-drying, wells were stained with 150 µl of 2% Hucker‘s crystal violet for 5 min. Excess crystal violet was removed by gently rinsing plates under running tap water. Dye bound to the adherent cells was resolubilized with 150 µl of 33% (v/v) glacial acetic acid and the optical density (OD) of each well was obtained at 595 nm using a Multiskan reader (Ascent F1, Thermolabsystems). Tests were done in triplicate, on two separate occasions and the results averaged. The cut-off OD (ODc) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control (Basson *et al*., 2008). MBICs were indicated by concentrations where the OD was ≤ 0.5.

2.2.4. Effect of varying antimicrobial agent concentrations on biofilm formation

The effect of the sub-MIC, MIC and supra-MIC exposures of the five selected antimicrobial agents (azithromycin, ceftazidime, ciprofloxacin, gentamicinand tetracycline) on initial attachment and/or biofilm detachment was determined using a modified microtiter assay(Basson *et al*., 2008). MIC values were determined as described in section 2.2.3. Two treatments were investigated, i.e., exposure of cultures at the time of attachment and exposure after 24 h biofilm formation. Bacterial cultures were grown overnight at 30 ºC for 16 h, and microtiter plate assays were set up as described in section 2.2.3. For the initial attachment assay, isolates were exposed to sub-MIC (0.5×MIC), MIC, and supra-MIC (2×MIC) amounts of antimicrobial agents at the time of inoculation.For the effect on mature biofilm, 24 h biofilms wereexposed to sub-MIC, MIC and supra-MICs of antimicrobial agent and incubated for a further 24 h.

Contents of each well were aspirated, washed three times with 250 µl of sterile distilled water and the remaining cells were fixed with 200 µl of methanol for 15 min. After air-drying, wells were stained with 150 µl of 2% Hucker’s crystal violet for 5 min. Excess crystal violet was removed by gently rinsing plates under running tap water. Dye bound to the adherent cells was resolubilized with 150 µl of 33% (*v*/*v*) glacial acetic acid, and the optical density (OD) of each was obtained at 595 nm using a Multiskan reader (Ascent F1, Thermolabsystems) with a 595 nm filter. Tests were done in triplicate, on two separate

occasions and the results were averaged (Basson *et al*., 2008). Optical density (OD_{595} nm) in the presence of sub-MIC, MIC or supra-MIC of each antimicrobial agent was compared to that of control wells without antimicrobial agent exposure, to determine the effect of antimicrobial agent on adhesion or detachment.A measure of efficacy called Percentage biofilm reduction was calculated from the blank, control, and treated absorbance values (Pitts *et al*., 2003):

Percentage reduction $= \left[\frac{(C-B)-(T-B)}{C-B}\right] \times 100$, where B denotes the average absorbance per well for blank wells (no biofilm, no treatment), C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

2.2.5. Statistical analysis

One-way repeated measures ANOVA and Student's t-tests (SigmaStat) were used to examine the statistical significance of treated vs untreated assays for initial attachment and biofilm detachment assays. A *p* value of <0.05 was considered significant.

2.3. Results

2.3.1. Determinationof minimum inhibitory concentrations for*Aeromonas*spp. And *P. shigelloides* isolates

While theazithromycin MICs of *Aeromonas* spp. ranged from 0.5-64 μg/ml, the ceftazidimeMICs ranged from 0.064-128 μg/ml (Table 2.2). The ciprofloxacin MICs ranged from 0.064-12 μg/ml and the gentamicin MICs ranged from 0.0048-32 μg/ml (Table 2.2). The tetracycline MICs ranged from 6-32 μg/ml, with the majority of isolates displaying MICs of 12 and 32 μg/ml (Table 2.2). The majority of the isolates displayed MIC levels ranging from 2-32 μg/ml.

Table 2.2: Minimum inhibitoryand minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolates

		Azithromycin		Ceftazidime		Ciprofloxacin		Gentamicin		Tetracycline	
Isolates	**Species**	**MIC***	**MBIC**	**MIC**	**MBIC**	**MIC**	**MBIC**	**MIC**	**MBIC**	**MIC**	**MBIC**
		(µg/ml)	***(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**	**(µg/ml)**
M2	*A. hydrophila*	12	4096	64	4096	12	4096	4	4096	32	4096
M17	*A. hydrophila*	32	4096	12	> 4096	12	> 4096	4	> 4096	32	4096
M51	*A. hydrophila*	0.5	4096	32	2048	4	4096	12	4096	12	2048
M64	*A. hydrophila*	64	4096	0.064	> 4096	4	4096	32	4096	12	4096
M94	*A. hydrophila*	2	4096	12	4096	2	4096	4	> 4096	32	4096
M95	*A. hydrophila*	12	4096	32	4096	2	4096	2	4096	12	> 4096
M70	*A. bestiarum*	64	4096	4	4096	4	4096	2	4096	12	4096
M88	*A. bestiarum*	12	4096	1	4096	4	4096	2	4096	32	4096
M90	*A. bestiarum*	2	1024	32	256	4	12	12	12	12	12
M96	*A. bestiarum*	12	4096	12	4096	12	2048	2	2048	32	2048
M23	*A. culicicola*	12	1024	64	2048	12	256	4	256	32	2048
M31	*A. culicicola*	12	4096	12	1024	4	2048	12	4096	32	4096
M38	*A. culicicola*	4	2048	64	256	12	256	4	2048	32	4096
M58	*A. culicicola*	12	1024	32	2048	0.064	256	0.008	256	12	2048
M55	*A. veronii*	12	4096	64	4096	4	4096	4	2048	32	4096
M57	*A. veronii*	0.5	4096	32	4096	4	4096	2	> 4096	32	> 4096
M63	*A. veronii*	64	4096	64	> 4096	12	4096	12	4096	32	4096
M18	*A. caviae*	12	4096	32	4096	12	2048	12	2048	32	4096
M59	*A. caviae*	12	2048	4	2048	1	4096	4	2048	32	4096
M76	*A. salmonicida*	32	4096	128	4096	12	2048	12	4096	32	2048

M77	*A. salmonicida*	64	4096	12	4096	4	4096	2	4096	12	4096
M41	*Aeromonas* spp.	12	1024	4	4096	4	> 4096	12	4096	12	2048
M92	*A. allosaccharophila*	12	1024	32	4096	4	> 4096	32	4096	32	1024
M28	*A. jandaei*	32	4096	32	4096	1	> 4096	4	4096	12	> 4096
M49	*A. sobria*	12	4096	1	2048	4	4096	2	4096	6	4096
M9	*P. shigelloides*	32	2048	4	2048	1	4096	12	2048	32	4096
M46	*P. shigelloides*	12	2048	12	2048	12	1024	32	4096	32	1024
M67	*P. shigelloides*	12	1024	32	4096	4	> 4096	1	4096	12	2048

*MIC = Mininimum inhibitory concentration; MBIC = Mininimum biofilm inhibitory concentration.

2.3.2. Determination of minimum biofilm inhibitory concentrations for *Aeromonas* and *P. shigelloides* spp. isolates

The MBICs for AZM, CAZ, CIP, CN, and TET of selected isolates is summarised in Table 2.2, together with the respective MICs. The least effective concentration against isolate M2 for azithromycin was 12 μg/ml as it induced biofilm formation (Fig. 2.1). However, from 1024 to 4096 μg/ml, inhibition of biofilm was observed to increase as the concentration increased. With ceftazidime, 0.008 μg/ml was observed to be more effective than 0.5, 12 and 32 μg/ml (Fig. 2.1). However, with an increase in concentration from 256 to 4096 μg/ml, ceftazidime inhibited biofilm formation. The least effective concentration for ciprofloxacin was 256 μg/ml and from 1024 to 4096 μg/ml it inhibited biofilm formation (Fig. 2.1). Gentamicin induced biofilmat 0.008 μg/ml and it was also less effective at 0.5 and 12 μg/ml. It was observed that gentamicin increased inhibition of biofilm formation as the concentration increased from 32 to 4096 μg/ml. Tetracycline induced biofilm formation at 0.5 μg/ml, however, it was observed that from 32 to 4096 μg/ml, tetracycline inhibited biofilm formation as the concentration increased (Fig. 2.1).

With isolate M17, it was observed that the most effective concentrations of azithromycin to inhibit biofilm formation were 32, 2048 and 4096, respectively (Fig. 2.2). The least effective concentration was 0.008 μg/ml. The efficiency of ceftazidme to inhibit biofilm formation was inconsistent, and 0.008 μg/ml was more effective than 0.5, 256 and 1024 μg/ml in inhibiting biofilm formation, but less effective than 12, 2048 and 4096 μg/ml which were the most effectiveconcentrations (Fig. 2.2). With ciprofloxacin, 4096 μg/ml was more effective in inhibiting biofilm formation and 12 μg/ml followed by 0.008 μg/ml was less effective. Gentamicin induced biofilm formation at 0.008 μg/ml, and it was more effective in inhibiting biofilm formation as the concentration increased from 0.5 to 4096 μg/ml. Tetracycline was more effective in inhibiting biofilm formation from 1024 to 4096 μg/ml, while at 0.008, 12 and 256 μg/ml it was observed that tetracycline was less effective (Fig. 2.2).

It was observed that azithromycin induced biofilm of isolate M51 at 0.008 μg/ml (Fig. 2.3). Inhibition of biofilm was observed to increase with the concentration starting from 32 to 4096 μg/ml. Ceftazidime increased inhibition of biofilm as the concentration increased (0.008 to 4096 μg/ml). It was observed that 0.008 μg/ml of ciprofloxacin was more effective in inhibiting biofilm of isolate M51than 0.5 and 12 μg/ml (Fig. 2.3). However, as the concentration increased from 32 to 4096 μg/ml, ciprofloxacin was more effective in

inhibiting biofilm formation. Gentamicin induced biofilm formation at 0.008 and 0.5 μg/ml. It was observed that from 12 to 4096 μg/m of gentamicin, biofilm inhibition increased with antimicrobial agents. Tetracycline was more effective in inhibiting biofilm formation as the concentration increased from 0.008 to 4096 μg/ml (Fig. 2.3).

For isolate M64, azithromycin was more effective at inhibiting the biofilm as the concentration increased (0.008-4096 μg/ml) (Fig. 2.4). With ceftazidime, 0.008 μg/ml and 12 μg/ml were less effective and inhibition of biofilm formation was observed from 256 to 4096 μg/ml. At 0.008 and 0.5 μg/ml of ciprofloxacin, induction of biofilm formation was observed. Inhibition of biofilm formation with ciprofloxacin was shown to be more effective as the concentration increased from 32 to 4096 μg/ml (Fig. 2.4). Gentamicin inhibited biofilm formation of M64 as the concentration increased from 256 to 4096 μg/ml. The concentrations of gentamicin that induced biofilm formation were 0.008 and 12 μg/ml. Tetracycline induced biofilm formation of M64 at 0.5μg/ml and it was more effective at 4096 μg/ml (Fig. 2.4).

All antimicrobial agents were effective against biofilm of isolate M94 (Fig. 2.5). For azithromycin, 0.008 and 0.5 μg/ml were less effective compared to other concentrations. The most effective concentration to inhibit biofilm formation was 4096 μg/ml. Ceftazidime and tetracycline behaved in a similar manner, and from 0.008 to 32 μg/ml inhibition of biofilm was observed (Fig. 2.5). However,at 256 μg/ml the antimicrobial agents became less effective compared to 32 μg/ml and from 1024 to 4096 μg/ml these two antimicrobial agents increased inhibition of biofilm formation (Fig. 2.5). For ciprofloxacin, the less effective concentrations were 0.5 μg/ml, followed by12 μg/ml. It was observed that the most effectiveconcentration of ciprofloxacin was 4096 μg/ml. Gentamicin was more effective in inhibiting biofilm formation as the concentration increased (0.008 to 4096 μg/ml) (Fig. 2.5).

Azithromycin and tetracycline induced biofilm formation of isolate M95 at 0.008 and 0.5 μg/ml, ceftazidime and ciprofloxacin induced biofilm formation at 0.008 and 0.5 μg/ml, respectively (Fig. 2.6). Tetracycline also induced biofilm formation at 12 μg/ml. From 32 to 2048 μg/ml all antimicrobial agents were effective and more effective at 4096 μg/ml in inhibiting biofilm formation (Fig. 2.6).

With azithromycin it was observed that only 0.008 μg/ml was the least effective concentration, while other concentrations were effective in inhibiting biofilm formation of *A. hydrophila* ATCC 7966^T (Fig. 2.7). The most effectiveconcentration of azithromycin was 2048 μg/ml. Ceftazidime was least effective against *A. hydrophila* ATCC 7966^T at 0.008, 1024 and 32 μg/ml. The most effectiveconcentration for ceftazidime was 12 μg/ml.

Ciprofloxacin induced biofilm formation at 0.5 μg/ml (Fig. 2.7). All other concentrations were observed to be less effective when compared to 4096 μg/ml which was the most effective concentration. Gentamicin induced biofilm formation of this isolate at 0.008 μg/ml, which was followed by 0.5 and 12 μg/mlwhich were also less effective. Gentamicin increased inhibition of biofilm formation as the concentration increased from 32 to 4096 μg/ml. The least effective concentration of tetracycline was 12 μg/ml followed by 256 μg/ml, and the most effectiveconcentration was 4096 μg/ml (Fig. 2.7).

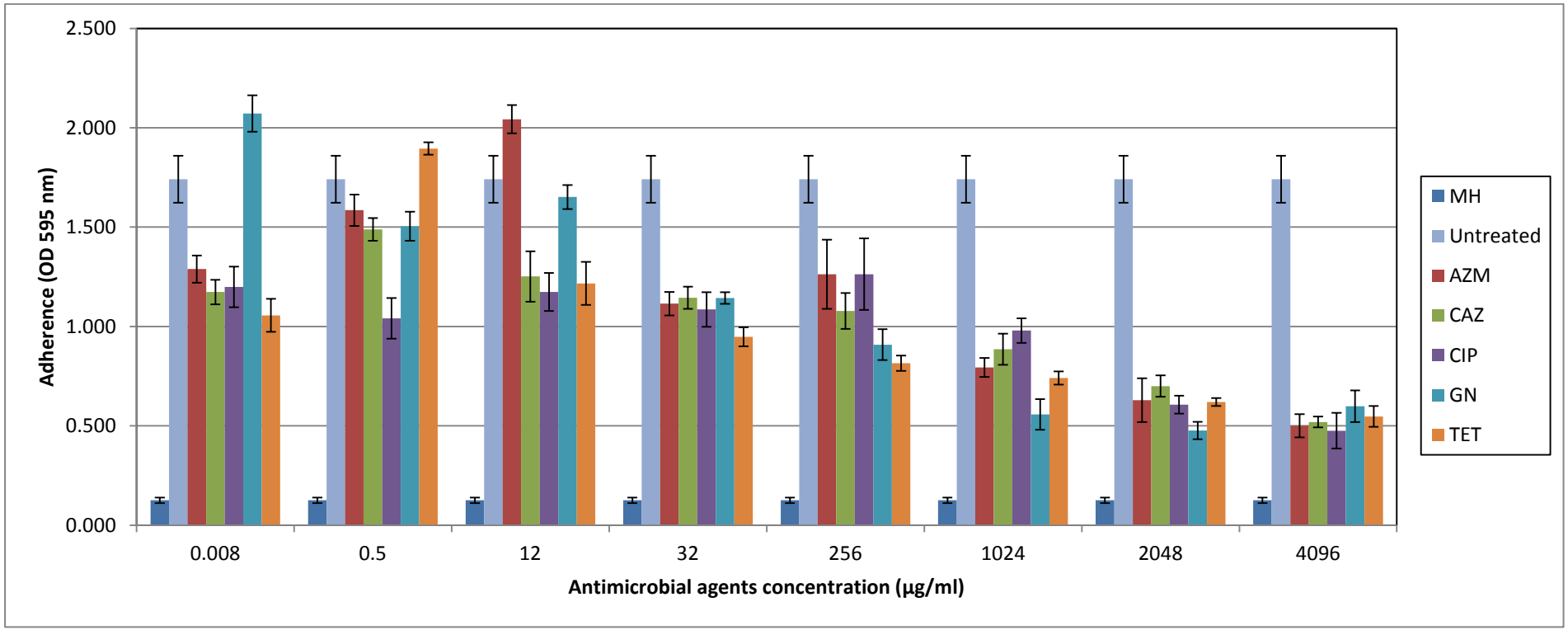

Figure 2.1: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M2 to identify minimum biofilm inhibitory concentrations.

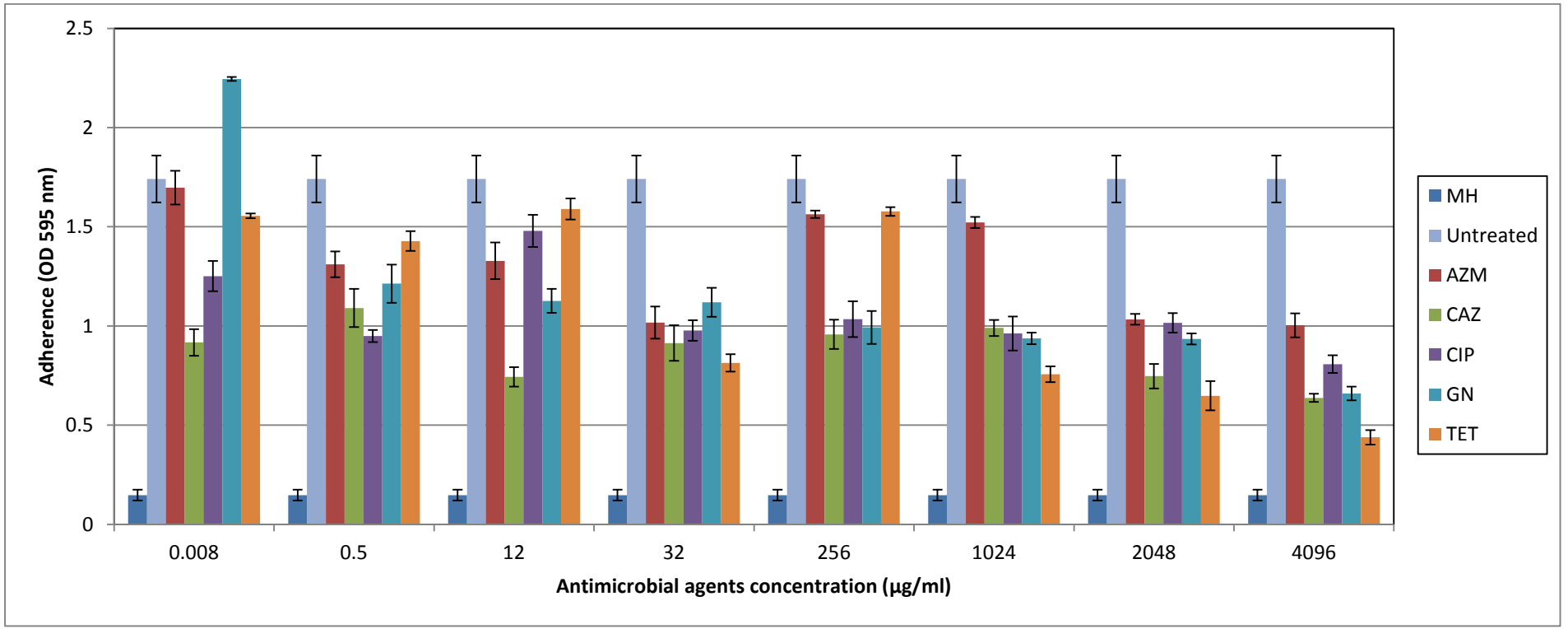

Figure 2.2: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M17 to identify minimum biofilm inhibitory concentrations.

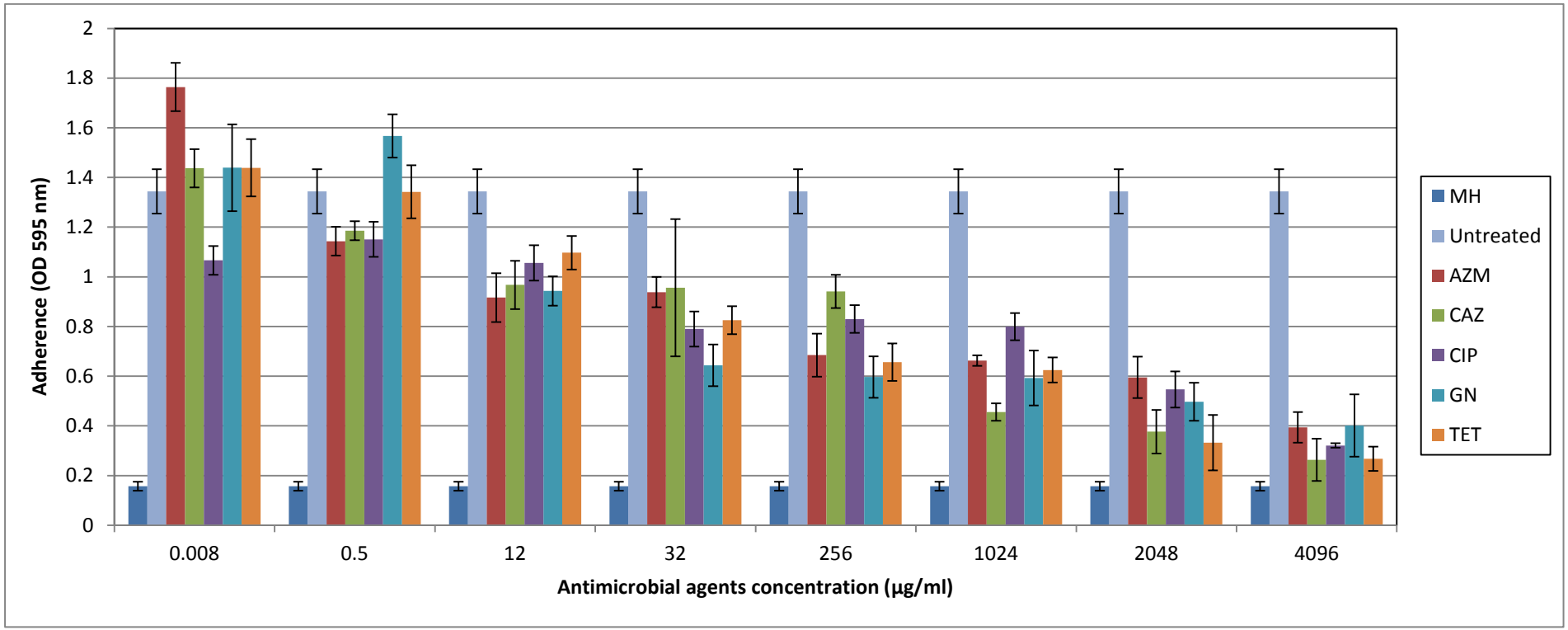

Figure 2.3: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M51 to identify minimum biofilm inhibitory concentrations.

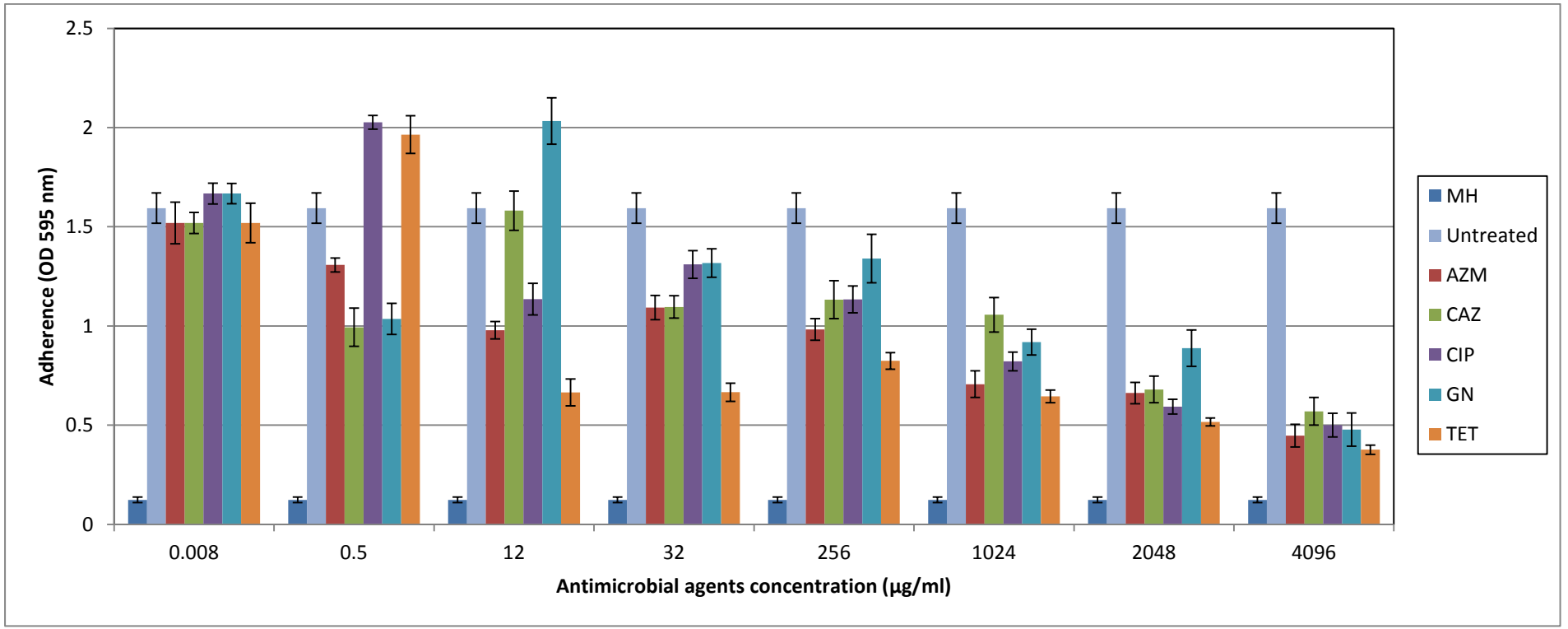

Figure 2.4: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M64 to identify minimum biofilm inhibitory concentrations.

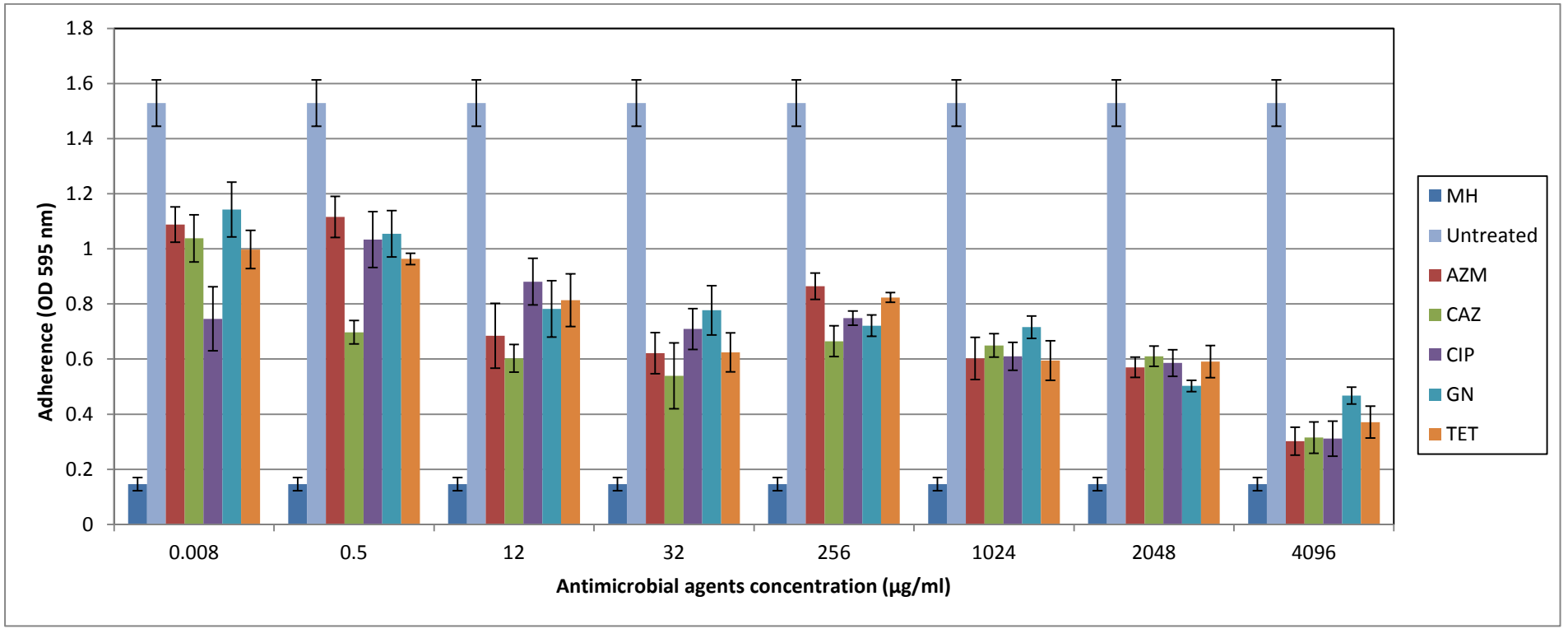

Figure 2.5: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M94 to identify minimum biofilm inhibitory concentrations.

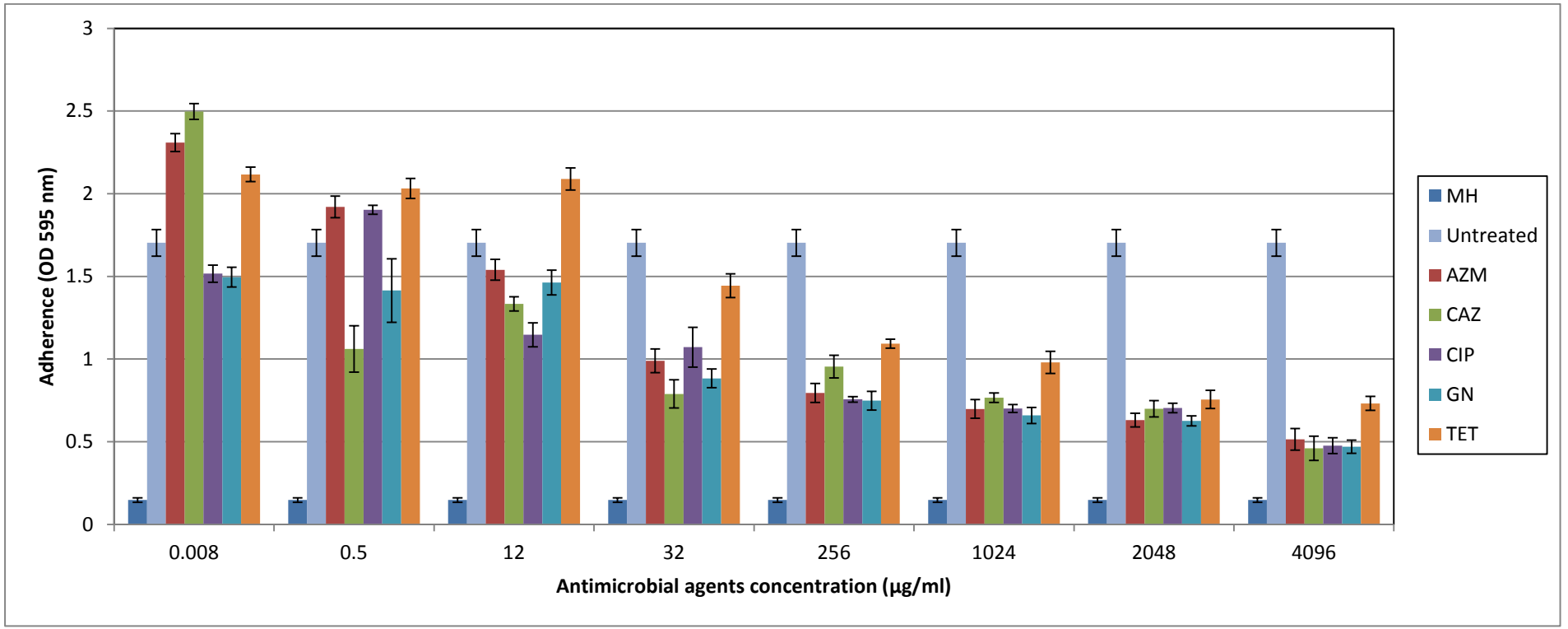

Figure 2.6: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate M95 to identify minimum biofilm inhibitory concentrations.

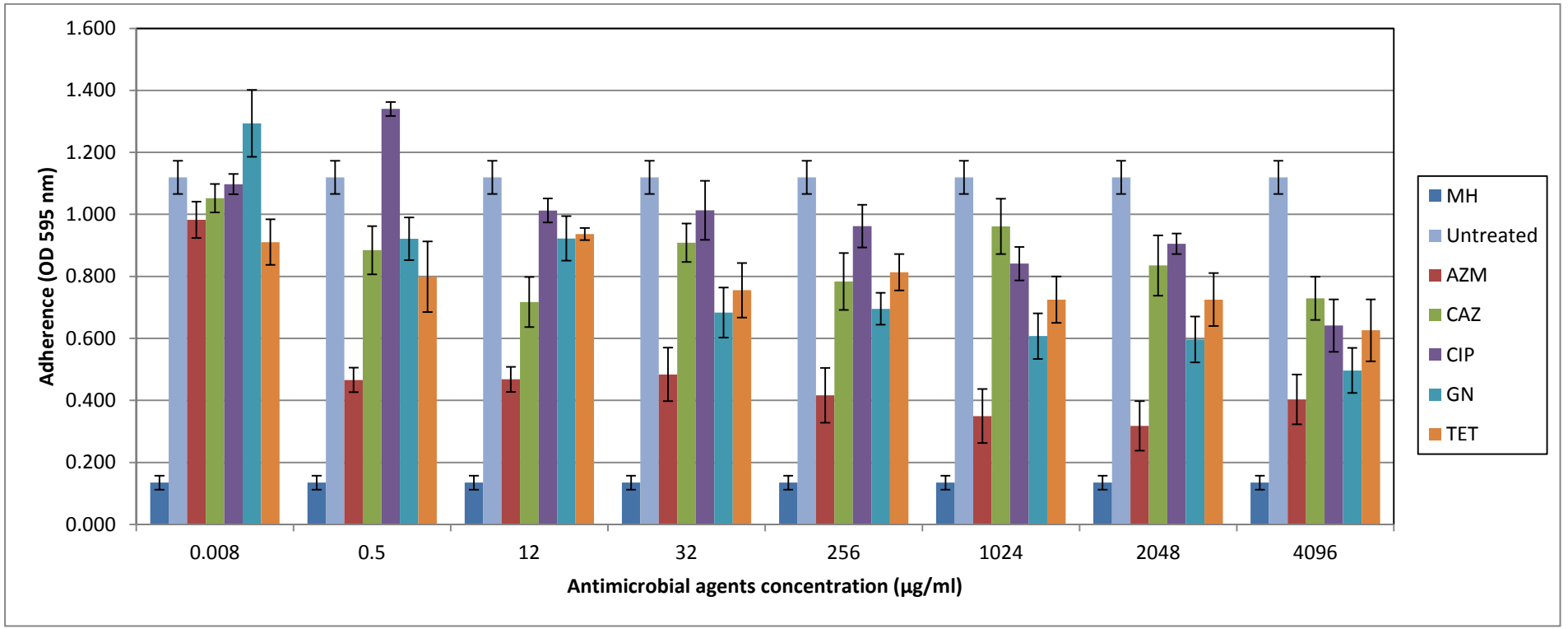

Figure 2.7: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. hydrophila* isolate ATCC 7966^{T} to identify minimum biofilm inhibitory concentrations.

It was observed that 0.5 μg/ml of azithromycin was less effective than 0.008 μg/ml in inhibiting isolate M18 biofilm and from 12 to 4096 μg/ml biofilm inhibition increased with increasing concentrations (Fig. 2.8). With ceftazidime, inhibition of biofilm increased with concentrations from 0.008 to 12 μg/ml. However, 32 μg/ml was less effective than 12 and 0.5 μg/ml and from 256 to 4096 μg/ml inhibition of biofilm was more effective as the concentration increased (Fig. 2.8). Ciprofloxacin and gentamicin exposures increased biofilm inhibition as the concentration increased from 0.008 to 4096 μg/ml. With tetracycline, it was observed that 0.5 and 12 μg/ml were less effective followed by 0.008 μg/ml, and from 32 to 4096 μg/ml this antimicrobial agent inhibited biofilm formation as the concentration increased (Fig. 2.8).

Azithromycin, ceftazidime and tetracycline were more effective in inhibiting biofilm formation of isolate M59 as the concentration increased from 0.008 to 4096 μg/ml, with the exception to tetracycline where 0.008 μg/ml induced biofilm formation (Fig. 2.9). With gentamicin it was observed that from 0.008 to 12 μg/ml inhibition of isolate M59 biofilm was obtained as the concentration increased, and 32 μg/ml was less effective than 12 μg/ml. However, from 256 to 4096 μg/ml biofilm inhibition increased as the concentration of the antimicrobial agents increased (Fig. 2.9).

Azithromycin induced biofilm formation of *A.caviae* ATCC 15468^{T} at 0.008 and 0.5 μg/ml (Fig. 2.10). Other concentrations that were less effective in inhibiting biofilm formation of *A.caviae* ATCC 15468^{T} included 12, 256 and 1024μg/ml, respectively. It was observed that azithromycin was effective and more effective at 32 μg/ml, than at 2048 and 4096 μg/ml. Ceftazidime induced biofilm formation of this isolate at 0.5 μg/ml (Fig. 2.10). It was observed that from 12 to 1024 μg/ml, ceftazidime was more effective in inhibiting biofilm as the concentration increased and from 1024 to 4096 it was *vice versa*. Ciprofloxacin was less effective at 0.008 and 0.5 μg/ml, respectively. This antimicrobial agent was effective at 12 to 4096 μg/ml, however, it was most effective at 1024 μg/ml (Fig. 2.10). Gentamicin was less effective at 0.008 and 0.5 μg/ml, however it was effective at 12 to 4096 μg/ml and most effective at 256 μg/ml. Tetracycline induced biofilm formation at 0.008 and 1024 μg/ml. Tetracycline was also less effective at inhibiting biofilm formation of this isolate at 0.5, 12 and 32 μg/ml. This antimicrobial agent was effective at 256 and 2048 μg/ml and most effective at 4096 μg/ml (Fig. 2.10).

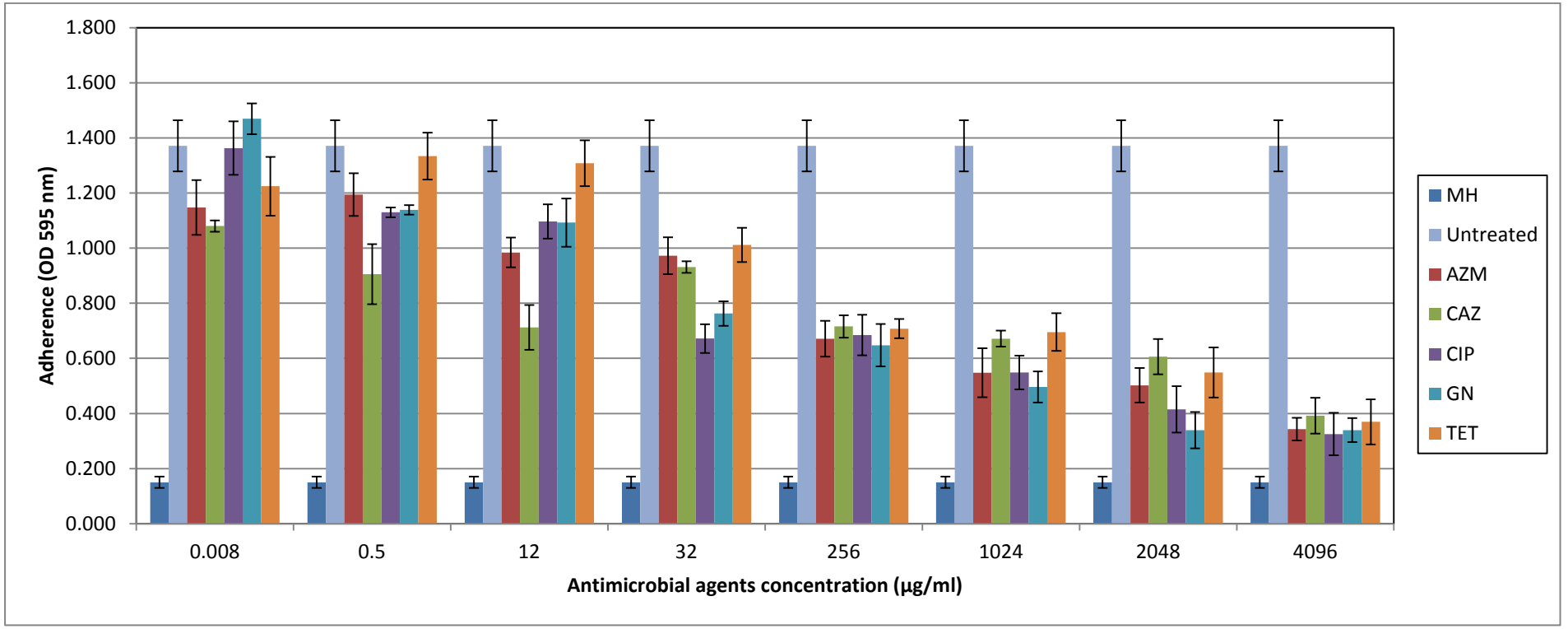

Figure 2.8: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. caviae* isolate M18 to identify minimum biofilm inhibitory concentrations.

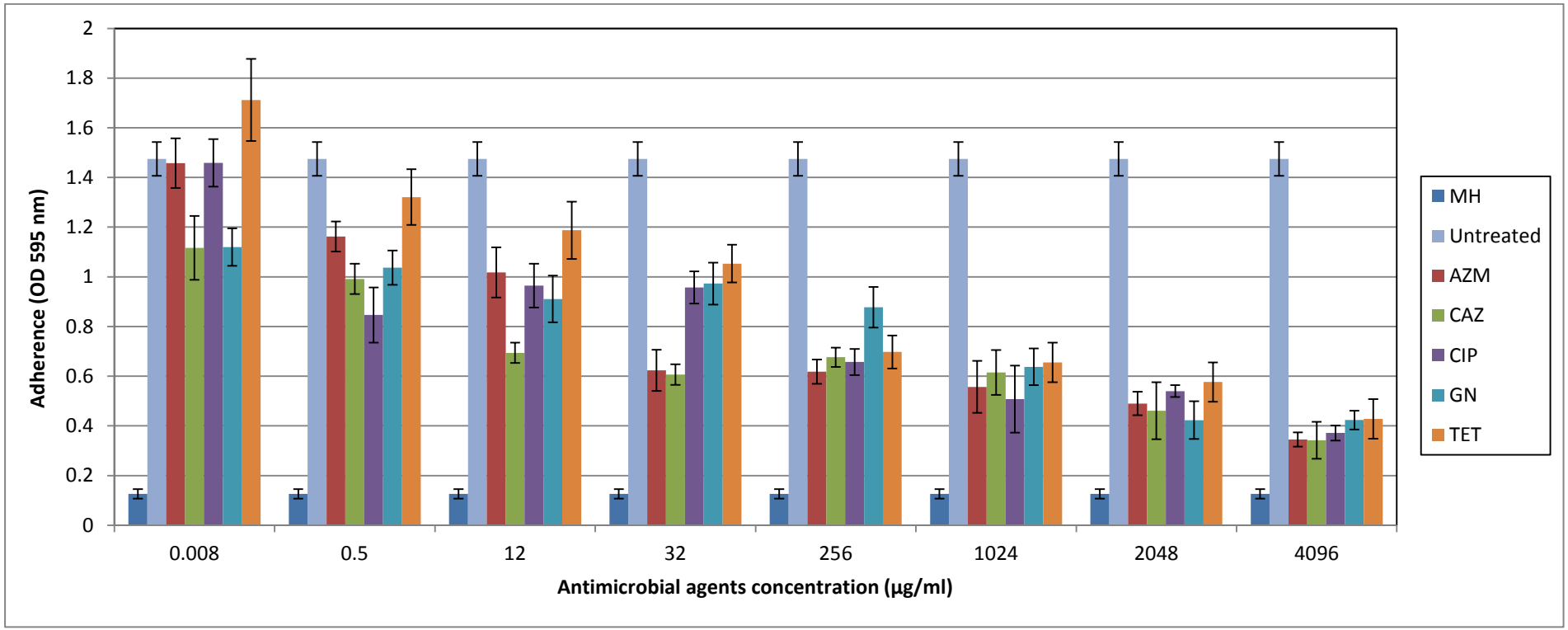

Figure 2.9: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. caviae* isolate M59 to identify minimum biofilm inhibitory concentrations.

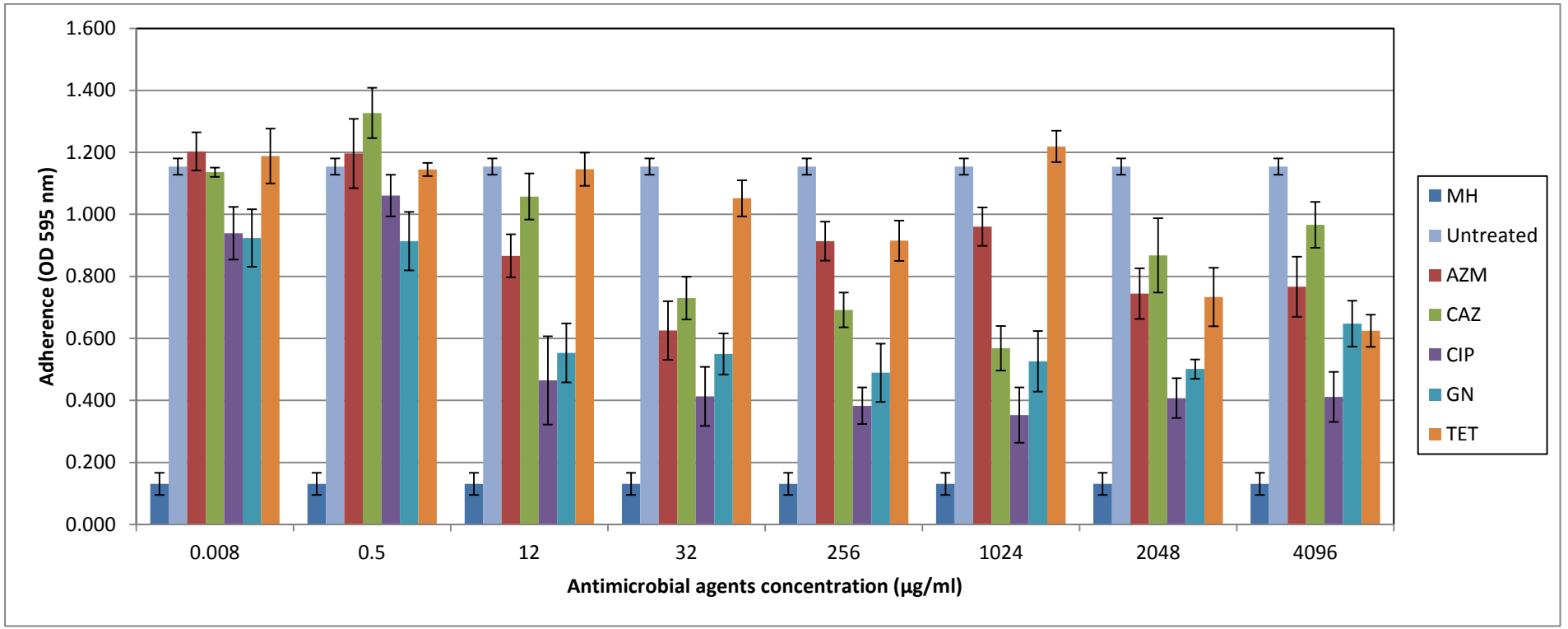

Figure 2.10: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. caviae* ATCC 15468^{T} to identify minimum biofilm inhibitory concentrations.

Azithromycin, ceftazidime, ciprofloxacin and gentamicin induced biofilm formation of isolate M70 at 0.008 µg/ml (Fig. 2.11). However, with azithromycin from 0.5 to 4096 µg/ml, inhibition of biofilm formation was observed as the concentration increased. With ceftazidime, 12 µg/ml was more effective when compared to 0.5 and 32 µg/ml but less effective than 256 µg/ml, where ceftazidime was more effective in inhibiting biofilm formation(Fig. 2.11). Ciprofloxacin was observed to be more effective in inhibiting biofilm as the concentration increased from 0.5 to 4096 µg/ml. Gentamicin increased biofilm inhibition as the concentration increased from 0.5 to 12 µg/ml, with the exception of 256 µg/ml which was the least effective. With tetracycline, 0.5 µg/ml was less effective compared to 0.008 µg/ml, and from 12 to 4096 µg/ml, this antimicrobial agent increased biofilm inhibition with the concentration (Fig. 2.11).

With isolate M88, it was observed that azithromycin, ceftazidime andciprofloxacin induced biofilm formation at 0.008 µg/ml, and only ciprofloxacin induced biofilm formation at 0.5 µg/ml (Fig. 2.12). With azithromycin it was observed that 32 µg/ml was more effective than 0.5, 12, 256 and 1024 µg/ml, but less effective than 2048 and 4096 µg/ml which were more effective concentration in inhibiting biofilm formation, respectively (Fig. 2.12). It was observed that while 0.5 µg/mlof ceftazidime was less effective, from 12 to 4096 µg/ml this antimicrobial agent decreased biofilm formation. With gentamicin0.5 µg/mlwas less effective than 0.008 µg/ml, which was more effective than 12 µg/ml (Fig. 2.12). However,from32 to 4096 µg/ml, biofilm inhibition increased as the concentrations of the antimicrobial agent increased. With tetracycline, it was observed that from 0.008 µg/ml to 4096 µg/ml inhibition of biofilm increased, except at 256 µg/ml, where tetracycline was less effective than 32 µg/ml (Fig. 2.12).

The efficiacy of antimicrobial agents against isolate M90 was inconsistent, with azithromycin, at 0.5 µg/ml being the less effective concentration and 32 µg/ml being the most effective concentration (Fig. 2.13). For ceftazidime, 32 µg/ml followed by 0.5 and 2048 µg/ml were less effective in inhibiting biofilm formation. The most effectiveconcentration for ceftazidime was 4096 µg/ml(Fig. 2.13). With ciprofloxacin, the most effectiveconcentration to inhibit biofilm formation was 12 µg/ml, the least effective concentrations were 0.008 and 0.5 µg/ml, respectively. From 256 to 4096 µg/ml, ciprofloxacin became less effective as the concentration increased (Fig. 2.13). The most effective concentrations of gentamicin ranged between 12 to 256 µg/m, with 32 µg/ml being the most effective concentration to inhibit biofilm formation. From 1024 to 4096 µg/ml, it was observed that gentamicin was less

effective compared to lower concentrations. Tetracycline induced biofilm formation at 0.5 μg/ml, followed by0.008 μg/ml, which was also less effective. The most effective concentration of tetracycline to inhibit biofilm formation was 12 μg/ml(Fig. 2.13).

It was observed that 0.008 μg/ml of azithromycin induced biofilm formation of isolate M96 (Fig. 2.14). However from 0.5 to 1024 μg/ml the azithromycin was effective in inhibiting biofilm formation. At 2048 μg/ml this antimicrobial agent became less effective compared to lower concentrations, and 4096 μg/ml was the most effective concentration to inhibit isolateM96 biofilm formation (Fig. 2.14). The efficiency of ceftazidime was inconsistent and the least effective concentration to inhibit biofilm formation was 0.008 μg/ml. From 256 to 4096 μg/ml, ceftazidime was more effective in inhibiting biofilm formation (Fig. 2.14). With ciprofloxacin, 0.008 μg/ml induced biofilm formation, however, from 0.5 to 4096 μg/ml this antimicrobial agent was more effective in inhibiting biofilm formation of isolate M96 (Fig. 2.14). It was observed that 0.008 μg/ml of gentamicin also induced biofilm formation, and from 0.5 to 32 μg/ml inhibition of biofilm was achieved. At 256 μg/ml gentamicin became less effective than 12 and 32 μg/ml, and from 1024 to 4096 μg/ml this antimicrobial agent was more effective in inhibiting biofilm formation. Tetracycline increased inhibition of biofilm as the concentration increased (Fig. 2.14).

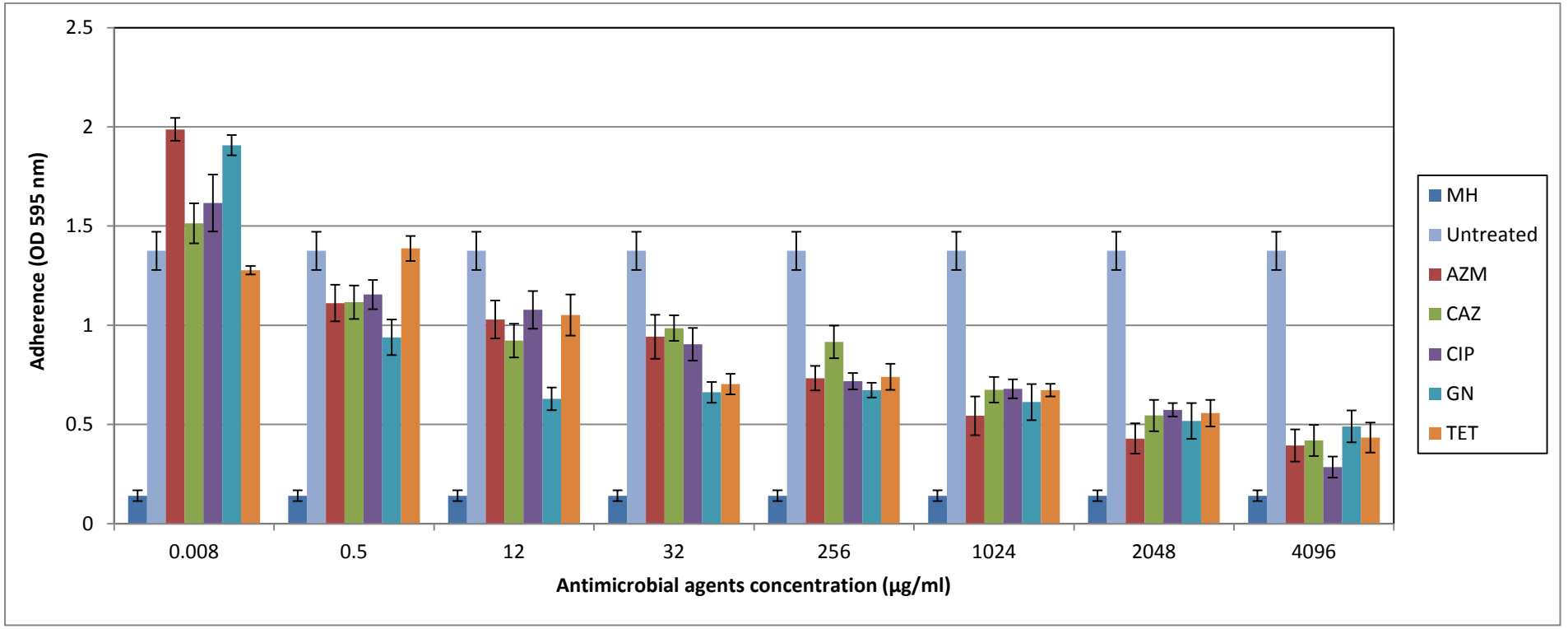

Figure 2.11: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. bestiarum* isolate M70 to identify minimum biofilm inhibitory concentrations.

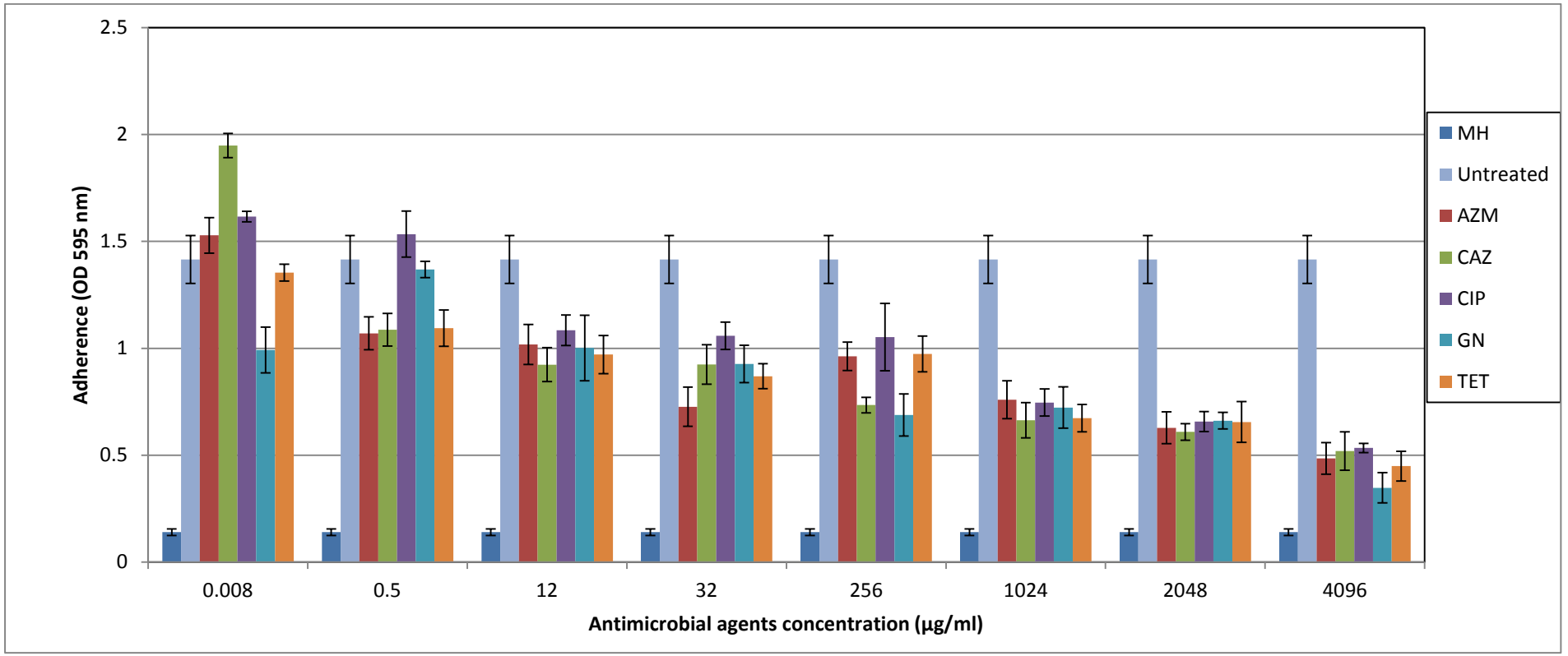

Figure 2.12: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. bestiarum* isolate M88 to identify minimum biofilm inhibitory concentrations.

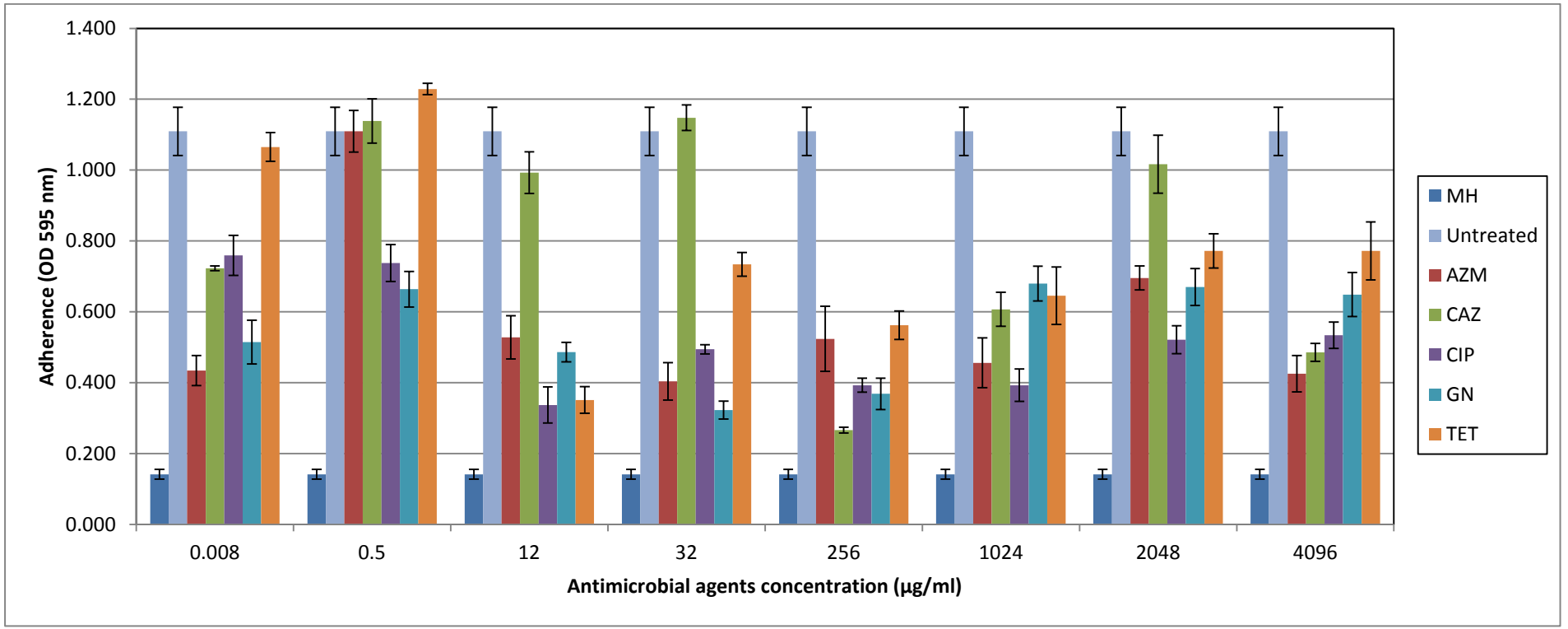

Figure 2.13: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. bestiarum* isolate M90 to identify minimum biofilm inhibitory concentrations.

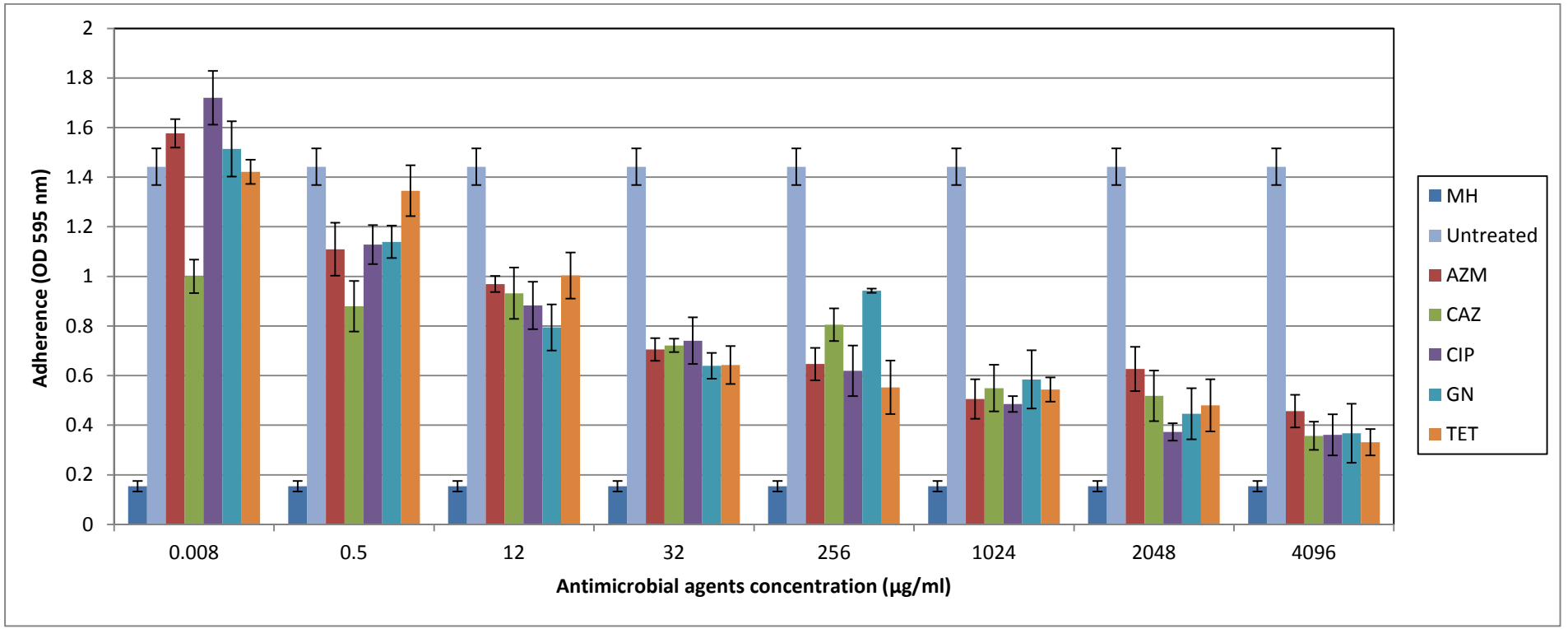

Figure 2.14: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. bestiarum* isolate M96 to identify minimum biofilm inhibitory concentrations.

Azithromycin, ceftazidime and tetracycline induced biofilm formation of isolate M23 at 0.008 μg/ml (Fig. 2.15). Azithromycin was effective at 0.5 and 12 μg/ml, however, from 32 to 4096 μg/ml inhibition of biofilm formation increased. With azithromycin it was observed that the most effective concentration was 4096 μg/ml, and the least effective concentration was 1024 μg/ml (Fig. 2.15). Ciprofloxacin and gentamicin were effective at inhibiting biofilm formation as the concentration increased from 0.008 to 4096 μg/ml. Tetracycline was effective in inhibiting biofilm formation as the concentration increased from 0.5 to 4096 μg/ml (Fig. 2.15).

Azithromycin, ciprofloxacin, gentamicin and tetracycline induced biofilm formation of isolate M31 at 0.008 μg/ml (Fig. 2.16). Azithromycin, ceftazidime, gentamicin and tetracycline were more effective in inhibiting biofilm as the concentration increased from 32 to 4096 μg/ml. The same trend was observed with ciprofloxacin with the exception of 12 μg/ml, which was more effective than 32-1024 μg/ml (Fig. 2.16).

With isolate M38, all five antimicrobial agents induced biofilm formation at 0.008 and 0.5 μg/ml, and only gentamicin induced biofilm formation at 12 μg/ml (Fig. 2.17). Biofilm inhibition was observed with all five antimicrobial agents from 256 to 4096 μg/ml, with the most effective inhibitionat2048 to 4096 μg/ml (Fig. 2.17).

Azithromycin, ceftazidime and tetracyclinewereobserved to induce biofilm formation of isolate M58 at 0.008 μg/ml (Fig. 2.18). However, azithromycin was more effective at inhibiting biofilm formation as the concentrations increased from 0.5 to 4096 μg/ml. With ceftazidime it was observed that 256 μg/ml was least effective concentration and from 1024 to 4096 μg/ml, ceftazidime was more effective in inhibiting biofilm formation (Fig. 2.18). With ciprofloxacin 0.008, 0.5, 12 and 32 μg/ml were less effective and from 256 to 4096 μg/ml the antimicrobial agent was more effective. With tetracycline, 0.5 μg/ml was more effective than 12 μg/ml, however, it was observed that from 32 μg/ml to 4096 μg/ml, this antimicrobial agent was more effective in inhibiting biofilm formation (Fig. 2.18).

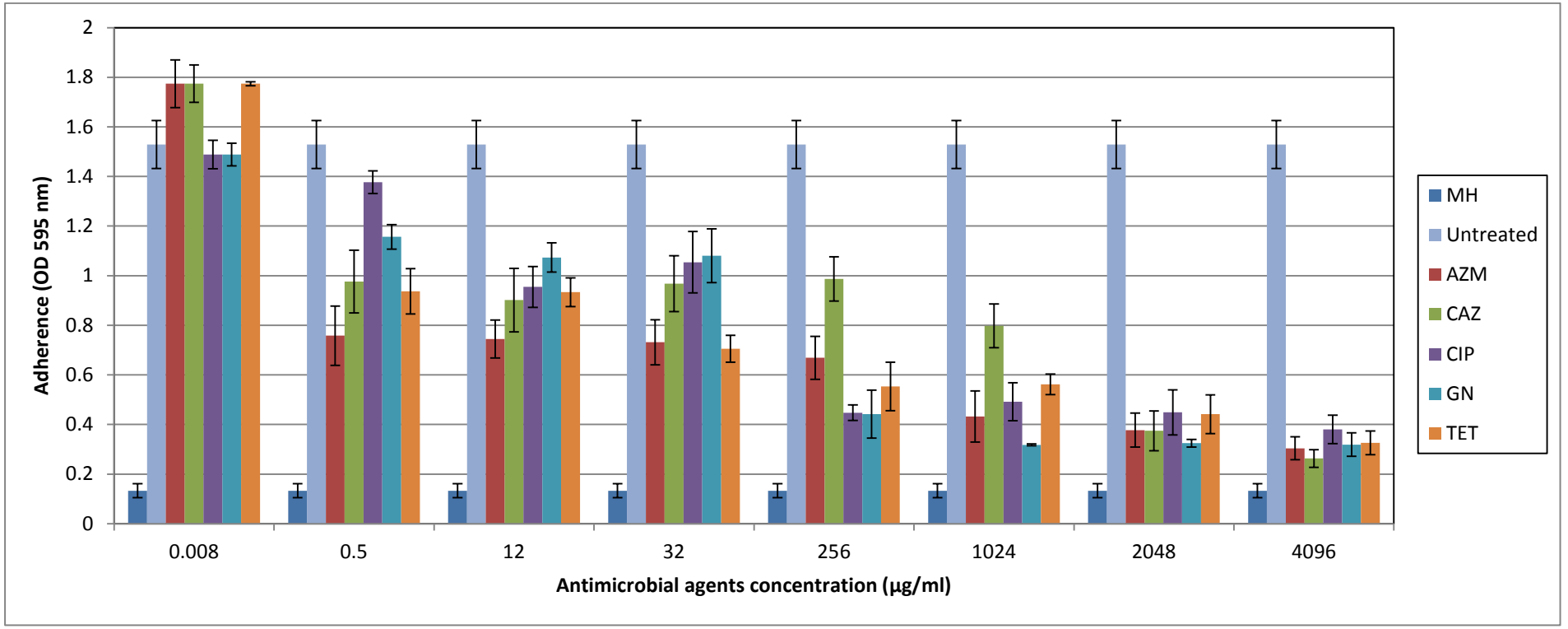

Figure 2.15: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. culicicola* isolate M23 to identify minimum biofilm inhibitory concentrations.

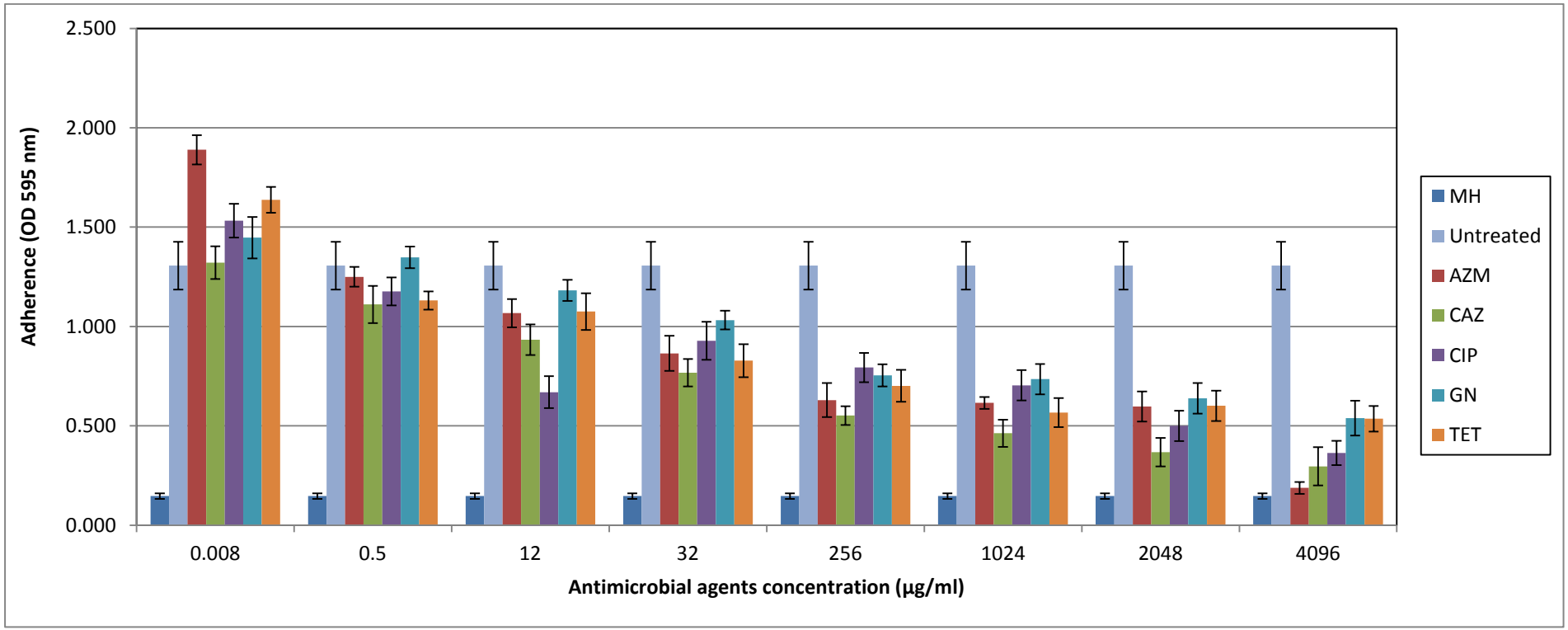

Figure 2.16: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. culicicola* isolate M31to identify minimum biofilm inhibitory concentrations.

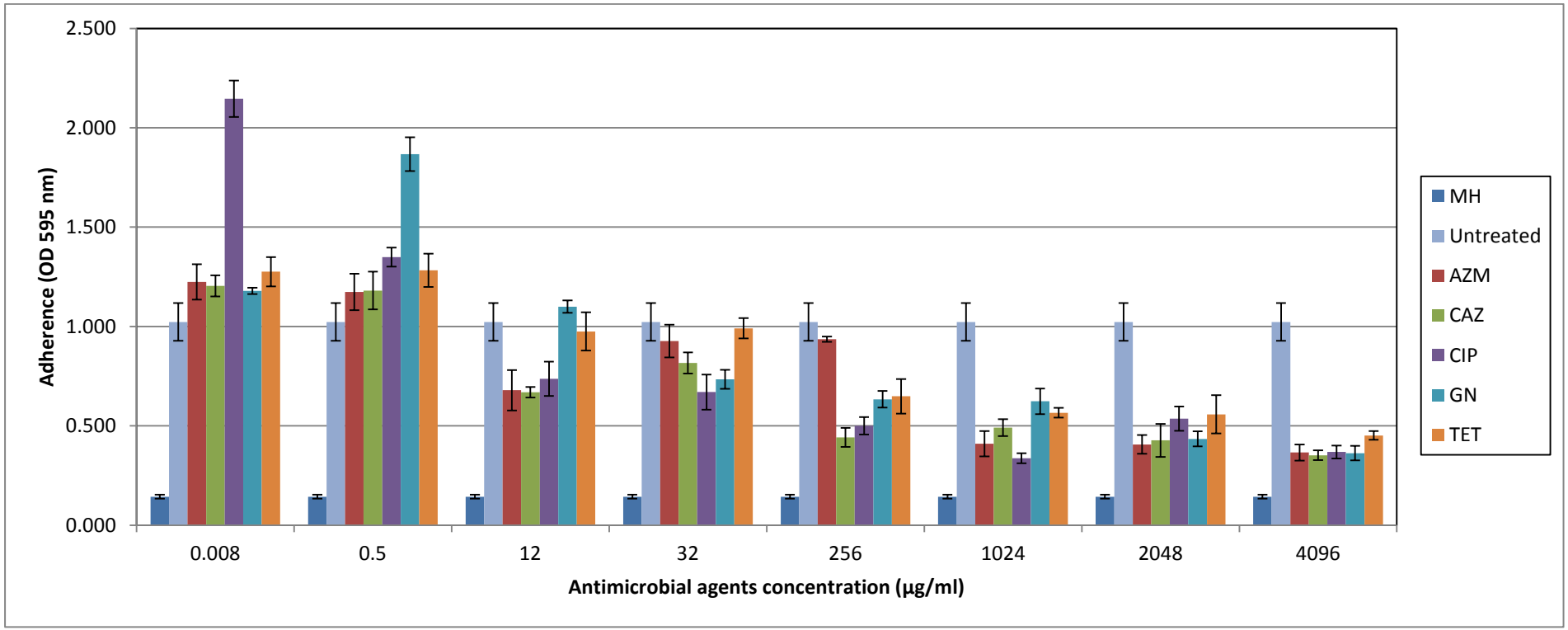

Figure 2.17: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. culicicola* isolate M38 to identify minimum biofilm inhibitory concentrations.

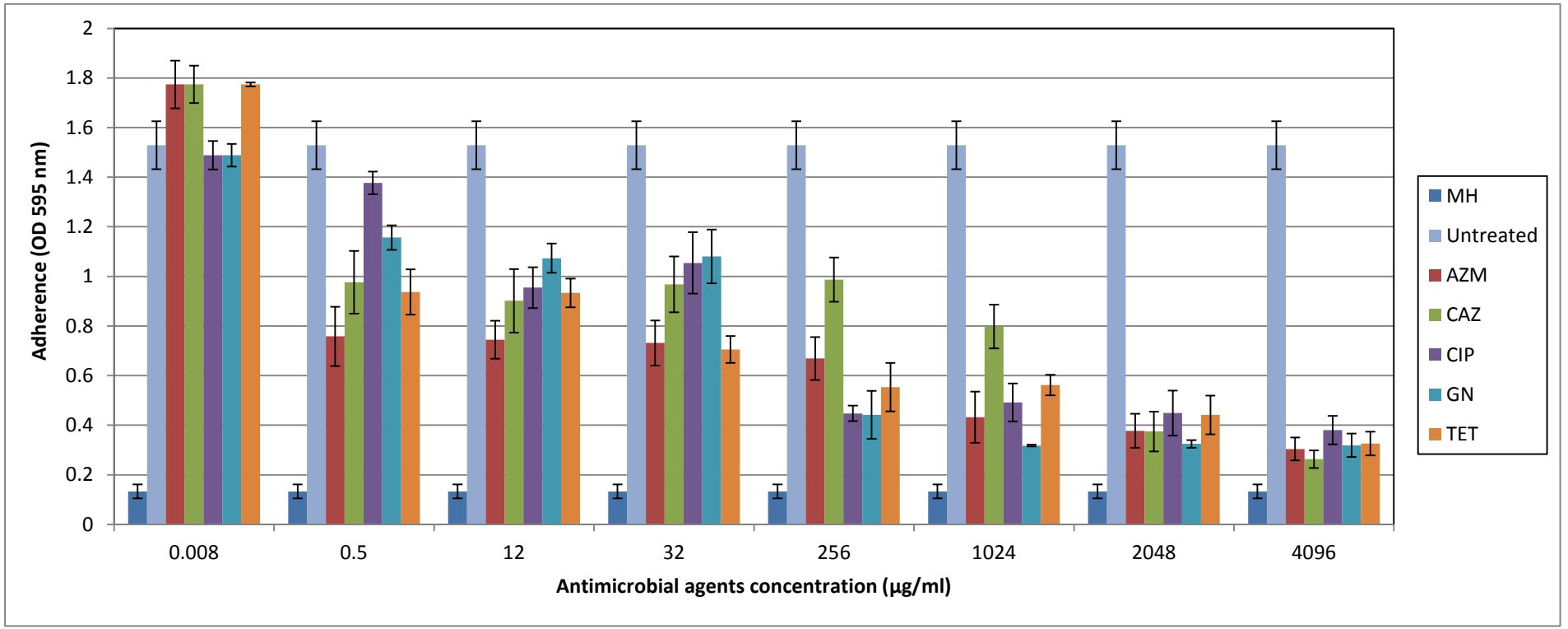

Figure 2.18: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. culicicola* isolate M58 to identify minimum biofilm inhibitory concentrations.

Azithromycin induced biofilm formation of isolate M55 at 0.5 and 12 µg/ml and became effective at 32 to 2048 µg/ml and mosteffective at 4096 µg/ml (Fig. 2.19). Ceftazidime induced biofilm formation from 0.5 to 256 µg/ml and was effective at inhibiting biofilm formation of isolate M55 at 1024 and 2048 µg/ml (Fig. 2.19). This antimicrobial agent was most effective at 4096 µg/ml. Ciprofloxacin induced biofilm formation at 12 and 256 µg/ml, these concentrations were followed by 0.008, 0.5, 1024 and 32 µg/ml which were less effective. Ciprofloxacin was more effective at 2048 and 4096 µg/ml(Fig. 2.19). Gentamicin induced biofilm formation of isolate M55 at 0.5 and 12 µg/ml. At 12 and 32 µg/ml, gentamicin was also observed to be less effective (Fig. 2.19). However, increased biofilm inhibition was showed as the concentration increased from 256 to 4096 µg/ml. Tetracycline induced biofilm at 0.5 µg/ml,and was followed by 0.008 which was also less effective. However, from 12 to 4096 µg/ml, tetracycline inhibited biofilm formation as the concentration increased (Fig. 2.19).

Biofilm formation of isolate M57 was induced by azithromycin at 0.008 µg/ml and from 0.5 to 4096 µg/ml it increased biofilm inhibition with the exception of 1024 µg/ml which was less effective compared to lower concentrations (Fig. 2.20). With ceftazidime, 0.008 µg/ml was less effective in inhibiting biofilm formation of isolate M57 compared to other concentrations. At 12 µg/ml, ceftazidime was more effective than other concentrations with the exception of 4096 µg/ml which was the most effective concentration (Fig. 2.20). The least effective concentration of ciprofloxacin to inhibit biofilm formation by isolate M57 was 32 µg/ml, followed by 0.008 and 0.5 µg/ml. The remaining concentrations were effective with 4096 µg/ml being the most effectiveconcentration. Gentamicin was less effective at 0.5 µg/ml, followed by 0.008 and 32 µg/ml, respectively (Fig. 2.20). Gentamicin was more effective at inhibiting biofilm formation as the concentration increased from 256 to 4096 µg/ml. Tetracycline induced biofilm formation of isolate M57 at 0.008 and 0.5 µg/ml, while from 12 to 4096 µg/ml it increased inhibition of biofilm formation (Fig. 2.20).

Azithromycin was less effective at 0.008 µg/ml ininhibiting biofilm formation by isolate M63, however, from 0.5 to 4096 µg/ml it was observed to be effective (Fig. 2.21). The most effective concentration of this antimicrobial agent was 4096 µg/ml. With ceftazidime, 0.5 µg/ml induced biofilm formation, and was followed by 0.008, 12 and 1024 which were also less effective compared to other concentrations. The most effective concentration of ceftazidime was 4096 µg/ml (Fig. 2.21). It was observed that ciprofloxacin was more effective in inhibiting biofilm formation from 0.5 to 4096 µg/ml, with the exception of 256

μg/ml which was less effective than 32 μg/ml. Gentamicin induced biofilm formation at 0.5 μg/ml, and 12 and 1024 μg/ml were less effective (Fig. 2.21). Gentamicin was observed to be effective at 32, 256, 2048 μg/ml and most effective at 4096 μg/ml. Tetracycline was less effective at 0.008 μg/ml, effective from 0.5 to 2048 μg/ml and most effective at 4096 μg/ml (Fig. 2.21).

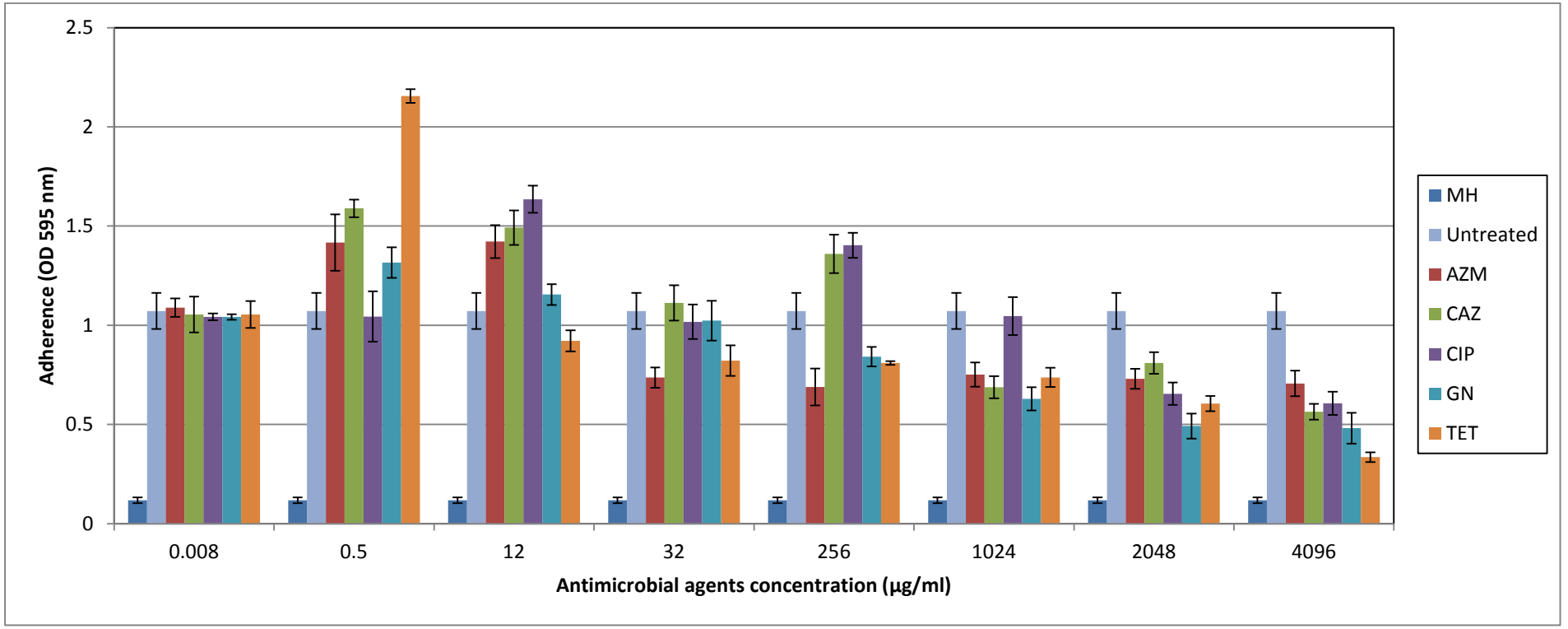

Figure 2.19: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. veronii* isolate M55 to identify minimum biofilm inhibitory concentrations.

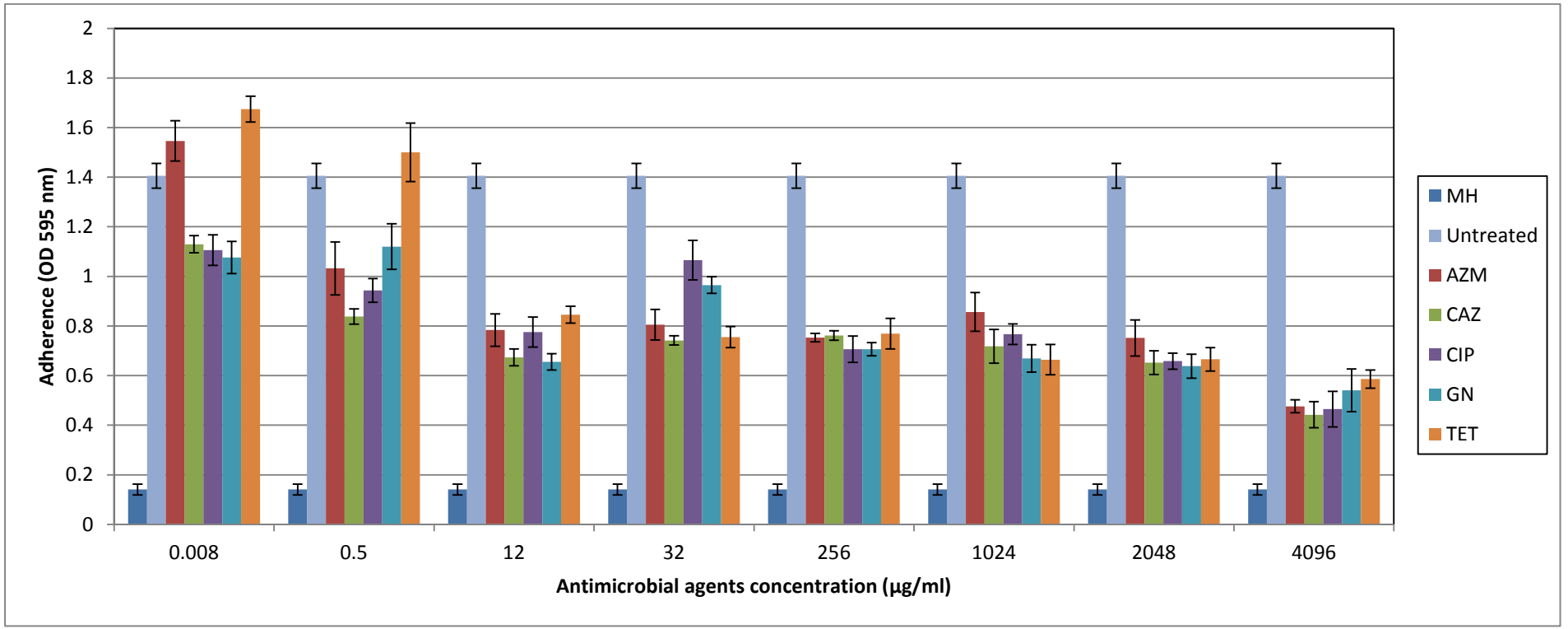

Figure 2.20: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. veronii* isolate M57 to identify minimum biofilm inhibitory concentrations.

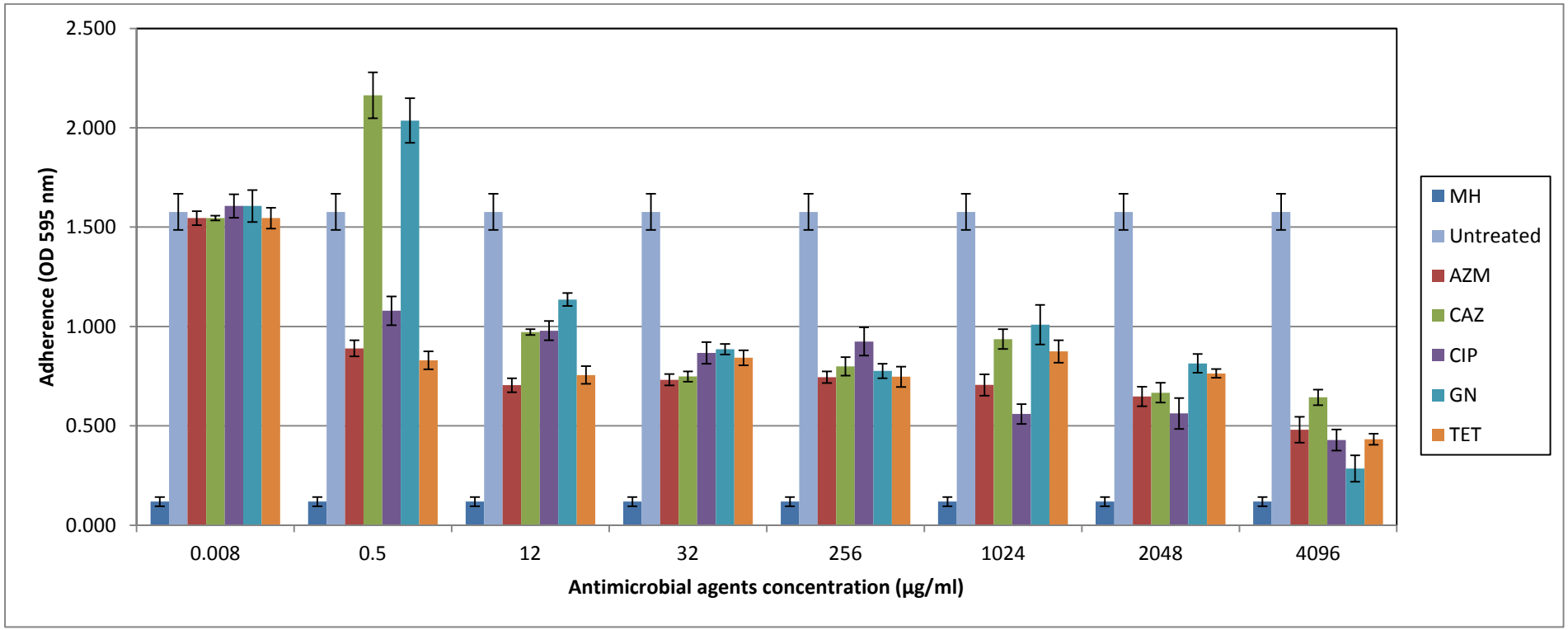

Figure 2.21: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. veronii* isolate M63 to identify minimum biofilm inhibitory concentrations.

Azithromycin, ceftazidime and tetracycline were more effective in inhibiting biofilm formation of isolate M76 as the concentration increased from 0.008 to 4096 µg/ml (Fig. 2.22). Ciprofloxacin induced biofilm formation at 0.008 µg/ml and from 0.5 to 4096 µg/ml it increased inhibition of biofilm formation (Fig. 2.22).Gentamicin induced biofilm formation at 0.008 and 0.5 µg/ml and from 12 to 4096 µg/ml it was more effective inhibiting biofilm formation (Fig. 2.22).

Azithromycin was most effective at inhibiting biofilm of isolate M77 as the concentration increased from 0.008 to 4096 µg/ml, with the exception of 32 µg/ml,which was less effective than 12 µg/ml (Fig. 2.23). The same trend was observed for ceftazidime, however, with ciprofloxacin, 1024 and 2048 µg/ml were less effective than 256 µg/ml. The most effectiveconcentration of ciprofloxacin to inhibit biofilm formation by isolate M77 was 4096 µg/ml, followed by 256 and 0.5 µg/ml, respectively (Fig.2.23). The least effective concentration was 0.008 µg/ml. Gentamicin was more effective at inhibiting biofilm as the concentration increased from 0.008 to 4096 µg/ml except with 12 µg/ml which was less effective than 0.5 µg/ml. Tetracycline induced biofilm formation at 0.008 µg/ml and was effective from 0.5 to 2048 µg/ml and most effective at 4096 µg/ml (Fig. 2.23).

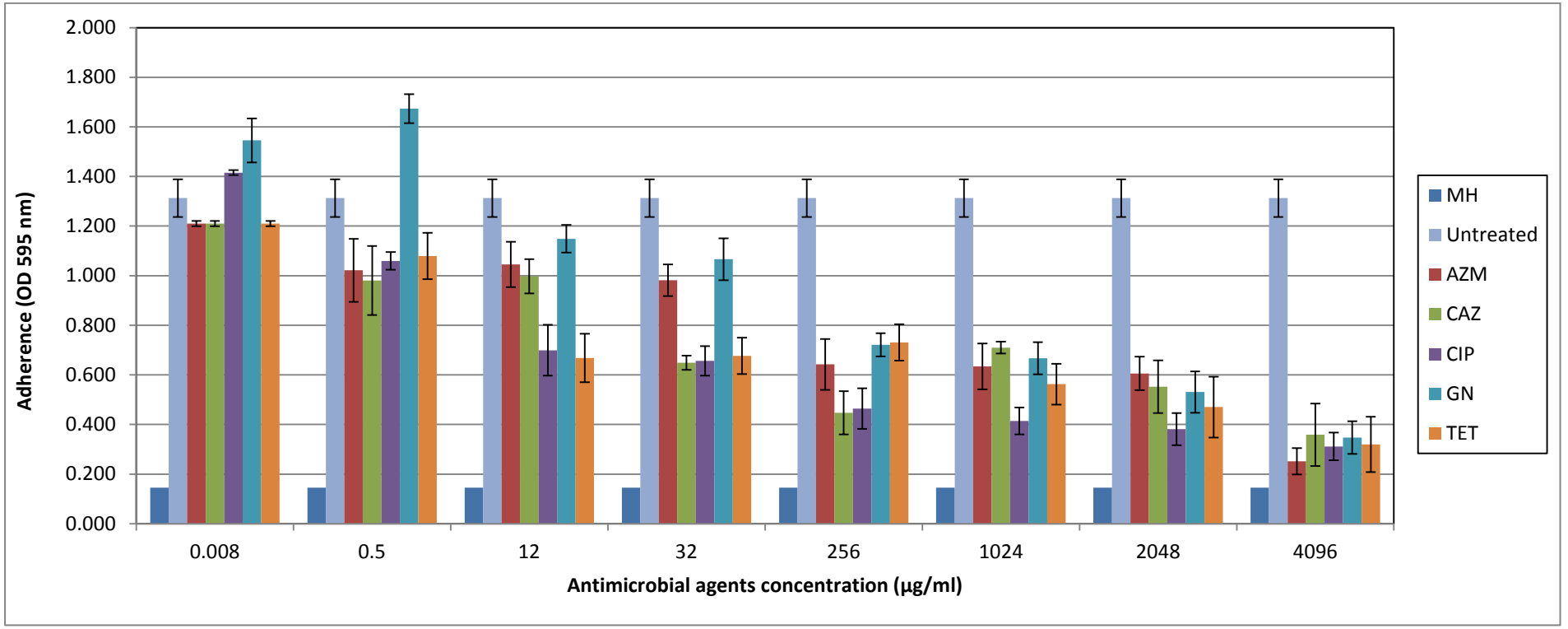

Figure 2.22: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. salmonicida* isolate M76 to identify minimum biofilm inhibitory concentrations.

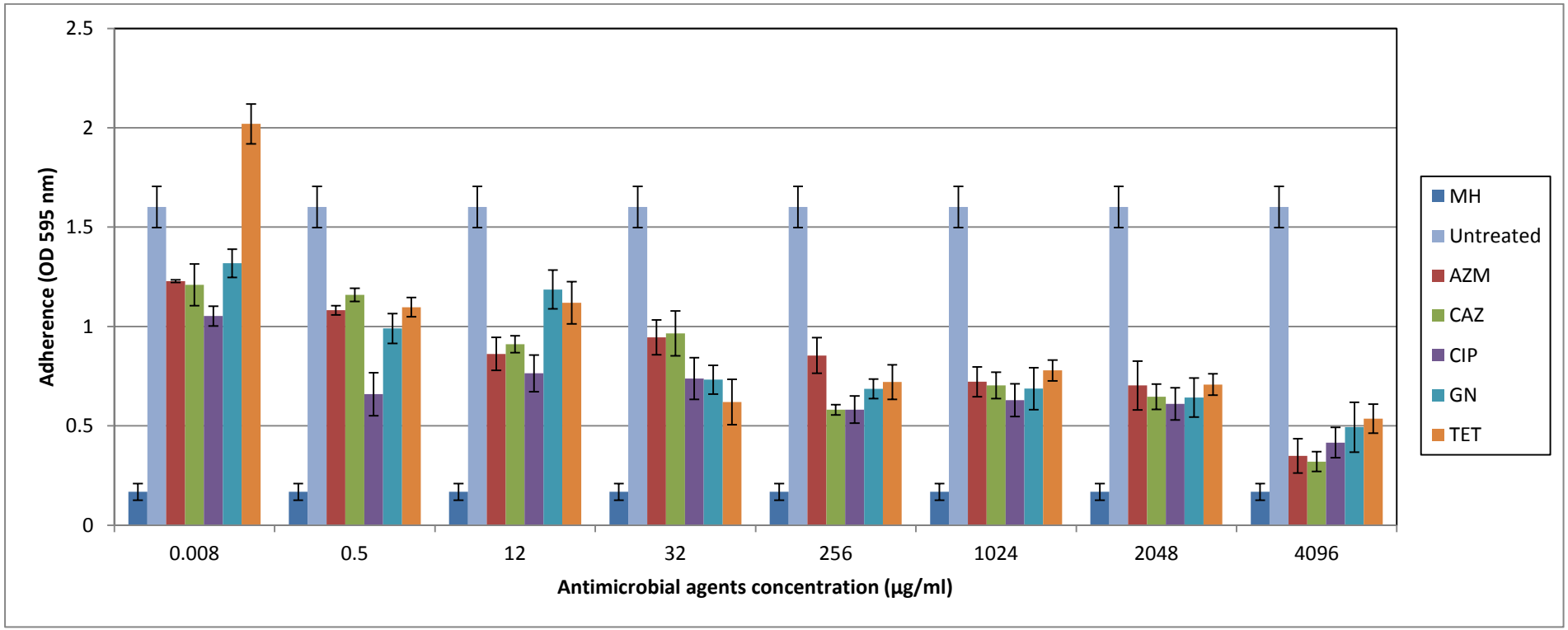

Figure 2.23: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. salmonicida* isolate M77 to identify minimum biofilm inhibitory concentrations.

Azithromycin induced biofilm formation of isolate M92 at 12 and 32 μg/ml, and at 0.008 and 0.5 μg/ml this antimicrobial agent was also observed to be less effective (Fig. 2.24). Azithromycin was effective from 256 to 4096 μg/ml and more effective at 1024 μg/ml. Ceftazidime induced biofilm formation at 0.008, 0.5 and 12 μg/ml. From 1024 to 4096 μg/ml, this antimicrobial agent increased inhibition of biofilm formation (Fig. 2.24). Ciprofloxacin induced biofilm formation at 0.5, 12 and 256 μg/ml, and 32 μg/ml as well as 1024 μg/ml were less effective in inhibiting biofilm formation. This antimicrobial agent was more effective at 0.008, 2048 μg/ml and most effective at 4096 μg/ml (Fig. 2.24). Gentamicin induced biofilm formation at 12 μg/ml and 0.008, 1024 and 2048 μg/ml were less effective, respectively. Gentamicin was most effective at 4096 μg/ml. Tetracycline induced biofilm formation at 0.008 μg/ml and 32 μg/ml. At 0.5 and 12 μg/ml, tetracycline was less effective in inhibiting biofilm formation by isolate M92. Tetracycline was effective at 256 and 2046 μg/ml, and most effective at 4096 μg/ml (Fig. 2.24).

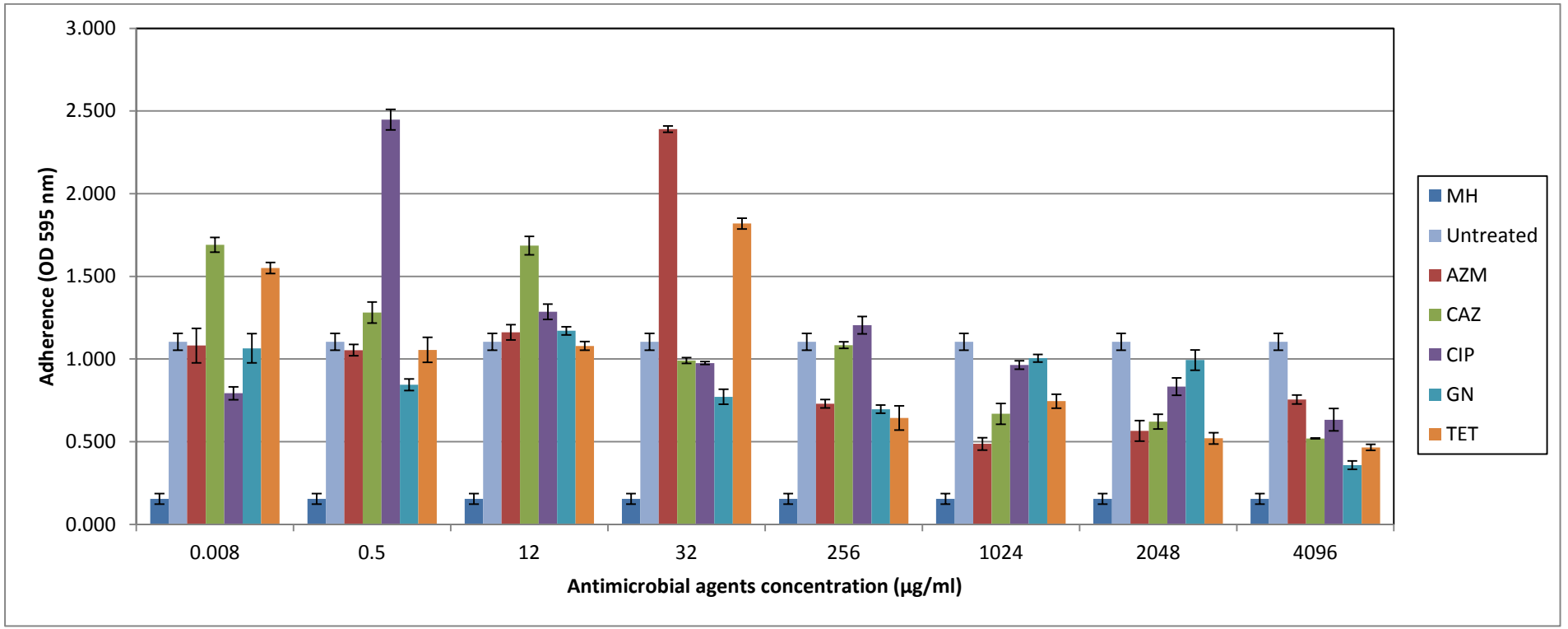

Figure 2.24: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. allosaccharophila* isolate M92 to identify minimum biofilm inhibitory concentrations.

Azithromycin was more effective inhibiting biofilm formation of isolate M41 from 0.008 to 4096 μg/ml as the concentration increased with the exception of 2048 μg/ml, which was less effective than 32, 256 and 1024 μg/ml (Fig. 2.25). Ceftazidime induced biofilm formation at 0.008 and from 0.5 to 4096 μg/ml it was more effective in inhibiting biofilm formation (Fig. 2.25). It was observed that 0.008 and 12 μg/ml of ciprofloxacin were less effective in inhibiting biofilm formation of M41 and the most effective concentration was 256 μg/ml (Fig. 2.25). Tetracycline induced biofilm formation at 0.008 μg/ml, and from 0.5 to 32 μg/ml the efficiency of this antimicrobial agent was more or less the same. However, from 256 to 4096 μg/ml, this antimicrobial agent was more effective (Fig. 2.25).

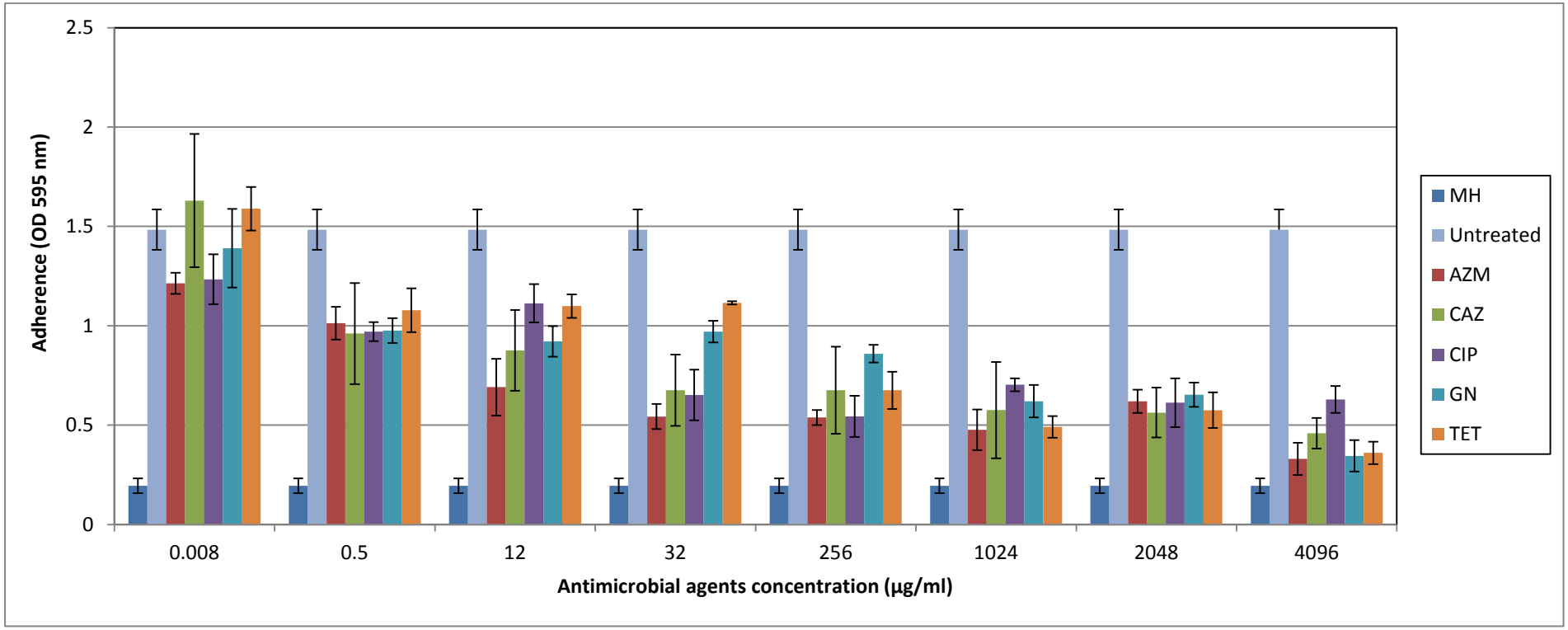

Figure 2.25: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against unspecified *Aeromonas* spp. isolate M41 to identify minimum biofilm inhibitory concentrations.

Azithromycin and ceftazidime induced biofilm formation of isolate M49 at 0.008 μg/ml (Fig. 2.26). However, they were both effective in inhibiting biofilm formation as the concentration increased from 0.5 to 4096 μg/ml. Ciprofloxacin displayed inhibition of biofilm formationfrom 0.008 to 256 μg/ml (Fig. 2.26). Ciprofloxacin increased inhibition of biofilm formation as the concentration increased from 1024 to 4096 μg/ml. Gentamicin was more effective in inhibiting biofilm formation of isolate M49 as the concentration increased from 0.008 to 4096 μg/ml (Fig. 2.26). The least effective concentration for tetracycline was 0.008, 0.5 and 256 μg/ml, and the most effectiveinhibition was observed from 1024 to 4096 μg/ml, with 4096 μg/ml being the most effectiveconcentration (Fig. 2.26).

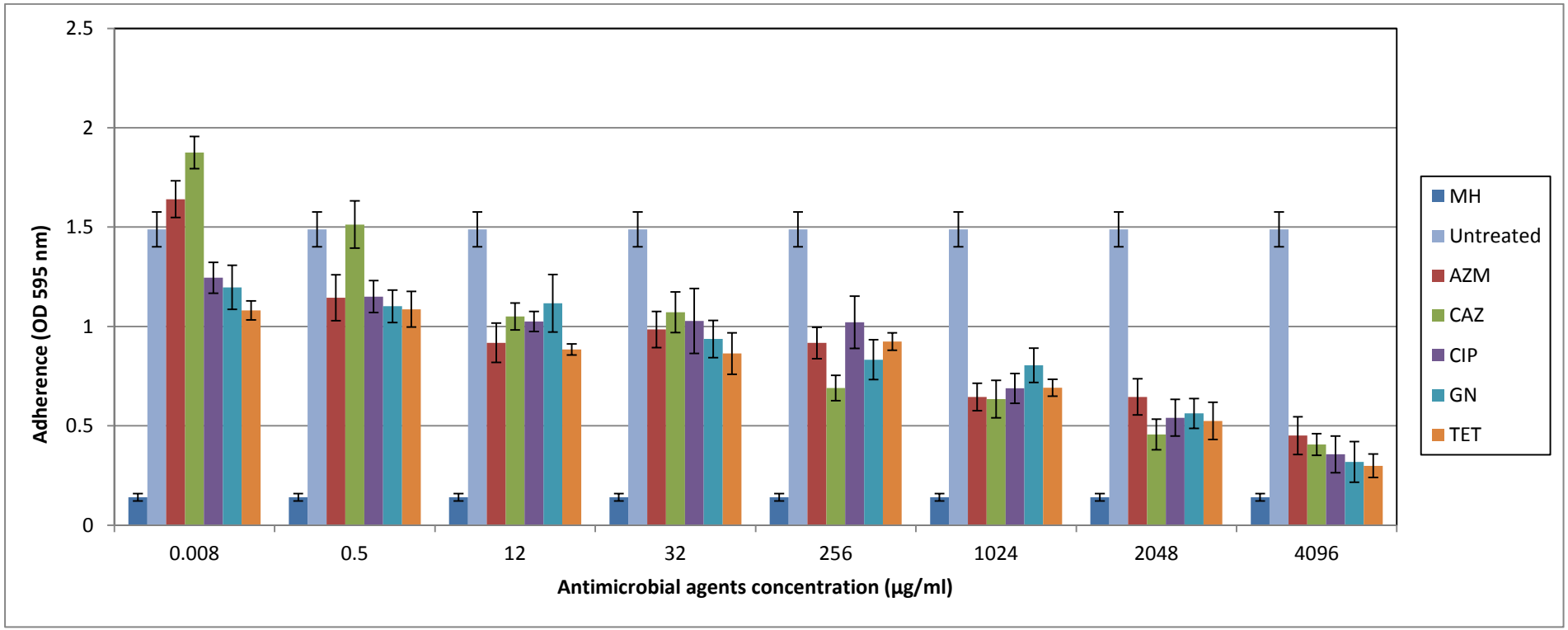

Figure 2.26: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. sobria* isolate M49 to identify minimum biofilm inhibitory concentrations.

Azithromycin was more effective in inhibiting biofilm formation of isolate M28 as the concentration increased from 0.008 to 4096 μg/ml (Fig. 2.27). Ceftazidime induced biofilm formation at 0.008 and from 0.5 to 4096 μg/ml it increased inhibition of biofilm formation. Ciprofloxacin induced biofilm formation at 0.008 and 0.5 μg/ml, and from 12 to 4096 μg/ml it was more effective at inhibiting biofilm formation (Fig. 2.27). Gentamicin was more effective in inhibiting biofilm formation as the concentration increased from 0.008 to 4096 μg/ml. Tetracycline induced biofilm formation at 0.008 and 0.5 μg/ml, and it was more effective in inhibiting biofilm formation as the concentration increased from 12 to 4096 μg/ml (Fig. 2.27).

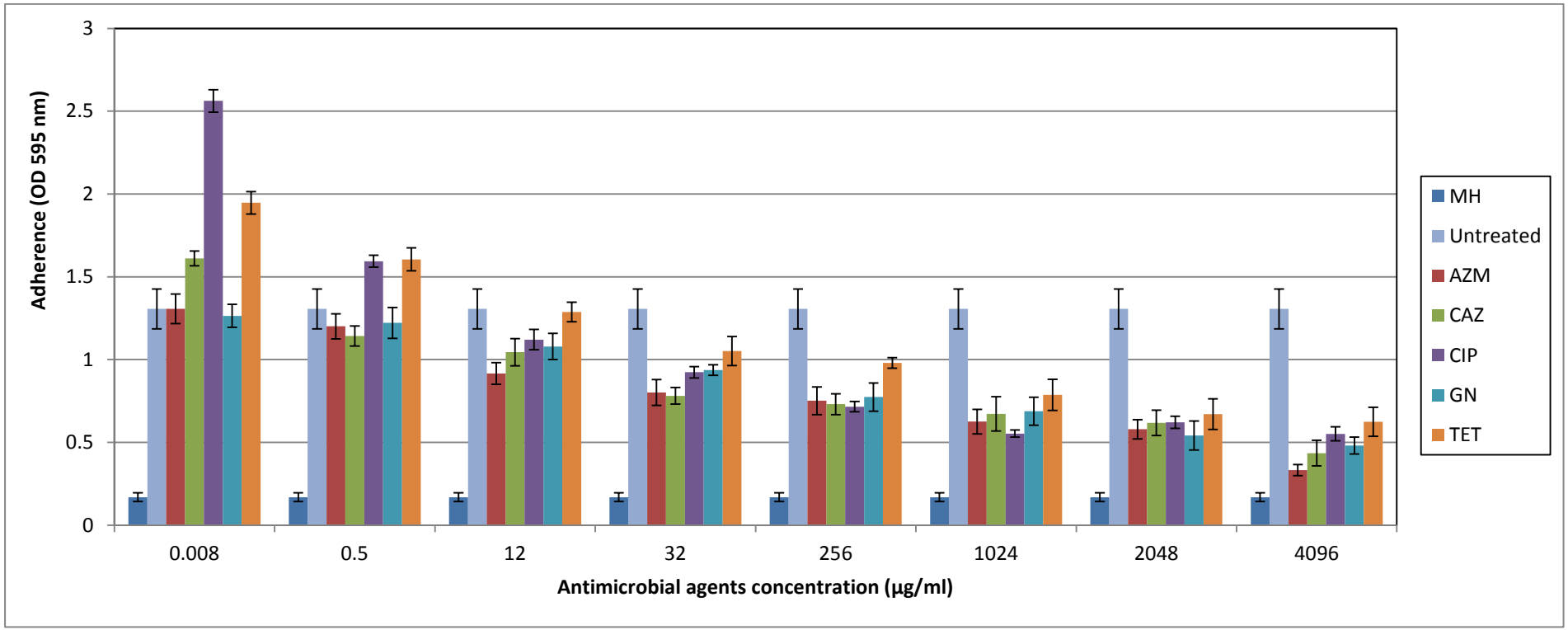

Figure 2.27: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *A. jandaei* isolate M28 to identify minimum biofilm inhibitory concentrations.

Azithromycin and ceftazidime were more effective in inhibiting isolate M9 biofilm formation as the concentration increased from 0.008 to 4096 μg/ml (Fig. 2.28). With ciprofloxacin, 0.008 μg/ml was less effective against isolate M9 biofilm, followed by 0.5, 12 and 32 μg/ml, respectively (Fig. 2.28). Ciprofloxacin was effective at 256 to 2048 μg/ml and more effective at 4096 μg/ml. Gentamicin was more effective in inhibiting biofilm formation as the concentration increased from 0.008 to 4096 μg/ml, with the exception of 12 μg/ml which was more effective than 32 μg/ml. Tetracycline induced biofilm formation at 0.008 μg/ml and became more effective in inhibiting biofilm formation as the concentrations increased from 0.5 to 4096 μg/ml (Fig. 2.28).

All five antimicrobial agents induced biofilm formation of isolates M46 at 0.008 μg/ml (Fig. 2.29). However, from 0.5 to 4096 μg/ml, all antimicrobial agents were more effective in inhibiting biofilm formation (Fig. 2.29).

Azithromycin induced biofilm formation of M67 at 12 and 32 μg/ml, and it was also observed to be less effective at 0.008 and 0.5 μg/ml (Fig. 2.30). Azithromycin was more effective at 256 to 4096 μg/ml, and the most effectiveconcentration was 1024 μg/ml. Ceftazidime induced biofilm formation at 0.008, 0.5 and 12 μg/ml, and it was also less effective at 256 and 32 μg/ml. Ceftazidime was more effective in inhibiting biofilm formation of isolateM67 as the concentration increased from 1024 to 4096 μg/ml (Fig. 2.30). Ciprofloxacin induced biofilm formation at 0.5, 12 and 256 μg/ml. At 32 and 1024 μg/ml, ciprofloxacin was also observed to be less effective. The most effective concentration of ciprofloxacin were 0.008, 2048 and 4096 μg/ml (Fig. 2.30). Gentamicin induced biofilm formation at 12 μg/ml, and 0.008, 1024 and 2048 μg/ml were also less effective, respectively. The most effectiveconcentration of gentamicin to inhibit biofilm formation was 4096 μg/ml. Tetracycline induced biofilm formation at 0.008 and 32 μg/ml. Tetracycline was effective at 256 to 2048 μg/ml and more effective 4096 μg/ml (Fig. 2.30).

Based on the responses of selected isolates to the varying concentrations of antimicrobial agents (Figs. 2.1-2.30), 4096 μg/ml appeared to be the most effective for biofilm inhibition. There was a ≥128-fold increase in MBICs (4096 μg/ml) compared to the determined MICs (Table 2.2) for all the antimicrobial agents.

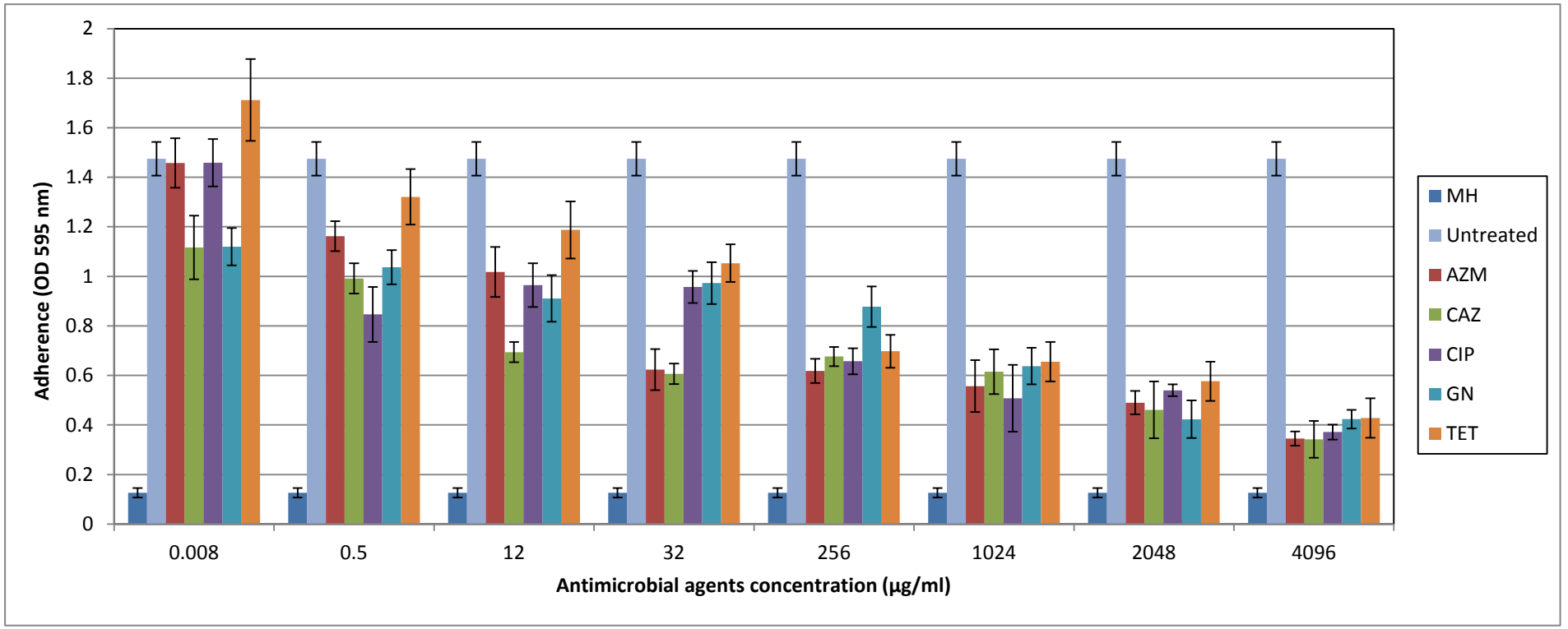

Figure 2.28: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *P. shigelloides* isolate M9 to identify minimum biofilm inhibitory concentrations.

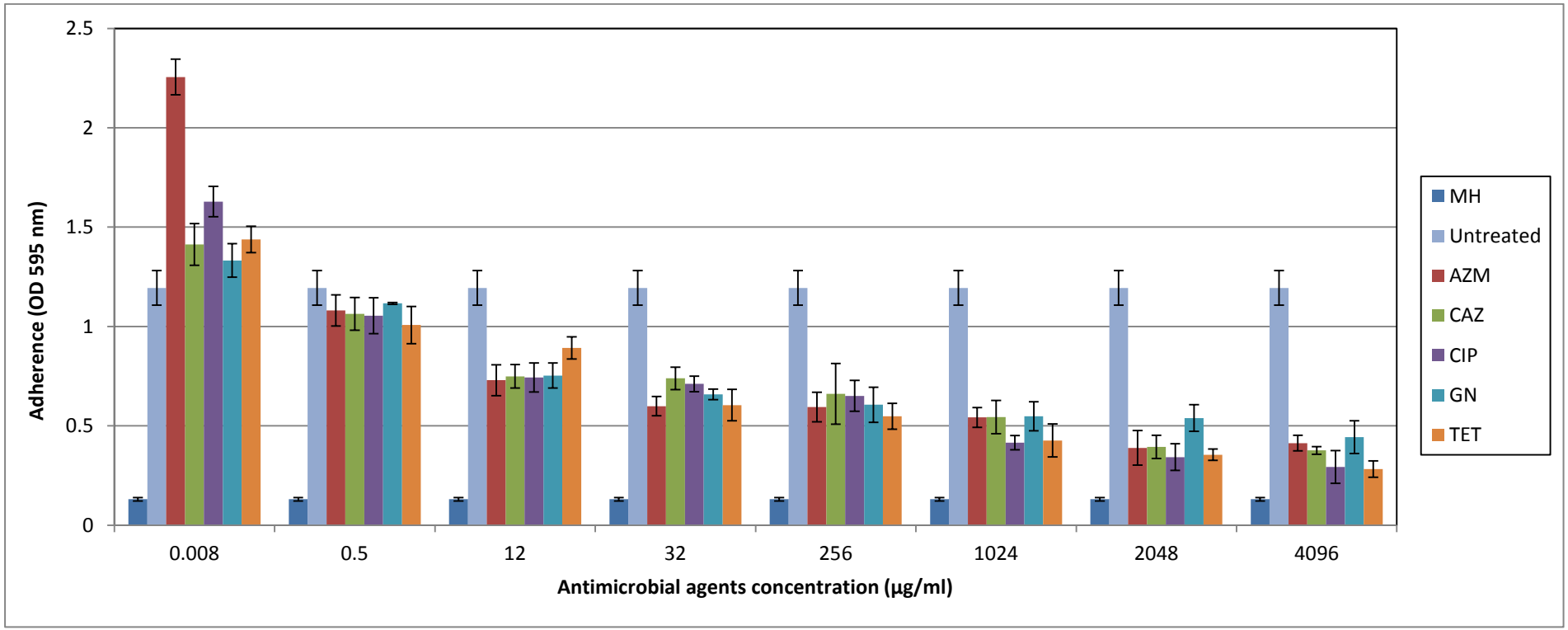

Figure 2.29: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *P. shigelloides* isolate M46 to identify minimum biofilm inhibitory concentrations.

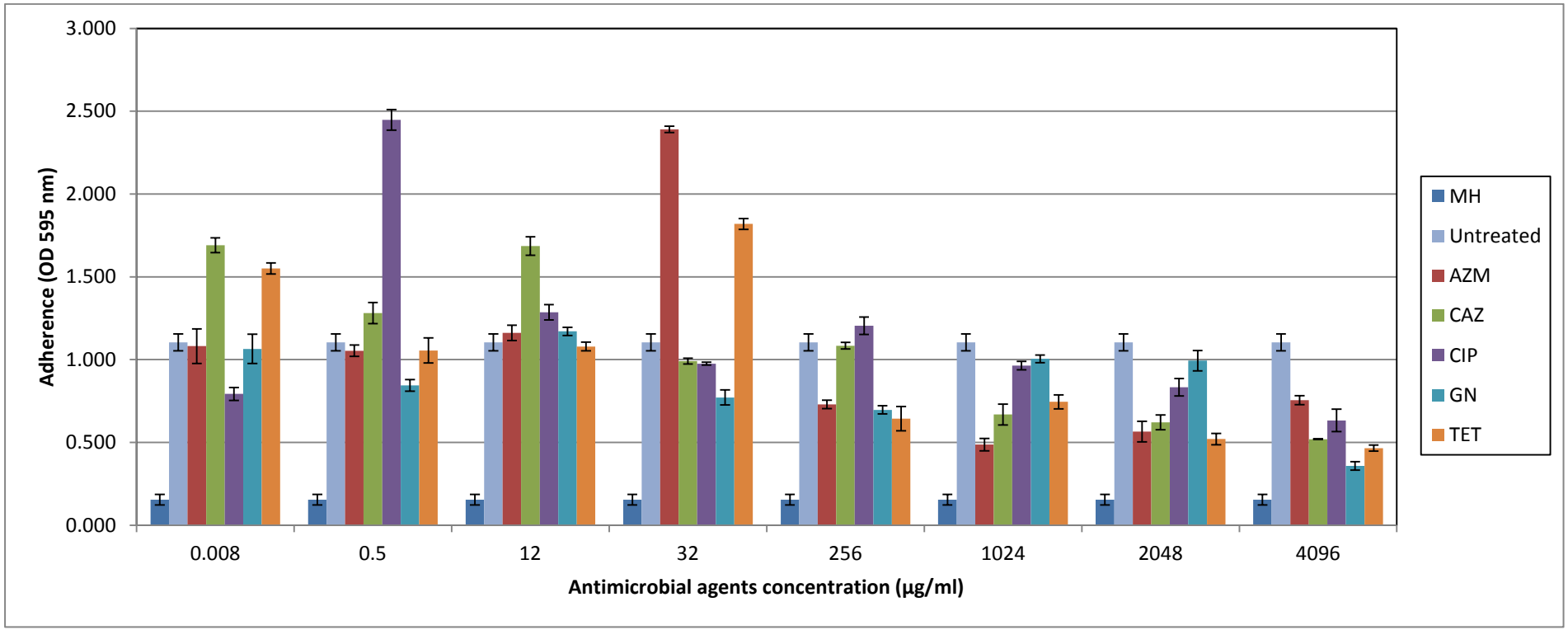

Figure 2.30: Effect of increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline against *P. shigelloides* isolate M67 to identify minimum biofilm inhibitory concentrations.

2.3.3. Effect of varying antimicrobial agent concentrations on biofilm formation

The effect of varying concentrations of AZM, CAZ, CIP, GN and TET (sub-MIC, MIC and supra-MIC) on both initial attachment and detachment was assessed for 25 *Aeromonas* spp. isolates and three *P. shigelloides*. In the initial attachment assays, sub-MIC, MIC and supra-MIC exposure to azithromycin reduced biofilm formation of all isolates, except for isolate M94 where sub- and supra-MIC exposure of azithromycin induced biofilm formation (Fig. 2.31). Sub-MIC, MIC and supra MIC exposure to azithromycin treatments were statistically significant ($p < 0.001$).

MIC exposure of isolates to ceftazidime induced biofilm formation of isolate M64 and reduced biofilm formation of all the remaining isolates (Fig. 2.32). While the sub- and supra-MIC exposuresto ceftazidime induced biofilm formation of isolate M30, only sub-MIC exposure to ceftazidime induced biofilm formation of *A. hydrophila* ATCC 7966^T (Fig. 2.32). The effect of sub-MIC, MIC and supra MIC exposure to ceftazidime treatments were statistically significant ($p < 0.001$).

Exposure to sub-MIC of ciprofloxacin induced biofilm formation of isolates M2, M95 and M90 and exposure to all concentrations (sub-MIC, MIC and supra-MIC) induced biofilm formation of isolates M57, M88 and M96, with the remaining isolates these concentrations reduced biofilm formation (Fig. 2.33). Sub-MIC and MIC exposure to ciprofloxacin induced biofilm formation of *A. hydrophila* ATCC 7966^T and *A. caviae* ATCC 15468^T (Fig. 2.33). Sub-MIC ($p = 0.006$), MIC ($p < 0.001$) and supra MIC ($p < 0.001$) exposure to ciprofloxacin treatments were statistically significant.

Sub-MIC exposure to gentamicin induced biofilm formation of isolates M2, M23, M31, M55 and M57and reduced biofilm formation all other isolates (Fig. 2.34). While the Supra-MIC exposure to gentamicin induced biofilm formation of isolate M2 and reduced biofilm formation of the remaining isolates, gentamicin MIC exposures reduced biofilm formation of all isolates (Fig. 2.34). Sub-MIC, MIC and supra MIC exposure to gentamicin treatments were statistically significant ($p < 0.001$).

Sub-MIC exposure to tetracycline induced biofilm formation of isolates M38, M55, M31, M96, M94 and *A. caviae* ATCC15468^T and exposure to MIC induced biofilm formation of isolates M17 and M90, while for the remaining isolates sub-MIC, MIC and supra-MICexposures reduced biofilm formation (Fig. 2.35). Sub-MIC, MIC and supra MIC exposure to tetracycline treatments were statistically significant ($p < 0.001$).

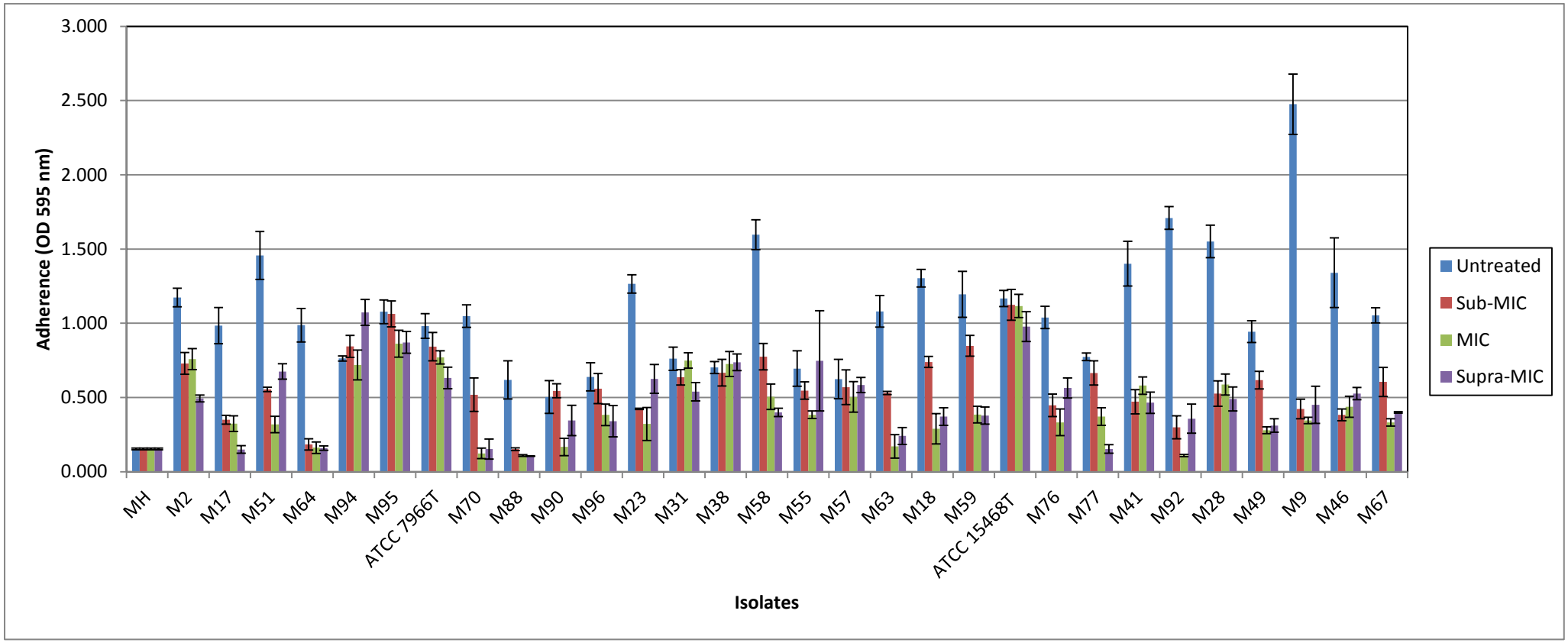

Figure 2.31: Effect of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

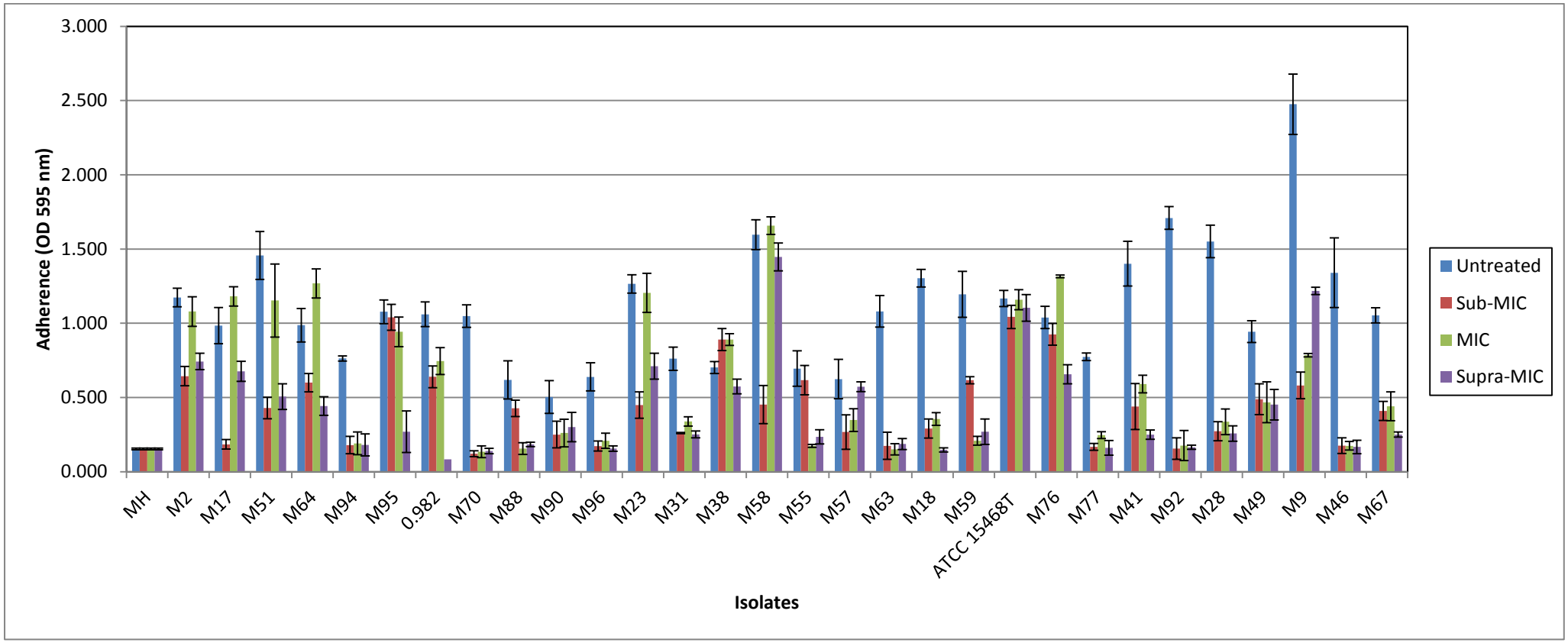

Figure 2.32: Effect of sub-MIC, MIC and supra-MIC exposures of ceftazidime (CAZ) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

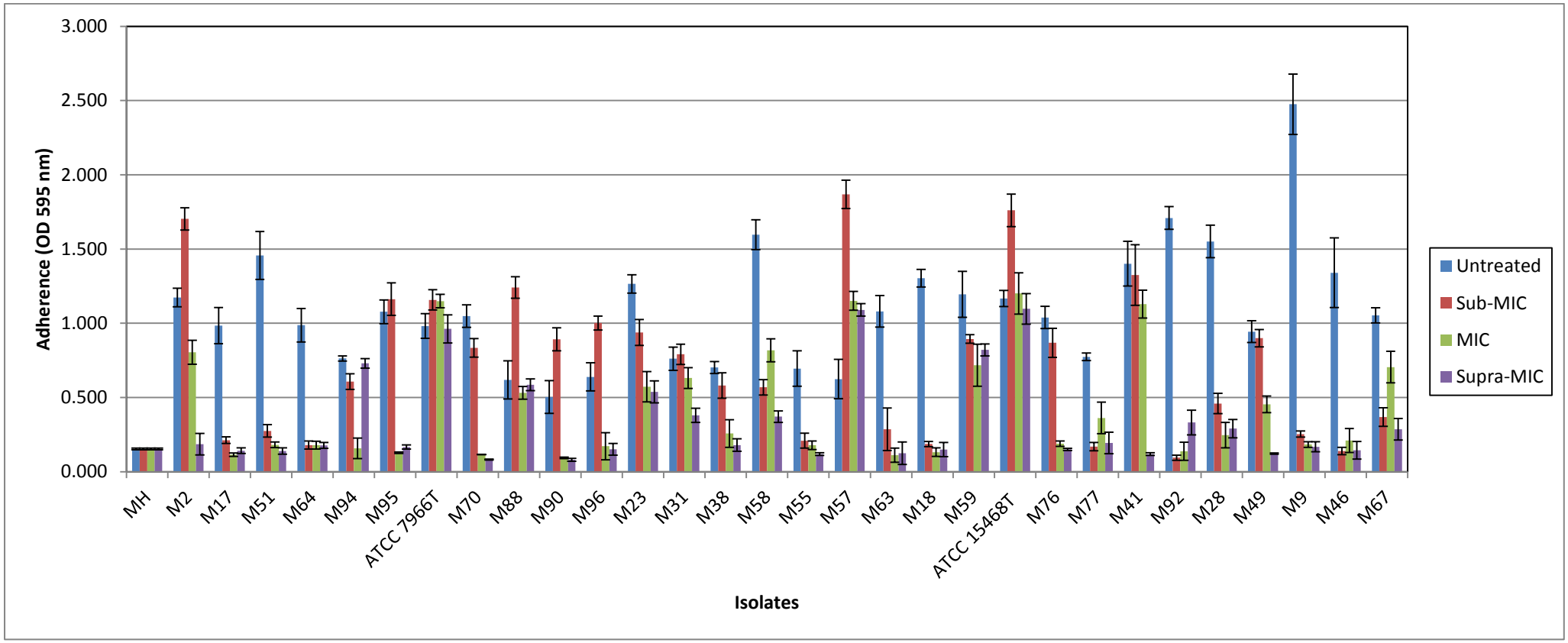

Figure 2.33: Effect of sub-MIC, MIC and supra-MIC exposures of ciprofloxacin (CIP) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

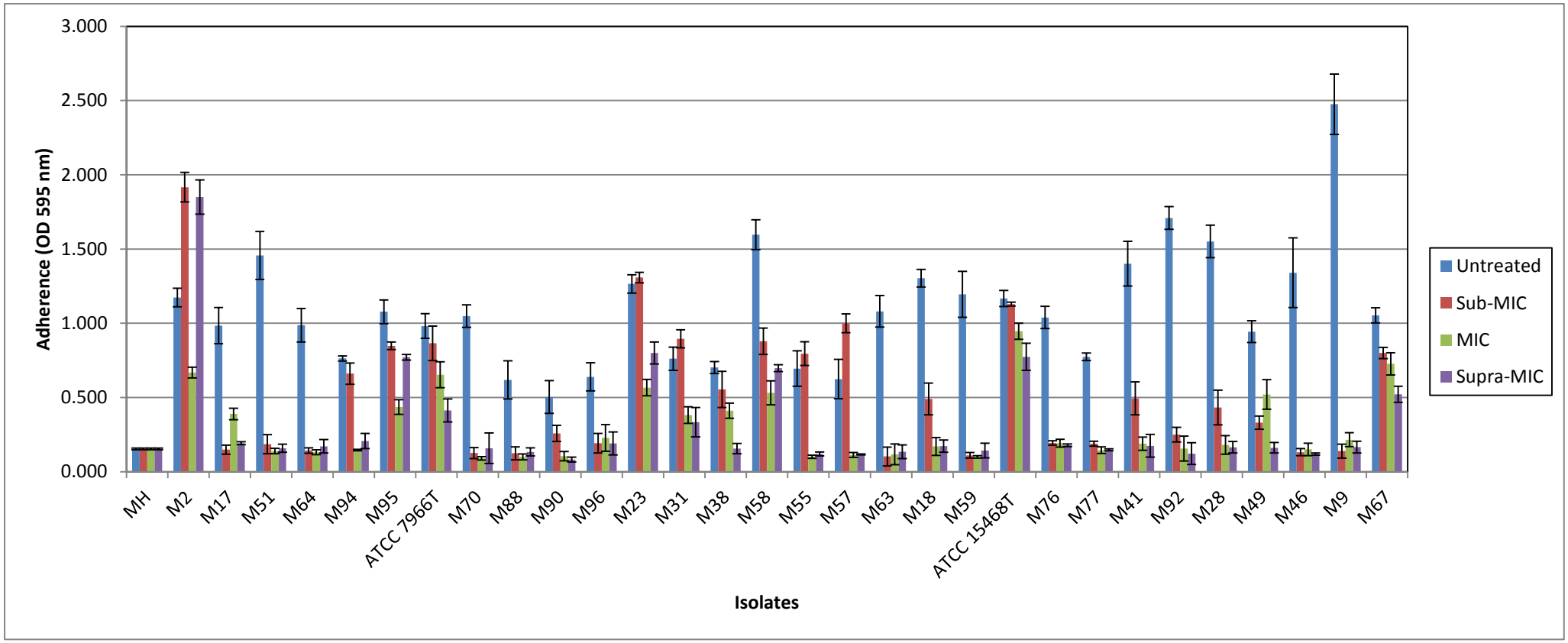

Figure 2.34: Effect of sub-MIC, MIC and supra-MIC exposures of gentamicin (GN) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

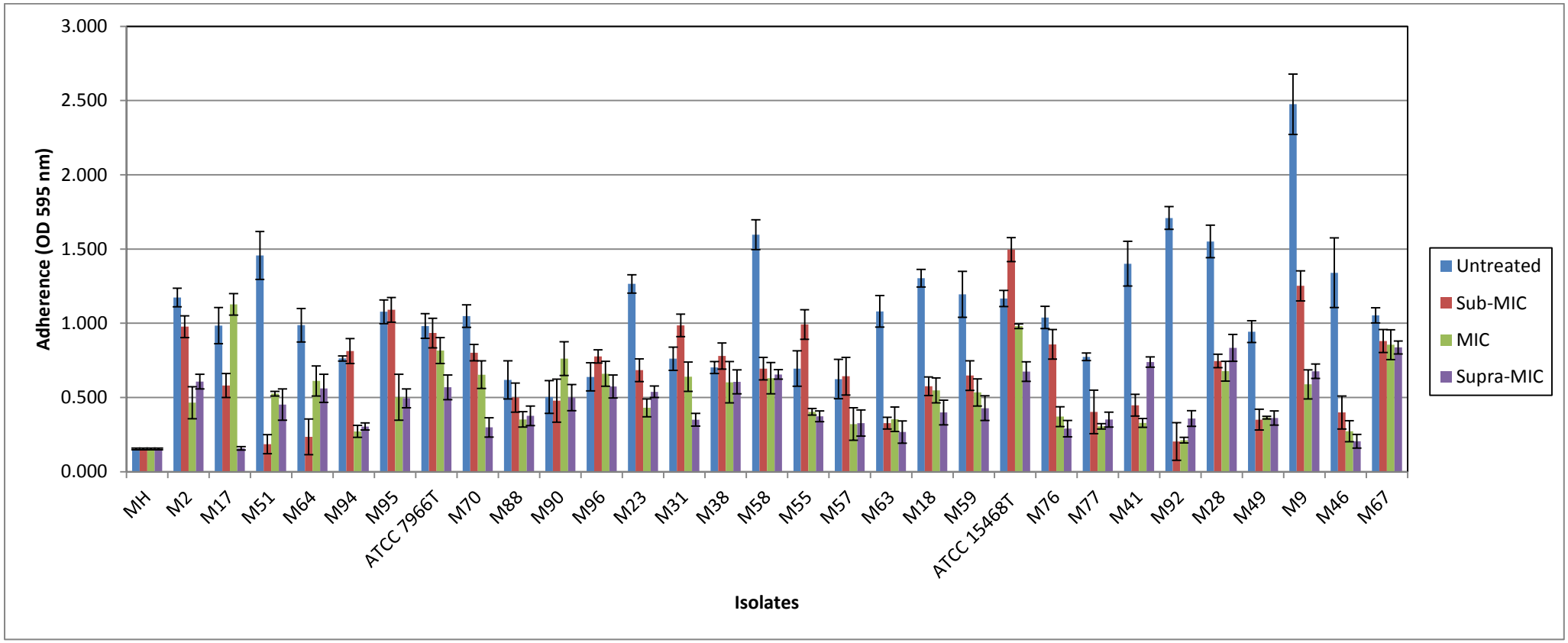

Figure 2.35: Effect of sub-MIC, MIC and supra-MIC exposures of tetracycline (TET) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

Sub-MIC, MIC and supra-MIC exposures of azithromycin inhibited initial attachment of 85.7% (24/28), 89.3% (25/28) and 89.3% (25/28) of isolates, respectively (Table 2.3). With sub-MIC, MIC and supra-MIC exposures of ceftazidime, it was observed that attachment of 92.9% (26/28), 82.1% (23/28) and 96.4% (27/28) of isolates, respectively, was inhibited (Table 2.3). While sub-MIC exposures of ciprofloxacin inhibited attachment of 75% (21/28) of isolates, MIC exposures inhibited attachment of 92.9% (26/28) of isolates, and supra-MIC exposures inhibited attachment of 89.5% (25/28) of isolates (Table 2.3). Sub-MIC, MIC and supra-MIC exposures of gentamicin inhibited attachment of 82.1% (23/28), 100% (28/28) and 96.4% (27/28) of isolates, respectively (Table 2.3). With tetracycline sub-MIC, MIC and supra-MIC exposures inhibited attachment of 75% (21/28), 89.3% (25/28) and 96.4% (27/54) of isolates, respectively (Table 2.3). The MIC exposure of gentamicin was observed to be more effective in inhibiting attachment of isolates followed by supra-MIC of both azithromycin and tetracycline (Table 2.3).

Table 2.3: Effect of antimicrobial agents on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates

Antimicrobial agents	% Decrease			% Increase			% No effect		
	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC
Azithromycin	85.7 (24/28)	89.3 (25/28)	89.3 (25/28)	3.6 (1/28)	0	3.6 (1/28)	10.7 (3/28)	10.7 (3/28)	10.7 (3/28)
Ceftazidime	92.9 (26/28)	82.1 (23/28)	96.4 (27/28)	3.6 (1/28)	7.1 (2/28)	0	3.6 (1/28)	10.7 (3/28)	3.6 (1/28)
Ciprofloxacin	75 (21/28)	92.9 (26/28)	89.3 (25/28)	21.4 (6/28)	7.1 (2/28)	10.7 (3/28)	3.6 (1/28)	0	0
Gentamicin	82.1 (23/28)	100 (28/28)	96.4 (2728)	17.9 (5/28)	0	3.6 (1/28)	0	0	0
Tetracycline	75 (21/28)	89.3 (25/28)	96.4 (2728)	14.3 (4/28)	7.1 (2/28)	0	10.7 (3/28)	3.6 (1/28)	3.6 (1/28)

*MIC = Minimum inhibitory concentration, *sub-MIC = 0.5×MIC, *supra-MIC = 2×MIC

In the detachment assays, sub-MIC exposure to azithromycin induced biofilm maturationof isolates M23, M46, M41, M55, M57, M70, M49, M94 and M77while the biofilms of the remaining isolates were reduced (Fig. 2.36). Supra-MIC exposure to azithromycin induced biofilm maturation of isolates M2,M23, M55, M57, M63, M70, M90 and M95promoted biofilm detachment of the remaining isolates (Fig. 2.36). Sub-MIC and MIC exposure to azithromycin induced biofilm maturation of isolate M70 and sub-MIC and supra-MIC exposures induced biofilm maturation of isolates M55 and M23. The exposure to all concentrations (sub-MIC, MIC and supra-MIC) only induced biofilm maturation of isolate M57 (Fig. 2.36). Sub-MIC exposure to azithromycin treatments were statistically insignificant (p = 0.122). MIC and supra MIC exposure to azithromycin treatments was statistically significant ($p < 0.001$, $p = 0.029$).

Sub-MIC exposure to sub-MIC of ceftazidime induced biofilm maturation by isolates M2, M17, M76, and M95 and promoted biofilm detachment of the remaining isolates (Fig. 2.37). While MIC exposure to ceftazidime induced biofilm maturationby isolates M18, M23, M28, M41, M55, M57 and M92, exposure to supra-MIC induced biofilm maturationof isolates M2, M23, M9, M92, M55 and M57. With the remaining isolates, these concentrations promoted biofilm detachment (Fig. 2.37). Sub-MIC (p = 0.003) and supra MIC (p = 0.031) exposure to ceftazidime treatments were statistically significantand MIC exposure to ceftazidime treatments were not statistically significant (p = 0.440).

Sub-MIC exposure to ciprofloxacin induced biofilm maturation by isolates M17, M23, M41, M46, M55, M57, M59, M63, M95 and M96 and *A. caviae* ATCC 15468^T (Fig. 2.38). MIC exposure to ciprofloxacin induced biofilm maturation of isolates M2, M23, M55, M57, M59 andM64, and *A. caviae* ATCC 15468^T as did supra-MIC exposure induced biofilm maturation of isolate M63 (Fig. 2.38). These concentrations induced the biofilm maturation of the remaining isolates (Fig. 2.38). Sub-MIC exposure to ciprofloxacin treatments were not statistically significant (p = 0.989), while MIC (p = 0.007) and supra-MIC (p<0.001) exposures to ciprofloxacin treatments were statistically significant.

While sub MIC exposure to gentamicin induced maturation of isolates M23, M55 and M95MIC exposure induced biofilm maturation of isolate M67, and supra-MIC exposure induced biofilm maturation of isolates M17, M23, M63, M94, and M90 (Fig. 2.39). However, sub-MIC, MIC and supra-MIC exposure to gentamicin promoted biofilm detachment of the remaining isolates (Fig. 2.39). Sub-MIC and MIC exposure to gentamicin treatments were statistically significant (p= 0.042, p = 0.001), while gentamicin supra-MIC exposures were

not statistically significant ($p = 0.086$).

Sub-MIC and supra-MIC exposure to tetracycline induced biofilm maturation of isolate M95, and exposure to MIC and supra-MIC induced biofilm maturation of isolate M88, while exposure to all antimicrobial agents induced biofilm maturation of isolates M23 and M55 (Fig. 2.40). While exposure to MIC of tetracycline induced biofilm maturation of isolate M2, exposure to supra-MIC induced biofilm maturation of isolates M41, M46,M59, M57, M77 and M92 (Fig. 2.40). These concentrations promoted biofilm detachment of the remaining isolates (Fig. 2.40). Sub-MIC, MIC and supra-MIC exposure to tetracycline treatments were statistically insignificant ($p = 0.052$, $p = 0.125$, $p = 0.482$).

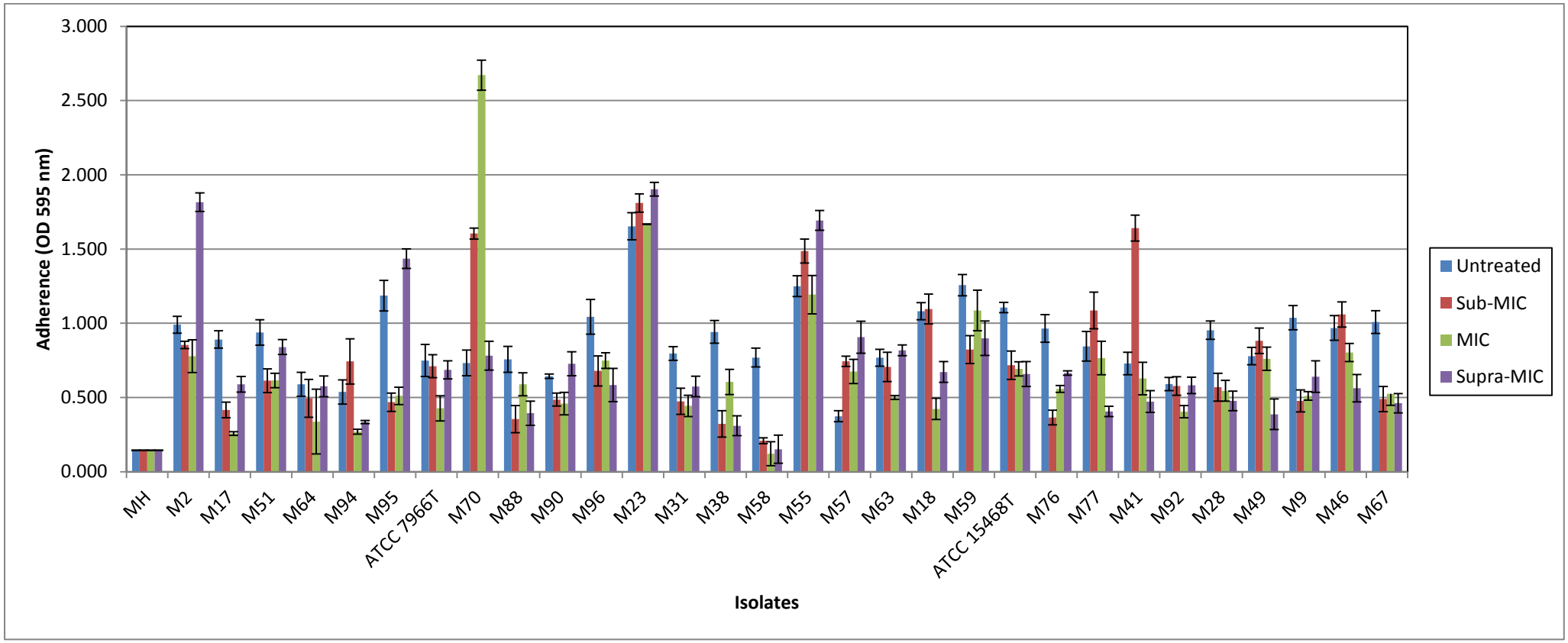

Figure 2.36: Effect of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

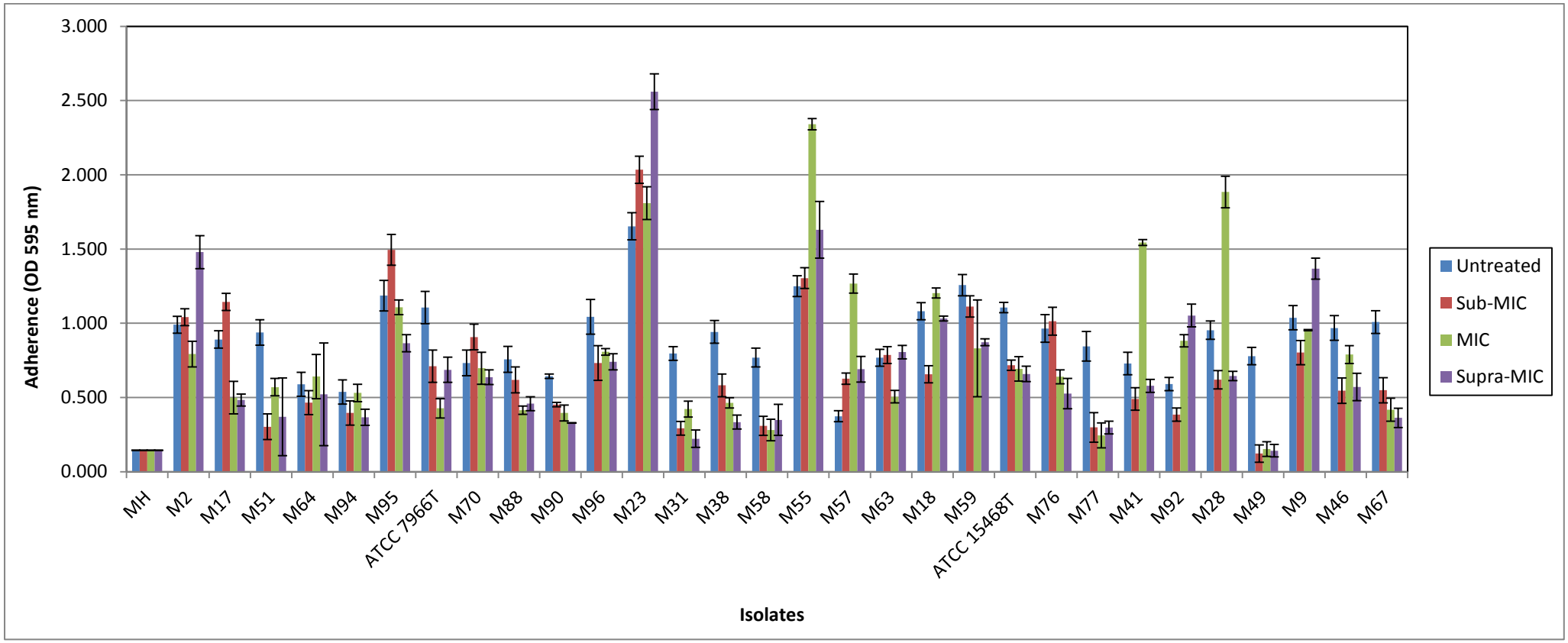

Figure 2.37: Effect of sub-MIC, MIC and supra-MIC exposures of ceftazidime (CAZ) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

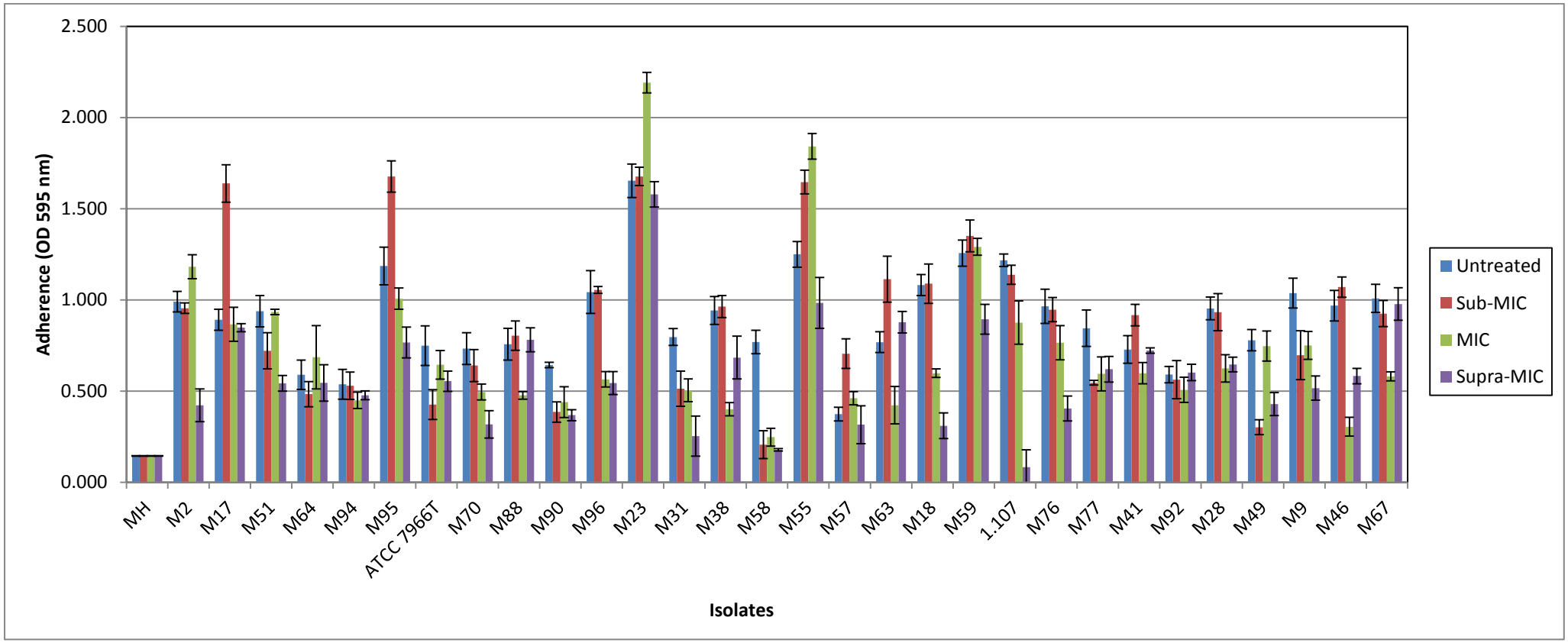

Figure 2.38: Effect of sub-MIC, MIC and supra-MIC exposures of ciprofloxacin (CIP) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

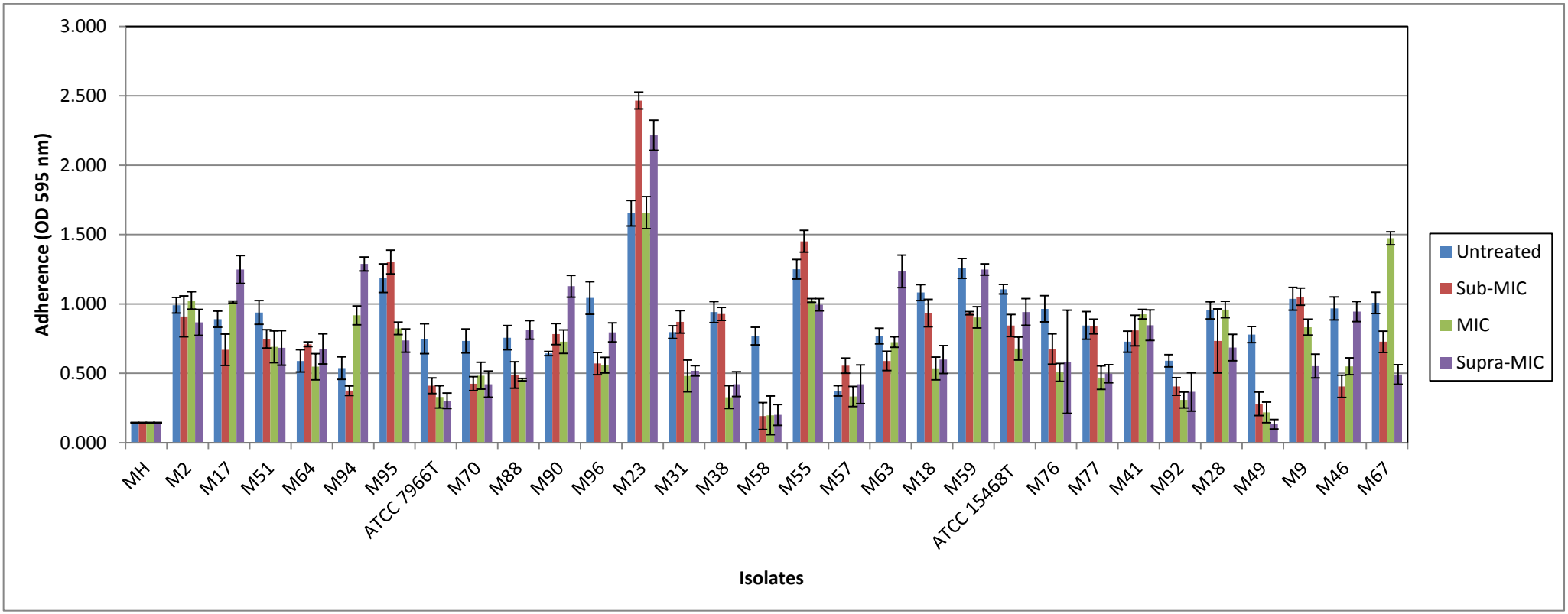

Figure 2.39: Effect of sub-MIC, MIC and supra-MIC exposures of gentamicin (GN) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

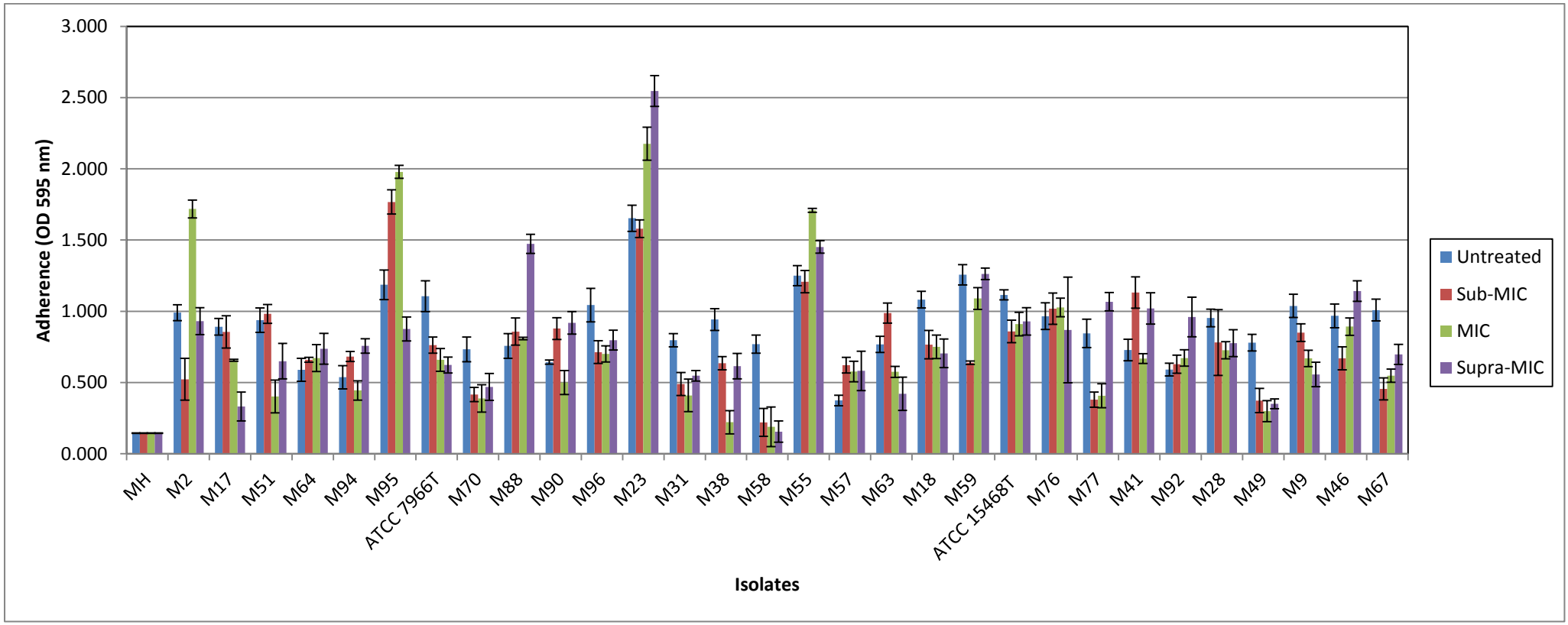

Figure 2.40: Effect of sub-MIC, MIC and supra-MIC exposures of tetracycline (TET) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M17, M51, M64, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M88, M90, M96); *A. culicicola* (M23, M31, M38, M58); *A. veronii* (M55, M57, M63); *A. caviae* (M18, M59, ATCC 15468^{T}); *A. salmonicida* (M76, M77); *Aeromonas* spp. (M41); *A. allosaccharophila* (M92); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides (*M9, M46, M67).

In the detachment assays, it was observed that sub-MIC, MIC and supra-MIC exposures of azithromycin increased detachment of 60.7% (17/28), 82.1% (23/28) and 71.4% (20/28) of isolates, respectively (Table 2.4). Sub-MIC, MIC and supra-MIC of ceftazidime increased detachment of 67.9% (19/28), 64.3% (18/28) and 67.9% (19/28), respectively (Table 2.4). It was observed that with sub-MIC exposures of ciprofloxacin, detachment was increased for only 35.7% (10/28) of isolates. MIC and supra-MIC of gentamicin exposures increased detachment of 60.7% (17/28) and 71.4% (20/28) of isolates, respectively (Table 2.4). Both sub-MIC and MIC exposures of gentamicin increased detachment of 57% (16/28) of isolates, respectively, and supra-MIC increased detachment of 60.7% (17/54) of isolates (Table 2.4). Sub-MIC, MIC and supra-MIC exposures of tetracycline increased detachment of 46.4% (13/28), 60.7 (17/28) and 57% (16/28) of isolates, respectively (Table 2.4). With the pre-formed biofilm assays, azithromycin was more effective when compared to other antimicrobial agents (Table 2.4).

Table 2.4: Effect of antimicrobial agents on detachment of *Aeromonas* spp. and *Plesiomonas* spp. isolates

Antimicrobial agents	% Decrease			% Increase			% No effect		
	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC
Azithromycin	60.7 (17/28)	82.1 (23/28)	71.4 (20/28)	28.6 (8/28)	27.1 (2/28)	21.4 (6/28)	10.7 (3/28)	10.7 (3/28)	7.1 (2/28)
Ceftazidime	67.9 (19/28)	64.2 (18/28)	67.9 (19/28)	14.3 (4/28)	28.6 (8/28)	21.4 (6/28)	17.9 (5/28)	7.1 (2/28)	10.7 (3/28)
Ciprofloxacin	35.7 (10/28)	60.7 (17/28)	71.4 (20/28)	35.7 (10/28)	21.4 (6/28)	3.6 (1/28)	28.6 (8/28)	17.9 (5/28)	25 (7/28)
Gentamicin	57.1 (16/28)	57.1 (16/28)	60.7 (17/28)	10.7 (3/28)	3.6 (1/28)	17.9 (5/28)	32.1 (9/28)	39.3 (11/28)	21.4 (6/28)
Tetracycline	24.1 (13/28)	60.7 (17/28)	57.1 (16/28)	10.7 (3/28)	17.9 (5/28)	32.1 (9/28)	42.9 (12/28)	21.4 (6/28)	10.7 (3/28)

*MIC = Minimum inhibitory concentration, *sub-MIC = 0.5×MIC, *supra-MIC = 2×MIC

2.3.4. Determination of percent biofilm reduction

When determining the percent reduction, the negative value represents induction of biofilm formation and the positive values represent reduction of biofilm formation. After calculating the percent reduction, it was observed that sub-MIC, MIC and supra-MIC exposures of azithromycin inhibited initial attachment of 92.8% (26/28), 96.4% (27/28) and 92.9% (26/28)of isolates, respectively (Table 2.5). Percent reduction for azithromycin sub-MIC exposure ranged from 1.5 to 100.3% and percent induction was 12 and 13.2%(Table 2.5). For MIC exposure, percent reduction ranged from 2 to 109.5% and percent induction was -4.2%. For supra-MIC exposure, percent reduction ranged from 8.6 to 110.5% and percent induction ranged from 6.4 to 50.8% (Table 2.5). Sub-MIC, MIC and supra-MIC exposures of ceftazidime inhibited initial attachment of 96.4% (27/28), 82.1% (23/28) and 100% (28/28) of isolates, respectively. Percent reduction for ceftazidime sub-MIC exposure ranged from 4 to 103.6% and percent induction was 34.3% (Table 2.5). For MIC exposure, percent reduction ranged from 5 to 102.1% and percent induction ranged from 4.2 to 34.3% (Table 2.5). For supra-MIC exposure, percent reduction ranged from 10.4 to 101.5%. With ciprofloxacin, sub-MIC, MIC and supra-MIC exposures inhibited initial attachment of 75% (21/28), 96.4% (27/28) and 96.4% (27/28) of isolates (Table 2.5). Percent reduction for ciprofloxacin sub-MIC exposure ranged from 6.1 to 103.8% and percent induction ranged from 4.9 to 264.3% (Table 2.5). For MIC exposure, percent reduction ranged from 0.9 to 117.3% and percent induction was 111.9%. For supra-MIC exposure, percent reduction ranged from 5.4 to 121.1% and percent induction was12.8 and 98.9% (Table 2.5). Sub-MIC, MIC and supra-MIC exposures of gentamicin inhibited adhesion of 82.1% (23/28), 100% (28/28) and 96.4% (27/28) of isolates. Percent reduction for gentamicin sub-MIC exposure ranged from 16.8 to 104.1% and percent induction ranged from 3.9 to 79.6% (Table 2.5). For MIC exposure, percent reduction ranged from 0.8 to 111.3% and percent induction was -3.4%. For supra-MIC exposure, percent reduction ranged from 6.9 to 120.6% and percent induction was 16.2% (Table 2.5). Adhesion of 75% (21/28), 89.2% (25) and 57.1% (16/28) of isolates was inhibited with sub-MIC, MIC and supra-MIC exposures of tetracycline, respectively (Table 2.5). Percent reduction for tetracycline sub-MIC exposure ranged from 5.7 to 97.5% and percent induction ranged from1.4 and 54.9% (Table 2.5). For MIC exposure, percent reduction ranged from 18.1 to 89.9% and percent induction ranged from 4.4 to 74.1%. For supra-MIC exposure, percent reduction ranged from 1 to 99.9% (Table 2.5).

Table 2.5: Percent reduction of sub-MIC, MIC and supra-MIC exposures to azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), and tetracycline (TET) on initial attachment of *Aeromonas* spp. isolates

Species designation	Isolates	% Reduction														
		AZM			CAZ			CIP			GN			TET		
		Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC
A. hydrophila	**M2**	43.5	40.6	66.7	52	9.3	42.2	-51.9	36.2	96.8	-72.8	49.5	-66.2	19.3	69.5	55.5
	M17	76.2	79.6	100.4	96.2	-23.8	37.1	92.9	104.7	101.4	100.6	71.6	95.4	48.5	-17.3	99.5
	M51	69.3	87.3	60	78.9	23.3	72.9	90.7	97.9	101	97.5	101.1	99.7	97.5	71.4	77.1
	M64	96.4	99	99.3	46.4	-33.8	65.3	96.9	96.9	97.1	101.3	102.6	97.8	90.2	45.1	51.1
	M94	-13.2	7.2	-50.8	95.7	93.8	95.5	25.5	99.2	5.4	16.8	101.2	91.2	-8.2	80.6	75.1
	M95	1.5	23.3	22.3	4	14.6	87.4	-9.2	102.7	98.6	24.8	69.4	33.1	-1.4	62.3	63
	ATCC 7966^{T}	14.1	39.6	68.6	16.7	10.6	22.4	-9.5	50.8	-12.8	-21.3	0.8	18.8	5.7	20	49.8
A. bestiarum	**M70**	59.2	103.3	100.1	103.6	102.1	101.5	24	104.2	108.1	103.1	107	99.5	27.6	44.1	83.8
	M88	100.3	109.5	110.5	41.2	99.5	93.5	-133.6	18.8	7.1	106.3	111.3	104.3	25.7	57.3	52.1
	M90	-12	96	45.2	72.3	69.3	57.9	-111.3	117.3	121.1	70.1	113.8	120.6	7	-74.1	1
	M96	16.3	52.7	61.4	96.1	88.7	99.5	-74.6	96.1	100.5	92	84.7	92.4	-28.4	-4.4	13.4
A. culicicola	**M23**	75.7	84.9	57.6	73.4	5.4	49.9	29.4	62.3	65.3	-3.9	62.8	41.9	52.3	75.1	65.3
	M31	20.5	2	36.5	82.4	69.5	84	-4.9	21.4	62.7	-22	62.5	70.3	-37	20	67.6
	M38	6.4	-4.2	-6.4	-34.3	-34.3	23.3	22.2	81.1	95.2	27	53	99.5	-14	18.1	17.6
	M58	57	75.6	82.9	79.3	-4.2	10.4	71.2	54	84.9	49.7	73.8	62.3	62.5	67	65.3

A. veronii	**M55**	27.3	57.4	-9.8	14.4	96.2	84.9	89.7	95.3	106.4	-18.6	109.6	106.2	-54.9	53.8	59.4
	M57	11.6	25.5	8.6	75.8	58.7	11	-264.3	-111.9	-98.9	-79.6	108.5	107.8	-4	64.4	63
	M63	59.3	98.1	90.6	97.8	100.3	96.4	85.7	104.5	103.1	105.5	103.9	102	81.2	78.4	87.7
A. caviae	**M18**	49.1	88.1	81	88	82.5	100.5	97.1	101.9	100.4	70.7	98.5	98.3	63.2	65.7	78.6
	M59	33.4	77.8	78.4	55.6	94.7	88.8	29	45.8	36	104.1	105.1	101	52.6	63.5	73.6
	ATCC 15468^{T}	3.7	21.7	38.7	4.2	5	18.7	12.2	0.9	6.2	-58.6	-3.4	6.9	-32.5	18.4	48.6
A. salmonicida	**M76**	66.8	79.7	53.7	12.9	-31.2	43.3	19.3	96	100.3	95.5	95.6	97.2	20.5	75.4	84.5
	M77	17.5	64.8	100	97.7	85	98.7	97.4	66.4	93.5	94.2	101.3	100.9	59.9	75.5	68.1
Aeromonas spp.	**M41**	74.5	65.8	75	77.1	65	92.2	6.1	21.8	102.8	72.7	97.2	98.3	76.4	85.9	53.1
A. allosacharophila	**M92**	90.6	102.8	86.9	99.8	98.5	99.3	103.8	101	88.5	93.8	99.8	102	96.8	96.2	86.8
A. jandae	**M28**	73.3	69	75.9	91.5	86.9	92.5	78.1	93.3	90.2	80	98	99.2	57.6	62.5	51.2
A. sobria	**M49**	41.3	84.1	80	57.6	60.2	62.2	5.5	61.9	104	77.5	53.5	99	75	73.3	73.6
P. shigelloides	**M9**	88.4	91.7	87.2	81.6	72.8	54.2	95.7	98.7	99.4	100.6	97.3	99.5	52.7	81.3	77.5
	M46	80.7	76.1	68.5	98.1	98.2	98.9	101.2	95.2	100.7	101.8	100.3	102.9	79.3	89.9	95.6
	M67	49.8	80.2	72.7	71.5	68	89.2	76.1	38.7	85.2	28.1	36.3	59	19.1	21.9	24

*Percent reduction=Percentage reduction = $[\,((C-B)-(T-B))/(C-B)\,]\times 100$, where B=average absorbance per well for blank wells, C=average absorbance per well for control wells, T=average absorbance per well for treated wells(Pitts *et al*., 2003),#AZM = azithromycin, #CAZ = ceftazidime, #CIP = #ciprofloxacin, #GN =gentamicin, #TET =tetracycline.^MIC = Minimum inhibitory concentration, ^sub-MIC = 0.5×MIC, ^supra-MIC = 2×MIC

In the pre-formed biofilm assays, sub-MIC, MIC and supra-MIC exposures of azithromycin detached biofilm of 64.2% (18/28), 89.2% (25/28) and 78.6% (22/28) of isolates (Table 2.6). Percent reduction for azithromycin sub-MIC exposure ranged from 3 to 89.8% and percent induction ranged from 1.6 and 161.3% (Table 2.6). For MIC exposure, percent reduction ranged from 5.1 to 103.8% and percent induction ranged from -0.9 to -329.6% (Table 2.6). For supra-MIC exposure, percent reduction ranged from 2 to 99% and percent induction ranged from 7.9 to 232.3%. Sub-MIC, MIC and supra-MIC of ceftazidime exposures detached biofilm of 67.9% (19/28), 71.4% (20/28) and 75% (21/28) of isolate(Table 2.6). Percent reduction for ceftazidime sub-MIC exposure ranged from 12.9 to 103.5% and percent induction ranged from 4.9 and 110.5% (Table 2.6). For MIC exposure, percent reduction ranged from 1.7 to 98.7% and percent induction ranged from -10.3 to -390% (Table 2.6). For supra-MIC exposure, percent reduction ranged from 5.2 to 100.5% and percent induction ranged from 6 to 138.2% (Table 2.6). It was observed that ciprofloxacin sub-MIC, MIC and supra-MIC exposures detached biofilm of 57.1% (16/28), 78.6% (22/28) and 89.3% (25/28) of isolates. Percent reduction for ciprofloxacin sub-MIC exposure ranged from 2.3 to 75.2% and percent induction ranged from 0.8 and 144.6% (Table 2.6). For MIC exposure, percent reduction ranged from 0.5 to 83.5% and percent induction ranged from 3.1 to 53.6%. For supra-MIC exposure, percent reduction ranged from 1 to 94.7% and percent induction ranged from 2.6 to 17.5% (Table 2.6). Sub-MIC, MIC and supra-MIC exposures of gentamicin inhibited attachment of 67.9% (19/28), 75% (21/28) and 67.9% (19/28) respectively (Table 2.6). Percent reduction for gentamicin sub-MIC exposure ranged from 1 to 56.2% and percent induction ranged from 1.7 and 79.1% (Table 2.6). For MIC exposure, percent reduction ranged from 6.9 to 91.5% and percent induction ranged from -0.3 to -96.9% (Table 2.6). For supra-MIC exposure, percent reduction ranged from 0.8 to 101.8% and percent induction ranged from 9 to 191.3%. Sub-MIC, MIC and supra-MIC exposure of tetracycline detached biofilm of 60.7% (17/28), 67.9% (19/28) and 57.1% (16/28) of isolates(Table 2.6). Percent reduction for tetracycline sub-MIC exposure ranged from 3.8 to 87.9% and percent induction ranged from 5.5 and 108.1% (Table 2.6). For MIC exposure, percent reduction ranged from 9.2 to 92.8% and percent induction ranged from 8.6 to 88.4%. For supra-MIC exposure, percent reduction ranged from 7 to 74.9% and percent induction ranged from 0.5 to 117.2% (Table 2.6).

Table 2.6: Percent reduction of sub-MIC, MIC and supra-MIC exposures to azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), and tetracycline (TET) of pre-formed biofilm of *Aeromonas* spp. isolates

Species designation	Isolates	% Reduction														
		AZM			CAZ			CIP			GN			TET		
		Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC	Sub-MIC	MIC	Supra-MIC
A. hydrophila	**M2**	16.2	25.1	-97.6	-6.1	23.3	-57.9	4.2	-22.7	67.2	9.4	-4.1	14.5	55.3	-86.2	7
	M17	63.6	84.9	40.5	-33.9	52.5	54.7	-100.2	3.3	5.8	29.7	-16.6	-47.9	4.9	31.5	74.9
	M51	41	40.8	12.4	80	46.4	71.7	27.4	0.5	49.8	24	31.2	32.1	-5.5	67.6	36.4
	M64	21.4	56.7	3.1	28	-11.6	15.2	23.9	-21.6	10	-26.9	9.4	-19.4	-15.8	-18.3	-32.9
	M94	-52.4	68.1	51.6	36	1.7	43.4	1.9	22.5	15.3	41.5	-96.9	-191.3	-37	23.7	-56
	M95	68.9	64.8	-23.9	-29.7	7.5	30.8	-47.2	17.2	40.3	-11.1	34.8	43.2	-55.8	-76.1	29.8
	ATCC 7966^{T}	6.4	53.3	10.4	41.1	70.6	43.6	53.5	17.4	32.3	56.2	69.3	74	35.7	46.5	50.2
A. bestiarum	**M70**	-148.2	-329.6	-8.3	-29.6	6.1	16.4	15.8	40.5	70.7	52.3	42.5	52.9	54	58.6	44.9
	M88	65.7	27.4	59.3	22.6	55.9	48.7	-7.8	45.8	-4	43.8	49.4	-9	-16.4	-8.6	-117.2
	M90	31.5	36.9	-16.9	38.4	49.7	63.1	51.7	40.8	55.2	-28	-17.1	-97.3	-47.3	28.8	-55.5
	M96	40.6	32.8	51.1	34.6	26.3	33.7	-1.3	53.3	55.6	52.6	53.9	27.6	36.7	38.1	27.3
A. culicicola	**M23**	-10.4	-0.9	-16.5	-25.2	-10.3	-60	-1.6	-35.7	5	-53.8	-0.3	-37.3	4.9	-34.7	-59.2
	M31	49.5	54.1	34.1	77.4	57.5	88.1	43.4	44.9	83.4	-11.4	48.4	42.8	47.1	59.4	38.3
	M38	77.8	42.3	79.3	45.2	60	76.3	-2.7	68	32.4	1.7	77	65.2	38.5	90.4	41.1
	M58	89.8	103.8	99	73.7	78.2	67.3	90.2	83.5	94.7	92.5	91.5	91.1	87.9	92.8	98.3
A. veronii	**M55**	-21.5	5.1	-40.1	-4.9	-98.8	-34.4	-35.9	-53.6	24	-18.3	20.3	23.1	3.8	-41.5	-18.3
	M57	-161.3	-131.6	-232.3	-110.5	-390	-138.2	-144.6	-38	25.5	-79.1	18.1	-20.7	-	-88.4	-90.7

														108.1		
	M63	10.1	43.1	-7.9	-2.8	42.1	-6	-55.5	55.5	-17.5	28.8	6.9	-74.9	-35.1	31	55.7
A. caviae	**M18**	-1.6	70.3	43.8	45.4	-13	5.2	-0.8	51.6	82.4	15.7	58.3	51.5	33.8	35.4	40.2
	M59	39	15.4	32.1	12.9	38.3	34.6	-8.5	-3.1	32.6	29.1	31.8	0.8	55.6	15	-0.5
	ATCC 15468^{T}	40.4	43	46.6	40.4	43	46.6	-11.5	-3.2	24	27.2	44.5	17	26.4	21.1	19.2
A. salmonicida	**M76**	73.2	49.7	36.6	-5.9	39.7	53.5	2.3	24.4	68.3	35.4	55.9	46.6	-6.4	-7.5	11.7
	M77	-34.5	11.2	62.6	78	85.7	78.1	42.7	35.8	32.1	1	53.7	49.7	66.4	62.5	-31.8
Aeromonas spp.	**M41**	-156.4	17.1	43.8	40.7	-139.9	25.7	-32.3	22.3	1	-13.8	-34	-20.3	-69.1	10.4	-50.1
A. allosacharophila	**M92**	3	41.7	2	46.2	-65.4	-103.5	6.2	18.7	-2.6	41.7	63.3	50.4	-8.5	-18.3	-83
A. jandae	**M28**	47.5	50.5	58.9	41.2	-115.1	38.1	2.6	40.7	38.1	27.1	-0.8	33.1	21.4	28.1	21.9
A. sobria	**M49**	-16.3	2.9	61.8	103.5	98.7	100.5	75.2	5	55.3	78.8	88.4	101.8	63.9	75.7	67.5
P. shigelloides	**M9**	62.8	59	44.4	26.3	9.3	-37.1	38.1	32.2	58.4	-1.7	22.9	54.4	21	41.2	53.9
	M46	-11.1	20.1	49.3	51.2	21.7	48.3	-12.4	80.6	46.9	68.3	50.7	2.8	36.3	9.2	-21.1
	M67	60.1	56	63.3	53.2	68.4	74.7	9.6	49.5	3.6	32.5	-53.9	59.7	64.1	53.3	36

*Percent reduction=Percentage reduction = $[\,((C-B)-(T-B))/(C-B)\,]\times 100$, where B=average absorbance per well for blank wells, C=average absorbance per well for control wells, T=average absorbance per well for treated wells(Pitts *et al*., 2003),#AZM = azithromycin, #CAZ = ceftazidime, #CIP = #ciprofloxacin, #GN = gentamicin, #TET = tetracycline.^MIC = Minimum inhibitory concentration, ^sub-MIC = 0.5×MIC, ^supra-MIC = 2×MIC

2.4. Discussion

Aeromonas spp. are suggested to contribute to severe economic loss in aquaculture since members of this genus cause disease in fish. Members of this genus have also been shown to cause disease in humans. Aeromonads are resistant to a wide variety of antimicrobial agents and their ability to form biofilm makes it difficult to eradicate them since biofilm cells are more resistant to antimicrobial agents compared to their planktonic counterparts (Presterl *et al*., 2009). The current study aimed atcomparing the MICs and MBICs of biofilm-associated *Aeromonas* spp. isolates. The effect of varying antimicrobial agent concentrations on biofilm formation was also determined.

The MICs forazithromycin ranged from 0.5-64 μg/ml and the MIC for ceftazidime and ciprofloxacin ranged from 0.064-64 μg/ml, while the MICs for gentamicin ranged from 0.0048-32 μg/ml, and the MIC for tetracycline ranged from 6-32 μg/ml. The observed MIC trend forazithromycin, ceftazidime ciprofloxacin was similar to that observed by Ramalivhana *et al*. (2009) where MIC of gentamicin, amikacin, isepamicin and netilmicin ranged from 1-64 μg/ml. High MIC levels were observed for tetracycline, azithromycin and ceftazidime. The high frequency of tetracycline resistance in *Aeromonas* spp. isolates has been reported by Jacobs and Chenia (2007). *A. allosaccharophila* was shown to be susceptible to ciprofloxacin at the MIC of greater than 1 mg/l (Picao *et al*., 2008). Castro-Escarpulli *et al*. (2003) reported that 44.1% of *Aeromonas* spp. isolates that were isolated from frozen fish were resistant to tetracycline. The lowest MIC in the current study was obtained with gentamicin. *Aeromonas* spp. isolates from a waste water treatment plant were susceptible to gentamicin (Igbinosa and Okoh, 2012). *Aeromonas* spp. isolates from India (Igbinosa *et al*, 2012), *Piaractus mesopotamicus* and *Oreochromis niloticus* (Belem-Costa and Cyrino, 2006) were also shown to be susceptible to gentamicin. Antimicrobial agents used in the present study (azithromycin, ciprofloxacin, ceftazidime, gentamicin, and tetracycline) were ineffective against the majority of *Aeromonas* spp. isolates as the MBICs ranged from 12μg/ml to ≥ 4096 μg/ml. The results obtained indicated that the effectiveness of the antimicrobial agents is not class-specific but concentration-dependent. The MBICs were~128-foldhigher compared to the MICs of antimicrobial agents (Table 2.2). Similar results were obtained by Sandoe *et al*. (2006) who observed that *Enterococcus faecalis* biofilm isolates displayed MBICs of 8192-, 4096- and 4096 mg/l for

ampicillin, vancomycin and linezolid, respectively, while the MIC of the same antimicrobial agents was 4 mg/l. The MBIC of bacitracin, vancomycin, gentamicin, rimfampin, nitrofurazone and enrofloxacin against *S.epidermidis* biofilm was observed to be 4096 µg/ml, while the MIC of the same antimicrobial agents was 512 µg/ml (Pettit *et al*., 2005).

Sub-inhibitory antimicrobial concentrations are lower than the MICs and have been suggested to be important in determining the resistance of bacteria to antimicrobial agents. This is due to the ability of these concentrations to affect cell functions without killing the cell (Dynes *et al*., 2009). In the current study, sub-MIC, MIC and supra-MIC of antimicrobial agents were effective in inhibiting initial attachment and detaching biofilm isolates. Percent reduction, whichmeasures the efficacy of treatments (Pitts *et al*., 2003), was used to confirm if sub-MIC, MIC and supra-MIC exposures indeed reduced the adhesion of the isolates on polystyrene surface. During initial attachment the biofilm is not fully matured, and the absence of the extracellular polymeric substances during this stage increases the susceptibility of the cells to antimicrobial agents (Takahashi *et al*., 2007). Among the sub-MICs exposures of all five antimicrobial agents tested, sub-MIC exposures of ceftazidime was the highest in the initial attachment and pre-formed biofilm and it inhibited attachment and detached 92.9% (26/28) and 67% (19/28) isolates, respectively. Sub-MICs exposures to ceftazidime are suggested to be capable of inhibiting QS in *P. aeruginosa* (Høiby *et al*., 2010). Pompilio *et al*. (2010) observed that moxifloxacin sub-MIC exposures affect cellular functions reducing cell hydrophobicity and biofilm formation of *St.maltophilia*. Even though the sub-MIC of ceftazidime was more effective, however, sub-MICs of all antimicrobial agents were more effective in inhibiting inital attachment as it has been observed by similar studies. Sub-MIC exposures of cefazolin, vancomycin and dicloxacillin were shown to inhibit initial attachment of *S.epidermidis* (Cerca *et al*. 2005). Cerca *et al*. (2005) suggested that sub-MIC exposures inhibit initial attachment of cells on surfaces which as a result prevents biofilm formation. Sub-MIC exposures of gentamicin and ciprofloxacin (0.5×MIC) were shown to reduce biofilm formation by *Salmonella typhimurium* (Majtan *et al*., 2007). Sub-MIC of gemifloxacin was shown to affect adhesiveness, hydrophobicity, haemagglutination and swarming of both *E. coli* and *S. aureus* at 1/32MIC and 1/8 MIC respectively (Dal Sasso *et al*., 2003). Sub-MICs were also observed to be associated with biofilm induction. Other studies have obtained similar results, e.g exposure of *S.*

*aureus*tosub-MICs of cefalexin was observed to induce biofilm formation (Haddadin *et al*., 2009). Cargill and Upton (2009) observed that sub-MIC of vancomycin increased cell density of *S. epidermidis*. Exposure to sub-MIC of cefotaxime was observed to induce biofilm formation of *S. typhimurium* (Majtan *et al*., 2007). All the above-mentioned studies suggested that induction of biofilm formation by sub-MIC might be due to inability of these concentrations to penetrate within the biofilm.

MIC testing is the most preferable method to measure the activity of the antimicrobial agents (Lim and Yun, 2001). However, the addition of higher doses of antimicrobial agents to the MIC is suggested to be effective in suppressing the growth of bacteria for a longer period and this is called supra-MIC (Cars and Odenbok-Toraqrist, 1993). MIC exposure using gentamicin was most effective in inhibiting initial attachment compared to MIC exposure of other antimicrobial agents and it inhibited 100% (28/28) of isolates, the MIC of azithromycin detached 82.1% (23/28) of isolates. The supra-MICs exposures of azithromycin and ciprofloxacin were more effective in the pre-formed biofilm assay as they both detached 71.4% (20/28) of isolates. The supra-MIC exposures of ceftazidime, gentamicin and tetracycline were more effective against initial attachment with all three antimicrobial agents inhibiting 96.4% (27/28) of the isolates. Both ceftazidime and ciprofloxacin have been shown to reduce the biofilm formation by *Burkholderia cepacia* (Peeters *et al*., 2009). Tetracycline was observed to reduce biofilm formation of *S.epidermidis* when used in combination with vancomycin. Liaqat *et al*. (2009) observed that 5×MIC of tetracycline reduced biofilm formation by *Klebsiella* spp., *P. aeruginosa*, *Achromobacter* spp. *K.pneumoniae*, and *Bacillus pumilis.* Supra-MIC exposure of gentamicin was observed to be effective in inhibiting *E. coli* biofilm alone and to be more effective when it was used in combination with ultrasound (Carmen *et al*., 2005). Non-typeable *Haemophilus influenzae*was observed to be resistant to the MIC of azithromycin, however, sub-MIC exposure of azithromycin reduced biofilm formation of the same strain (Starner *et al*., 2008). The MIC exposure of ciprofloxacin against *K. pneumoniae*was observed to be 0.18 mg/ml, however 10× the concentration was observed to be more effective in reducing the biofilm formation (Anderl *et al.*, 2002). The most effective concentration among the different concentrations (sub-MIC, MIC and supra-MIC) used was the MIC. MIC exposures of gentamicin inhibited initial attachment of 100% (28/28) of isolates and MIC exposures of azithromycin

detached biofilms of 82.1% (23/28) of isolates. However, all five antimicrobial agents were more effective in the intial attachment assays and this might be due to the absence of resistance mechanismsthat are present in the matured biofilm.

CHAPTER 3

Identification of efflux pump-associated antimicrobial resistance and determination of the effect of efflux pump inhibitors and DNase I on *Aeromonas* spp. biofilm formation

3.1. Introduction

Aeromonas spp. has been shown to form biofilms in different aquatic environments, where they infect fish and cause different infections (Bomo *et al*., 2004). Biofilms are also associated with different diseases in humans, and the innate resistance to antimicrobial agentsmakes it hard to treat infections caused by these bacterial species (Alcaide *et al*., 2010). The increase in resistance to antimicrobial agents by bacteria is caused by different mechanisms, of which the presence of efflux pumps is one of the main mechanisms. The resistance-nodulation-cell division (RND) is the major type of efflux pump observed in Gram-negative bacteria, where it provides resistance to different classes of antimicrobial agents. These efflux pumps have been identified in common bacterial species such as *E. coli, K.pneumoniae, Enterobacter* spp.,and *P.aeruginosa*(Lupo *et al*., 2012). Hernould *et al*. (2008)observedthat *A. hydrophila* possessedan AheABC pump belonging toRND system by blocking it with phenylalanine arginine β-naphthylamide (PAβN), an efflux pump inhibitor. They also observed that cefuroxime, cefoperazone, erythromycin, lincomycin, pristinamycin, minocycline, trimethoprim, fusidic acid and rifampin are the substrates of the AheABC system. Amoxicillin, carbenicillin, ciprofloxacin, enrofloxacin, erythromycin, kanamycin, minocycline, oxytetracycline, streptomycin, sulphamethoxazole, tetracycline, and trimethoprim were also observed to be the substrates of the AheABC system in *A. hydrophila*(Lukkana *et al*., 2011).*aheA* encodes a membrane fusion protein, and *aheB* for inner membrane transporter, while *aheC* encodes an outer membrane protein (Hernould *et al*., 2008).

Since the resistance of biofilms to antimicrobial agents is increasing, the use of efflux pump inhibitors has been shown to be the most promising strategy (Kvist *et al*., 2008). By blocking the efflux pumps, EPIs inhibit them from pumping antimicrobial agents out. Efflux pump inhibitors can either be used directly to inhibit biofilm formation or to increase susceptibility of the bacteria to certain antimicrobial agents (Pagès and Amaral, 2009). Two commonly used efflux pump inhibitors, PAβN or 1-(1-naphthylmethyl)-piperazine (NMP) have been shown to block the activity of RND family of efflux pumps (Bina *et al*., 2009). Blockage of

the RND pumps reduced biofilm formation (Bina *et al*., 2009) and also decreased bacterial pathogenicity since RND pumps have been suggested to be involved with pathogenicity in some Gram-negative bacteria species (Blair and Piddock, 2009).

The application of PAβN and NMP against *Aeromonas* spp. is still limited, however, various studies are providing evidence of their effectiveness against other bacterial species. Both PAβN and NMPincreasedsusceptibilityof *V.cholerae* to Triton X-100, deoxycholate, cholate and erythromycin and PAβN was more effective than NMP (Bina *et al*., 2009). Increased activity of levofloxacin against *E. coli* was observed only when it was used with either PAβN and NMP (Pagès and Amaral, 2009). Hannula and Hanninen (2008) observed that PAβN increased susceptibility of *Campylobacter jejuni* and *Campylobacter coli* to erythromycin and rifampicin. While these inhibitors affected the pathogenicity of *P. aeruginosa*by reducing its invasiveness (Hirakata *et al.*, 2009), Kvist *et al*. (2008) observed that they inhibited biofilm formation by *E. coli*. CCCP which affects the bacteria indirectly by inhibiting the energy required by the efflux pump to function has also been identified as one of the best EPI candidates (Ramón-García*et al*., 2006). CCCP was shown to increase the susceptibility of tetracycline by inhibiting the energy required by the Tap protein (efflux pump) of *Mycobacterium fortuitum* (Ramón-García *et al*., 2006).

The use of DNase I hasalso been shown to be an effective strategy to inhibit biofilm formation. DNase I digests extracellular DNA (eDNA) via its exonuclease activity and disruptsthe extracellular matrix which then affects biofilm formation (Tetz and Tetz, 2010). The presence of eDNA in the extracellular matrix makes it a better target because its digestion will inhibit biofilm formation since it is important in the adhesion of biofilm (Das *et al.*, 2010) and biofilm development (Qin *et al.*, 2007). Biofilm formation of *S. aureus* was reduced after digesting eDNA with DNase (Tetz and Tetz, 2010). Tetz *et al*. (2009) observed that digestion of eDNA by DNase I reduced the biomass of *E. coli* biofilm.

Therefore, this chapter aimed at identifying efflux pump-associated antimicrobial resistance and detecting the effect of different EPIs on initial attachment and biofilm detachment by *Aeromonas* spp. Furthermore, the effect of DNase I on attachment and biofilmdetachment on *Aeromonas* spp. was also investigated as a strategy to limit biofilm formation.

3.2. Materials and Methods

3.2.1. Identification of efflux pump-associated antimicrobial resistance

To determine the presence of the efflux mechanism in *Aeromonas* spp. isolates, Mueller-Hinton (M-H) agar plates were prepared with or without efflux pump inhibitors CCCP, PAβN or NMP (Sigma, SA)] (Magnet *et al*., 2001; Shi *et al*., 2005). The final concentration of the efflux inhibitors in the M-H agar was 20 µg/ml. M-H agar with or without efflux inhibitors were inoculated with standardized cell suspensions equivalent to a 0.5 McFarland standard and amikacin (AK30), ampicillin (AMP10), azithromycin (AZM15), cefpodoximine (CPD10), chloramphenicol (C30), ciprofloxacin (CIP5), enrofloxacin (Baytril-ENR5), erythromycin (E15), gentamicin (CN10), nalidixic acid (NA30), norfloxacin (NOR10), ofloxacin (OFX5), streptomycin (S10), sulphamethoxazole (RL25), tetracycline (TE30) and trimethoprim (W1.25)discs (Oxoid, Basington, UK) were placed onto the inoculated plates. Plates were then incubated at 30 °C for 24 h. Inhibition zone diameters were measured and the resistance or susceptibility profiles of the isolates were determined in the presence/absence of the efflux pump inhibitor. If the efflux pumps were present and active in isolates, zone diameters on the efflux inhibitor-containing plates were greater than corresponding zone diameters on plates without the inhibitor (Magnet *et al*., 2001). A difference of ≥ 5 mm between a plate without EPI and a plate with EPI was considered a inhibition-positive result. Resistance, susceptibility and intermediate susceptibility to antimicrobial agents were established according to CLSI criteria (CLSI, 2007).

3.2.2. Effect of efflux pump inhibitors on biofilm formation

EPIs (CCCP, PAβN or NMP)were used to determine their effect on initial attachment and pre-formed biofilm using modified microtiter assays (Basson *et al*., 2008).

Aeromonas spp. isolates including *A. hydrophila* ATCC 7966^{T} and *A. caviae* ATCC 15468^{T}, were grown overnight in TSB, washed three times with sterile distilled water and the turbidity of the cell suspensions was adjusted to that equivalent to a 0.5 McFarland standard. The first assay investigated the effect of EPIs on initial attachment of cells. EPIsto a final concentration of20 µg/mlwere added to 90 µl TSB and 10 µl of cell suspension and incubated for 24 h at 30 ºC with agitation. For the effect on mature biofilm, 24 h biofilms were exposed to EPIs to a final concentration of 20 µg/ml and incubated for a further 24 h. The negative

controlscontainedonly TSB broth and positive controls contained the respective cell suspensions only with no EPIs added. Staining and determination of OD values was done as described previously in section 2.2.4, according to Basson *et al*. (2008). The $OD_{595\ nm}$of the control wells without EPIs were compared to wells with EPIs to determine their effect on biofilm formation. All experiments were done in triplicate, on two separate occasions. Percentage reduction was calculated as described in section 2.2.4.

3.2.3. Effect of DNase I on initial attachment and biofilm detachment

Bovine DNase I (Sigma) was added prior to initial attachment and to pre-formed biofilm to determine if *Aeromonas* spp. isolates use eDNA as an adhesin to attach to the surface or to maintain their biofilm structure, respectively. Sixteen hour-old cultures were used to prepare cell suspensions which were standardized equivalent to a 0.5 McFarland standard (Basson *et al*., 2008). For initial attachment assays, bovine DNase I (Sigma) was added to 90 µl TSB and 10 µl of cell suspension, at a final concentration of 1 mg/ml (Izano *et al.*, 2009) and microtitre plates were incubated for 24 h at 30 ˚C with agitation (Basson *et al*., 2008).

For pre-formed biofilm detachment assays, 24 h biofilms were established following addition of 90 µl TSB and 10 µl of standardized cell suspension to microtitre plate wells, which were incubated at 30 ºC for 24 h. After a 24 h incubation period, microtitre plates were washed three times with sterile deionised water and allowed to air-dry. Following the addition of 90 µl TSB and DNase I (to a final concentration of 1 mg/ml), microtitre plates wereincubated for a further 24 h with agitation at 30˚C.

For both initial attachment and biofilm detachment assays, the negative controls contained TSB broth only and positive controls contained respective cell suspensions with no DNase I added. Staining and determination of OD values was done as previously described in section 2.2.4, according to Basson *et al*. (2008). All assays were done in triplicate on two separate occasions. Percentage reduction was calculated as described in section 2.2.4.

3.2.4. Statistical analysis

One-way repeated measures ANOVA and Student's t-tests (SigmaStat) were used to examine the statistical significance of treated vs untreated assays for initial attachment and biofilm detachment assays. A *p* value of <0.05 was considered significant.

3.3. Results

3.3.1. Identification of efflux pump-associated antimicrobial resistance

When antimicrobial susceptibility was examined in the absence of EPIs, 100% (54/54) of isolatesdisplayed susceptibility to OFX5 (Table 3.1).Susceptibility of 98.1% (53/54) of isolates to NOR10, CIP5 and AK30, respectively was also observed. While 96.3% (52/54), 81.5% (44/54), and 79.6% (43/54) of isolates displayed susceptibility to CN10, NA30 and ENR5, respectively, 70.4% (38/54)) of isolates displayed susceptibility to both cefpodoxime (CPD10) and C30 (Table 3.1). Susceptibility to AZM15 was observed for 50% (27/54) isolates and withthe remaining antimicrobial agentsa small number of isolates (<50%) were susceptible (Table 3.1). Isolates were more resistant to W1.25 and RL25.With the former, it was observed that100% (54/54) of isolates displayed resistance and with the latter, 98.1% (53/54) of isolates displayed resistance (Table 3.1). With AMP10 or TE30, 94.4% (51/54) of isolates displayed resistance, while with erythromycin 90.7% (49/54) of isolates were resistant (Table 3.1).

In order to determine the efflux phenotypes of isolates, zone diameters on EPI-containing plates were compared to control plates without EPIs. When zone differences of ≥5 mm were observed, the R, I, S criteria of the isolates was assessed. Changes noted included: resistant (R→R), partial inhibition (R→I), complete inhibition (R→S), intermediate susceptibility to susceptibility (I→S) and susceptibility (S→S). R→R and S→S indicate that although the zone diameter difference was ≥5 mm; there however, was no change in the phenotype. Therefore, the current study will focus on (R→I), (R→S) and (I→S).

Table 3.1: Susceptibility of 48 *Aeromonas* spp. and six *P. shigelloides*isolates to 16 antimicrobial agents.

Antimicrobial agents	%Susceptibility	%Intermediate susceptibility	% Resistance
Ampicillin (AMP10)	1.9 (1/54)	3.7 (2/54)	94.4 (51/54)
Cefpodoxime (CPD10)	70.4 (38/54)	5.6 (3/54)	24.1 (13/54)
Chloromphenicol (C30)	70.4 (38/54)	20.4 (11/54)	9.3 (5/54)
Trimethoprim (W1.25)	0	0	100 (54/54)
Sulphamethoxazole (RL25)	1.9 (1/54)	0	98.1 (53/54)
Norfloxacin (NOR10)	98.1 (53/54)	0	1.9 (1/54)
Enrofloxacin (ENR5)	79.6 (43/54)	20.4 (11/54)	0
Ofloxacin (OFX5)	100 (54/54)	0	0
Ciprofloxacin (CIP5)	98.1 (53/54)	0	1.9 (1/54)
Nalidixic acid (NA30)	81.5 (44/54)	7.4 (4/54)	11.1 (6/54)
Tetracycline (TE30)	1.9 (1/54)	3.7 (2/54)	94.4 (51/54)
Gentamicin (CN10)	96.3 (52/54)	1.9 (1/54)	1.9 (1/54)
Streptomycin (S10)	98.1 (53/54)	1.9 (1/54)	0
Amikacin (AK30)	98.1 (53/54)	0	1.9 (1/54)
Azithromycin (AZM15)	50 (27/54)	38.9 (21/54)	11.1 (6/54)
Erythromycin (E15)	1.9 (1/54)	7.4 (4/54)	90.7 (49/54)

With CCCP, varying levels of efflux pump inhibition was observed when it was used in combination with 11 of the 16 antimicrobial agents tested (Table 3.2). Partial inhibition of the efflux pump of a single isolate was observed when CCCP was used in combination with ampicillin, cefpodoxime, sulphamethoxazole, ciprofloxacin and tetracycline. Complete inhibition of efflux pump was obtained when CCCP was used in combination with cefpodoxime,

chloramphenicol, trimethoprim, nalidixic acid, amikacin and azithromycin. Complete inhibition of the efflux pump was obtained for 14.8% (8/54) of isolates when CCCP was used with cefpodoxime. Finally, intermediate susceptibility to complete inhibition was observed when CCCP was used in combination with cefpodixime, chloromphenicol, enrofloxacin, nalidixic acid and azithromycin. It was observed that 13% (7/54) of isolates changed from intermediate susceptibility to complete susceptibility when CCCP was used with chloromphenicol.

Table 3.2: Alteration in susceptibility of 48 *Aeromonas* spp. and six *P. shigelloides*to 16 antimicrobial agents following exposure to CCCP

Antibiotics	R→R*	R→I*	R→S*	I→S*	S→S*
Ampicillin (AMP10)	29.6 (16/54)	1.9 (1/54)	0	0	0
Cefpodoxime (CPD10)	1.9 (1/54)	1.9 (1/54)	14.8 (8/54)	1.9 (1/54)	1.9 (1/54)
Chloromphenicol (C30)	0	0	5.6 (3/54)	13 (7/54)	7.4 (4/54)
Trimethoprim (W1.25)	0	0	1.9 (1/54)	0	0
Sulphamethoxazole (RL25)	0	1.9 (1/54)	0	0	0
Norfloxacin (NOR10)	0	0	0	0	25.9 (14/54)
Enrofloxacin (ENR5)	0	0	0	3.7 (2/54)	9.3 (5/54)
Ofloxacin (OFX5)	0	0	0	0	5.6 (3/54)
Ciprofloxacin (CIP5)	0	1.9 (1/54)	0	0	22.2 (12/54)
Nalidixic acid (NA30)	0	0	3.7 (2/54)	1.9 (1/54)	11.1 (6/54)
Tetracycline (TE30)	22.2 (12/54)	1.9 (1/54)	0	0	0
Gentamicin (CN10)	0	0	0	0	9.3 (5/54)
Streptomycin (S10)	0	0	0	0	7.4 (4/54)
Amikacin (AK30)	0	0	1.9 (1/54)	0	1.9 (1/54)
Azithromycin (AZM15)	0	0	1.9 (1/54)	1.9 (1/54)	0
Erythromycin (E15)	9.3 (5/54)	0	0	0	1.9 (1/54)

*R→R=resistant to resistant, *R→I=resistant to intermediate, *R→S=resistant to susceptible, *I→S=intermediate to susceptible, *S→S=susceptible to susceptible

NMP increased susceptibility of isolates to six different antimicrobial agents (Table 3.3). Partial inhibition of efflux pump was observed forcefpodoxime and erythromycin, in the presence of NMP (Table 3.3). Complete inhibition of efflux pump activity was observed when NMP was used in combination with cefpodoxime, nalidixic acid, and amikacin.Complete susceptibility to cefpodoxime was observed for 7.4% (4/54) of isolates. It was also observed that

5.6% (3/54) of isolates, which had intermediate susceptibility to chloromphenicol, became completely susceptible when NMP was used.

Table 3.3: Alteration in susceptibility of 48 *Aeromonas* spp. and six *P. shigelloides* to 16 antimicrobial agents following exposure to NMP

Antibiotics	R→R*	R→I*	R→S*	I→S*	S→S*
Ampicillin (AMP10)	14.8 (8/54)	0	0	0	0
Cefpodoxime (CPD10)	5.6 (3/54)	3.7 (2/54)	7.4 (4/54)	0	1.9 (1/54)
Chloromphenicol (C30)	1.9 (1/54)	0	0	5.6 (3/54)	1.9 (1/54)
Trimethoprim (W1.25)	0	0	0	0	0
Sulphamethoxazole (RL25)	0	0	0	0	0
Norfloxacin (NOR10)	0	0	0	0	13 (7/54)
Enrofloxacin (ENR5)	0	0	0	0	1.9 (1/54)
Ofloxacin (OFX5)	0	0	0	0	1.9 (1/54)
Ciprofloxacin (CIP5)	0	0	0	0	1.9 (1/54)
Nalidixic acid (NA30)	0	0	3.7 (2/54)	1.9 (1/54)	0
Tetracycline (TE30)	3.7 (2/54)	0	0	0	0
Gentamicin (CN10)	0	0	0	0	1.9 (1/54)
Streptomycin (S10)	0	0	0	1.9 (1/54)	3.7 (2/54)
Amikacin (AK30)	0	0	1.9 (1/54)	0	0
Azithromycin (AZM15)	0	0	0	0	0
Erythromycin (E15)	1.9 (1/54)	1.9 (1/54)	0	0	0

*R → R = resistant to resistant, *R → I = resistant to intermediate, *R → S = resistant to susceptible, *I → S = intermediate to susceptible, *S → S = susceptible to susceptible

PAβN increased susceptibility to 10 different antimicrobial agents (Table 3.4). Partial inhibition was obtained when PAβN was used in combination with cefpodoxime and erythromycin. PAβN in combination with erythromycin resulted in partial efflux pump inhibition for 7.4% (4/54) of isolates, unlike thePAβN-cefpodoximecombination in which partial inhibition was obtained for a single isolate only. PAβN resulted in complete efflux pump inhibition when used in combination with ampicillin, cefpodoxime, choloramphenicol, nalidixc acid, tetracycline, amikacin, azithromycinand erythromycin. Complete inhibition of the efflux pump was obtained for 5.6% (3/54) of isolateswhen PAβN was used with cefpodoxime. Finally, it was observed that

9.3% (5/54) of isolates that were intermediate susceptible became complete susceptible when PAβN was used with either chloromphenicol or azithromycin, respectively.

Table 3.4: Alteration in susceptibility of 48 *Aeromonas* spp. and six *P. shigelloides* to 16 antimicrobial agents following exposure to PAβN

Antibiotics	R→R*	R→I*	R→S*	I→S*	S→S*
Ampicillin (AMP10)	9.3 (5/54)	0	1.9 (1/54)	0	0
Cefpodoxime (CPD10)	3.7 (2/54)	1.9 (1/54)	5.6 (3/54)	0	0
Chloromphenicol (C30)	0	0	3.7 (2/54)	9.3 (5/54)	5.6 (3/54)
Trimethoprim (W1.25)	0	0	0	0	0
Sulphamethoxazole (RL25)	0	0	0	0	0
Norfloxacin (NOR10)	0	0	0	0	9.3 (5/54)
Enrofloxacin (ENR5)	0	0	0	1.9 (1/54)	3.7 (2/54)
Ofloxacin (OFX5)	0	0	0	0	0
Ciprofloxacin (CIP5)	0	0	0	0	7.4 (4/54)
Nalidixic acid (NA30)	0	0	3.7 (2/54)	3.7 (2/54)	0
Tetracycline (TE30)	11.1 (6/54)	0	1.9 (1/54)	0	0
Gentamicin (CN10)	0	0	0	0	0
Streptomycin (S10)	0	0	0	1.9 (1/54)	1.9 (1/54)
Amikacin (AK30)	0	0	1.9 (1/54)	0	0
Azithromycin (AZM15)	0	0	3.7 (2/54)	9.3 (5/54)	1.9 (1/54)
Erythromycin (E15)	7.4 (4/54)	7.4 (4/54)	1.9 (1/54)	0	0

*R → R = resistant to resistant, *R → I = resistant to intermediate, *R → S = resistant to susceptible, *I → S = intermediate to susceptible, *S → S = susceptible to susceptible.

The antimicrobial agent that was observed to be effluxed the most by all EPIs was cefpodoxime (displayed greatest levels of complete inhibition with EPIs than any of the other antimicrobial agents tested).Alterations in susceptibility for isolates M65, M80 and M90 were observed with CCCP and NMP and with CCCP and PAβN, alterations in susceptibility wereobserved for isolates M2, M8, M18, M23 and M80. Alterations in susceptibility with PAβN and NMP were observed forisolates M50, M26 and M51. Isolates for which alterations in susceptibility were observed with all three EPIs (CCCP, NMP and PAβN) included: isolates M23, M34, M51, M62, and M72.

3.3.2. Effect of EPIs on initial attachment of *Aeromonas* and*Plesiomonas* spp. isolates

CCCPinhibited attachment of92.6% (50/54) of isolates and increased attachment ofisolates M23, M41 as well as*A.hydrophila* ATCC 7966^{T}and *A. caviae* ATCC 15468^{T}(Fig. 3.1).It was observed that NMP inhibited attachment of 98.1% (53/54) of isolates and increased attachment of a single isolate (M94) (Fig. 3.2). NMP also increased attachment of *A. hydrophila* ATCC 7966^{T}and *A. caviae* ATCC 15468^{T} type strains (Fig. 3.2). The least effective inhibitor was PAβN, which decreased adherence of 61.1% (33/54) of isolates (Fig.3.3).PAβN increased attachment of isolates M8, M26, M22, M41, M50, M57, M58, M59, M60, M62, M63, M64, M66, M68, M86, M90, M92, M94,*A. hydrophila* ATCC 7966^{T}and*A. caviae* ATCC 15468^{T}(Fig. 3.3). Inhibition decreased in the following order: NMP>CCCP>PAβN (Figs 3.1-3.3, Table 3.5). Treatments of all EPIs were statistically significant ($p < 0.001$).

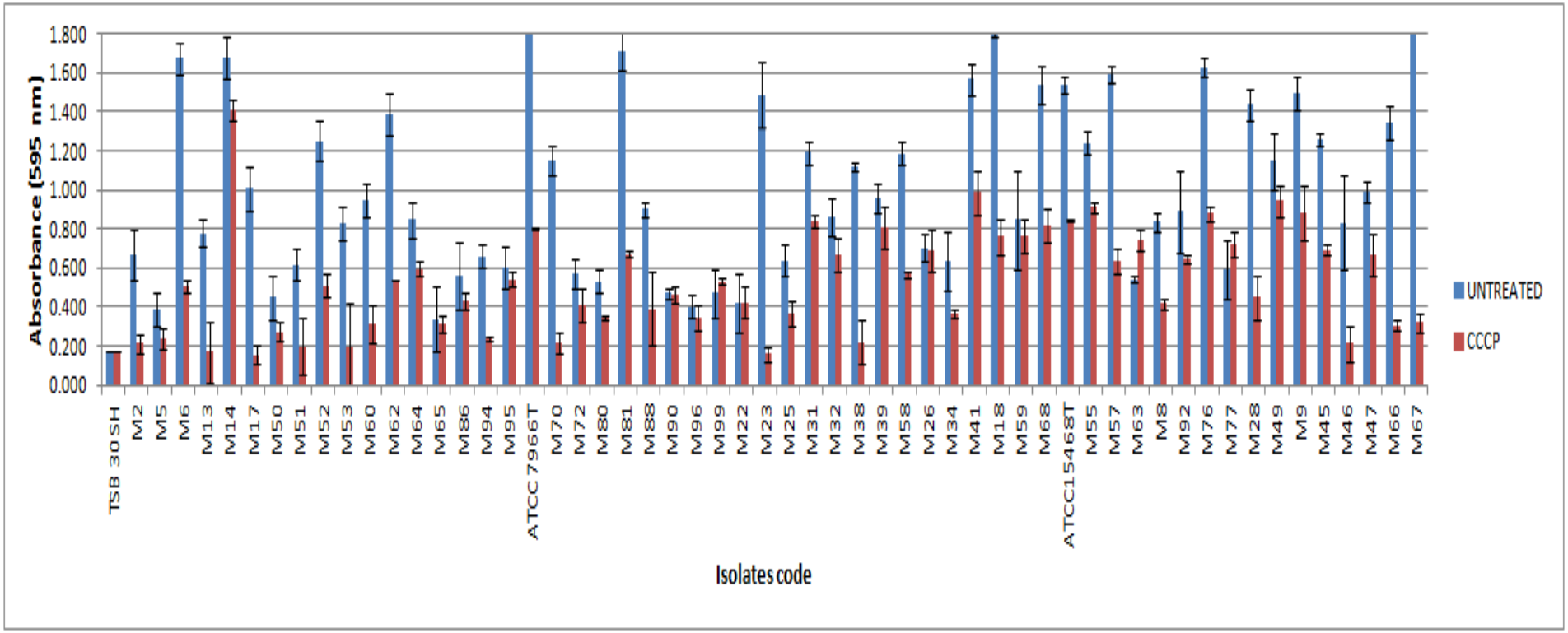

Figure 3.1: Effect of 20 µg/ml carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A.caviae* (M18, M59, M68, ATCC15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

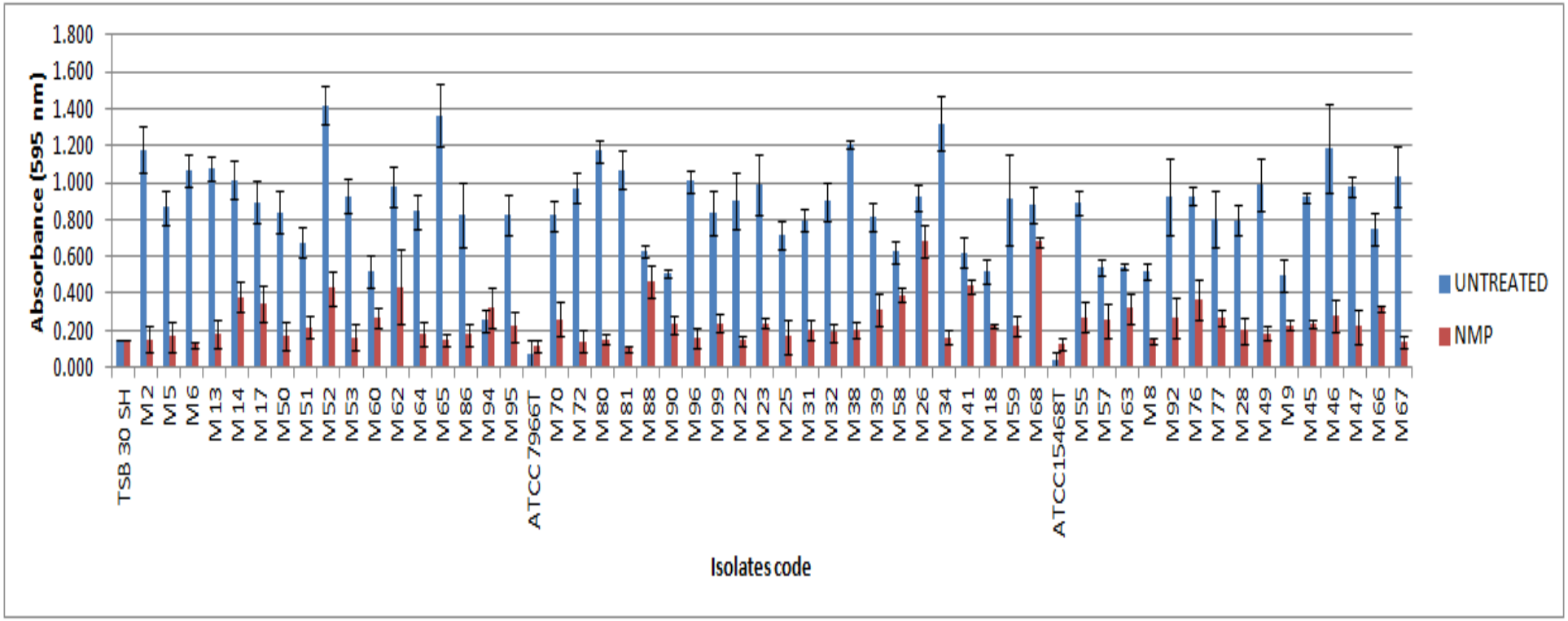

Figure 3.2: Effect of 20 µg/ml 1-(1-naphthylmethyl)-piperazine (NMP) on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99,); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

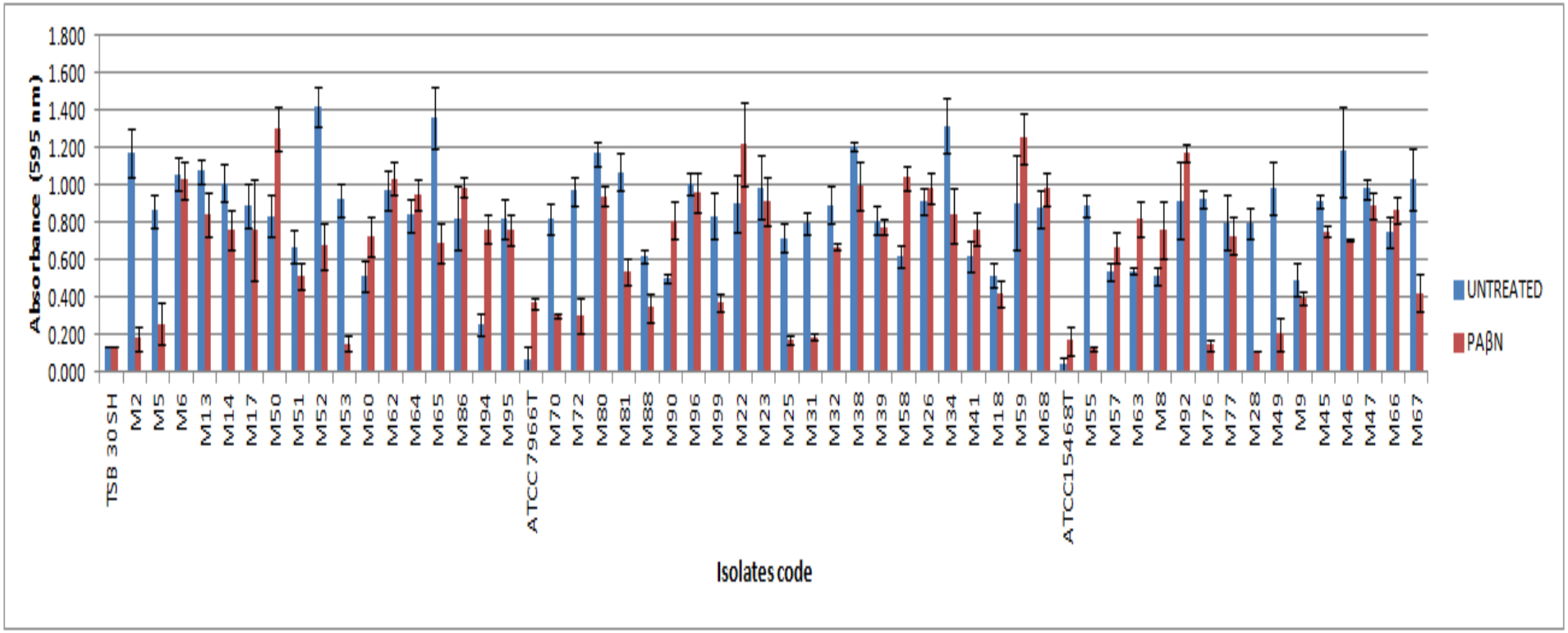

Figure 3.3: Effect of 20 µg/ml phenylalanine arginine β-naphthylamide (PAβN) on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

The most effective EPI in initial attachment was NMP (Table 3.5). NMP inhibited attachment of 98.1% of isolates. CCCP inhibited attachment of 92.6% of isolates whilst PAβNwas the least effective (Table 3.5).

Table 3.5: Effect of CCCP, NMP and PAβNon initial attachment and pre-formed biofilm of *Aeromonas* spp. and *Plesiomonas* spp. isolates

EPIs*	Initial attachment			Pre-formed biofilm		
	% Decrease	% Increase	% No effect	% Decrease	% Increase	% No effect
CCCP# (20 µg/ml)	92.6 (50/54)	3.7 (2/54)	3.7 (2/54)	85.2 (46/54)	7.4 (4/54)	7.4 (4/54)
NMP# (20 µg/ml)	98.1 (53/54)	1.9 (1/54)	0 (0/54)	100 (54/54)	0 (0/54)	0 (0/54)
PaβN# (20µg/ml)	61.1 (33/54)	33.3 (18/54)	5.6 (3/54)	90.7 (49/54)	1.9 (1/54)	7.4 (4/54)

*EPIs=efflux pump inhibitors, #CCCP=carbonyl cyanide 3-chlorophenylhydrazone, #NMP=1-(1-naphthylmethyl)-piperazine, #PAβN=phenylalanine arginine β-naphthylamide.

3.3.2.1. Species-specific effect of EPIs on initialattachment

NMP inhibited attachment of 100% of isolates in all the species except *A. hydrophila* where it was observed to inhibit 94.1% (16/17) of the isolates (Table 3.6). CCCP was observed to inhibit attachment of 100% of *A. hydrophila* and *A. caviae*isolates (Table 3.6). PAβN inhibited attachment of 100% of isolates for species with ≤ 2 isolates (Table 3.6).

Table 3.6: Species-specific effect of EPIs on initial attachment

Species designation	% Inhibition		
	CCCP*	**NMP***	**PaβN***
***A. hydrophila* (n=17)**	100 (17/17)	94 (16/17)	64.7 (11/17)
***A. culicicola* (n=8)**	87.5 (7/8)	100 (8/8)	62.5 (5/8)
***A. bestiarum* (n=8)**	87.5 (7/8)	100 (8/8)	75 (6/8)
***Aeromonas* spp. (n=3)**	66.7 (2/3)	100 (3/3)	33.3 (1/3)
***A. caviae* (n=3)**	100 (3/3)	100 (3/3)	33.3 (1/3)
***A. veronii* (n=3)**	66.7 (2/3)	100 (3/3)	33.3 (1/3)
***A. allosaccharophila* (n=2)**	100 (2/2)	100 (2/2)	0
***A. salmonicida* (n=2)**	100 (2/2)	100 (2/2)	100 (2/2)
***A. jandaei* (n=1)**	100 (1/1)	100 (1/1)	100 (1/1)
***A. sobria* (n=1)**	100 (1/1)	100 (1/1)	100 (1/1)
***P. shigelloides* (n=6)**	83.3(5/6)	100 (6/6)	83.3 (5/6)

*CCCP=carbonyl cyanide 3-chlorophenylhydrazone, *NMP=1-(1-naphthylmethyl)-piperazine, *PAβN=phenylalanine arginine β-naphthylamide.

3.3.3. Effect of EPIs on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates

CCCP increased detachment of 85.2% (46/54) of the isolates including the type strains, and increased attachment of isolates M22, M26, M63 and M99(Fig. 3.4). This EPI demonstrated no effect on isolates M22, M65, M90 and M95 (Fig. 3.4). NMP increased detachment of 100% of the isolates, and unlike in the initial attachment assays, NMP also increased detachment of *A.hydrophila* ATCC 7966^T and *A. caviae*ATCC 15468^T (Fig. 3.5). While PAβN increased detachment of 90.7% (46/54) of isolates and increased attachment of isolate M17, it was had no effect on isolates M13, M63, M94 and M95 (Fig. 3.5). With *A.hydrophila* ATCC 7966^T and *A. caviae*ATCC 15468^T, PAβN increased detachment of both strains (Fig. 3.6). An increase in biofilm detachment was observed in the following order: NMP > PAβN > CCCP (Figs. 3.4-3.6, Table 3.5). The treatments of all EPIs were statistically significant ($p < 0.001$).

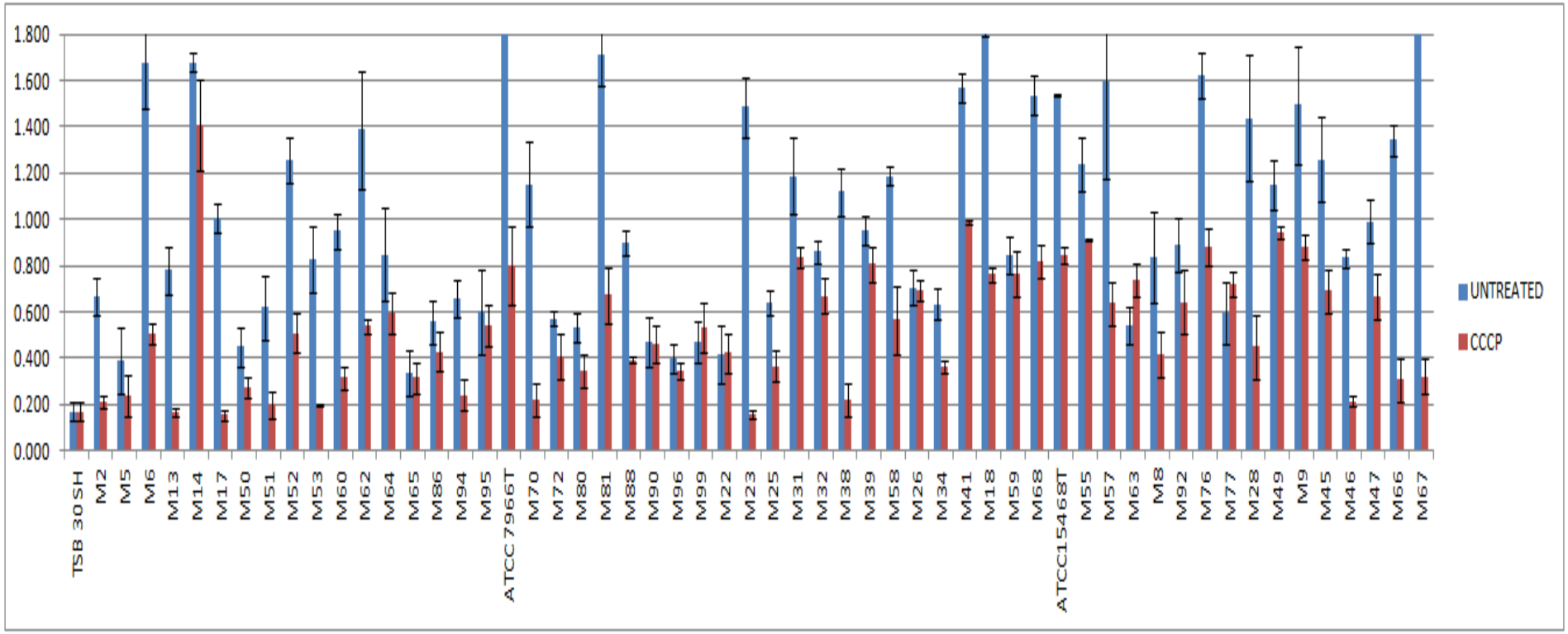

Figure 3.4: Effect of 20 µg/ml carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

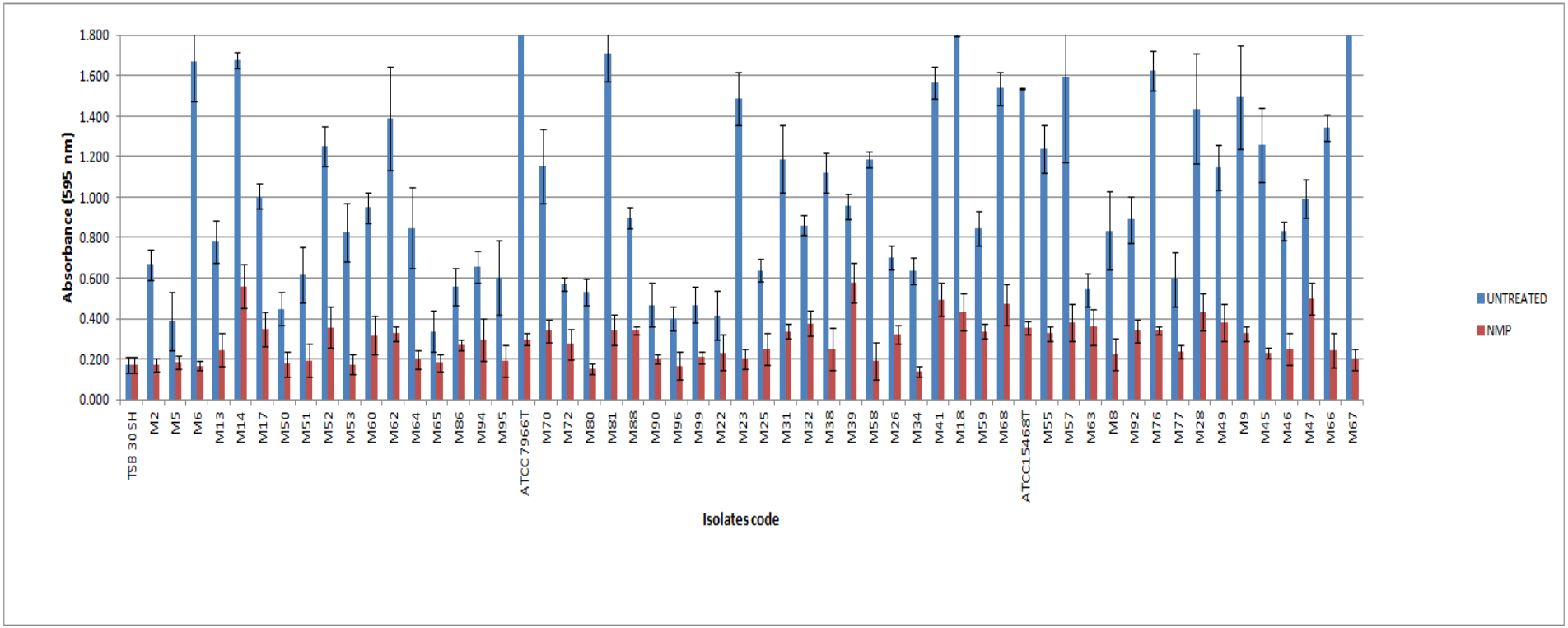

Figure 3.5: Effect of 20 µg/ml 1-(1-naphthylmethyl)-piperazine (NMP) on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

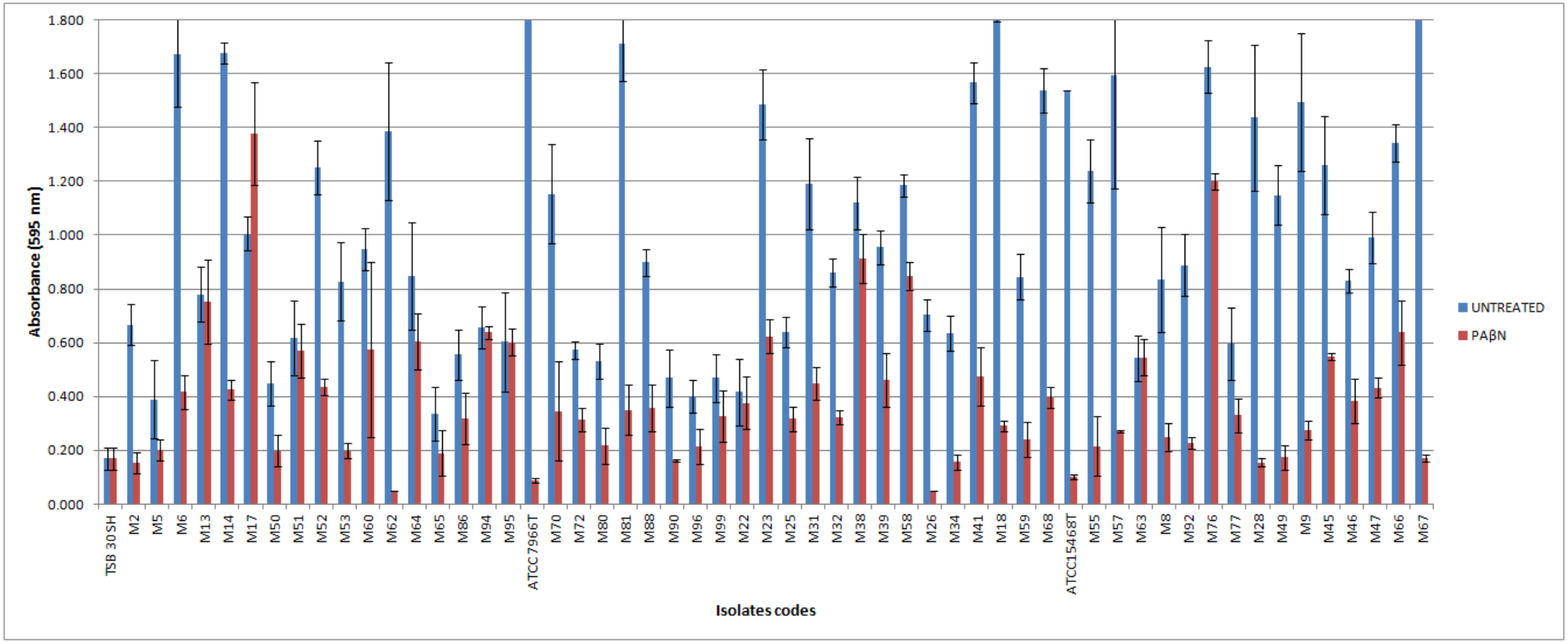

Figure 3.6: Effect of 20 µg/ml phenylalanine arginine β-naphthylamide (PAβN) on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays. *A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

NMP was also the most effective EPI in the pre-formed biofilm assays and it detached 100% of isolates (Table 3.5). PAβN was the second best EPI, and it detached 90.7% of isolates, whilst CCCP was the least effective (Table 3.5).

3.3.3.1. Species-specific effect of EPIs on pre-formed biofilm

NMP detached100% of the isolates in all 11 species that were investigated (Table 3.7). PAβN detached 100% of *A. culicicola, A. bestiarum*, unspecified *Aeromonas* spp. and *A. caviae* isolates (Table 3.7). All three EPIs increased detachment in 100% isolates of *P. shigelloides* (Table 3.7).

Table 3.7: Species-specific effect of EPIson pre-formed biofilm

Species designation	% Inhibition		
	CCCP*	**NMP***	**PAβN***
***A. hydrophila* (n=17)**	88.2 (15/17)	100 (17/17)	70.6 (12/17)
***A. culicicola* (n=8)**	87.5 (7/8)	100 (8/8)	100 (8/8)
***A. bestiarum* (n=8)**	62.5 (5/8)	100 (8/8)	100 (8/8)
***Aeromonas* spp. (n=3)**	33.3 (1/3)	100 (3/3)	100 (3/3)
***A. caviae* (n=3)**	66.7 (2/3)	100 (3/3)	100 (3/3)
***A. veronii* (n=3)**	66.7 (2/3)	100 (3/3)	66.7 (2/3)
***A. allosaccharophila* (n=2)**	100 (2/2)	100 (2/2)	100 (2/2)
***A. salmonicida* (n=2)**	50 (1/2)	100 (2/2)	100 (2/2)
***A. jandaei* (n=1)**	100 (1/1)	100 (1/1)	100 (1/1)
***A. sobria* (n=1)**	100 (1/1)	100 (1/1)	100 (1/1)
***P. shigelloides* (n=6)**	100 (6/6)	100 (6/6)	100 (6/6)

*CCCP=carbonyl cyanide 3-chlorophenylhydrazone, *NMP=1-(1-naphthylmethyl)-piperazine, *PAβN=phenylalanine arginine β-naphthylamide.

3.3.4. Determination of percent reduction

When determining the percent reduction, following EPI exposure, the negative value represents inductionof biofilm formation and the positive values represent reductionof biofilm formation. The percent reduction as shown in Table 3.8indicates that CCCP reduced biofilms of 92% (50/54) of isolates at the time of inoculation.For CCCP, percent reduction ranged from 6.7 to 119.7% and percent induction ranged from 1.3 to 907.5%. PAβN and NMP reduced biofilm formation of 66.7% (53/54) and 98.1% (53/54) of isolates, respectively,following addition at the

time of inoculation (Table 3.8).For PAβN percentreduction ranged from 3.5 to 536.2% and percentinduction ranged from 7.0 to 457.4%. While the percent reduction for NMP ranged from 30.3 to 109.4%, percent induction ranged from 58.3 to 214.5% (Table 3.8).

In the pre-formed assays it was observed that CCCP reduced biofilm formation of 85.2% (46/54) of isolates (Table 3.8). The percent reduction for CCCP ranged from 12 to 101.8% and percent induction ranged from 4.9 to 53.4%.Biofilms of 98.1% (53/54) and 100% (54/54) of isolates were reduced by PAβN and NMP, respectively (Table 3.8). For PAβN, percent reduction ranged from 0.2 to 122.8% and percent induction ranged from 1.1 to 44%. For NMP, the percent reduction ranged from 47.9% to 109.7% and no induction was observed.

Table 3.8: Percent reduction of EPIs on initial attachment and pre-formed biofilm of *Aeromonas* spp.and *P. shigelloides* isolates

Species designation	Isolates	% Reduction					
		Initial attachment			Mature biofilm		
		CCCP#	PaβN#	NMP#	CCCP#	PAβN#	NMP#
A. hydrophila	M2	99.7	96.1	99.2	91.2	103.1	99.7
	M5	102.9	83.7	97.1	68.5	84.9	94.1
	M6	101.7	3.5	102.3	83.6	94.7	98.4
	M13	96.3	24.5	95.8	100.3	4.3	87.5
	M14	53.4	28.6	72.8	17.8	83	74.1
	M17	98.2	17.8	73.3	101.8	-44.4	78.4
	M50	101.7	-67	96.1	63.1	89.5	98.2
	M51	93.6	29.2	85.9	93.4	10.6	57
	M52	94.9	20.8	88	52.2	65.3	94.3
	M53	75.5	98.5	97.5	95.7	95.5	99.4
	M60	91.2	-57.5	65.8	83.5	48.1	80.9
	M62	104.4	56.9	104.6	67.4	88.3	88.7
	M64	100.2	-15.1	94.4	37	35.6	96
	M65	101.1	55	99.6	-4.9	71.7	109.7
	M86	100.1	-23.4	95.3	61.5	99.5	92.2
	M94	118	-457.4	-58.3	85.4	4.1	74.3
	M95	94.6	9.3	88.8	14	0.2	95.1
	ATCC 7966T	119.7	19.5	109.4	69.6	103.9	93.8
A. bestiarum	M70	54.5	76.5	82.8	95.1	82.1	82.8
	M72	86.3	80.4	100.3	40.5	64.1	74.5
	M80	93.8	22.4	99.1	45.8	103.3	108.5

	M81	92.8	58.2	77.8	68.8	75.3	82.7
	M88	-2.8	57.3	33.3	69.4	74.5	76.4
	M90	90.2	-83.8	75.4	2.6	102.1	89.6
	M96	97.2	5.7	98.3	-12.9	35.5	56
	M99	91.8	66.5	86.1	-21.5	47.6	86.8
A. culicicola	**M22**	92.7	-42.3	100.1	40.9	15.9	75
	M23	-9.9	8.7	88.8	100.9	65.5	97.7
	M25	84.3	93.8	95.7	57.9	68.4	82.8
	M31	31.2	92.5	91.4	34.4	72.6	83.7
	M32	77.1	29.9	94.2	27.9	77.8	70.2
	M38	18.2	20	94.5	94.6	21.7	91.4
	M39	73.8	5.3	74.9	18.8	62.8	47.9
	M58	103.5	-87	48.8	61	33.2	97.8
Aeromonas spp.	**M26**	95.4	-7	65	69.7	109.8	87.2
	M34	101.4	40.8	98.4	62.7	90.5	100.9
	M41	-103.2	-31.4	37.8	62.7	78.3	76.7
A. caviae	**M18**	87.2	25.3	78.7	64.6	92.8	84.3
	M59	69.6	-45.1	89.9	12.4	89.5	75.4
	M68	89.7	-14.5	27.1	52.5	83.4	78.1
	ATCC 15468^{T}	-907.5	536.2	-214.5	50.6	104.9	62.1
A. veronii	**M55**	76.1	101.5	82.7	30.9	95.7	85.4
	M57	6.7	-32.2	72.3	67.3	93	85.3
	M63	86.5	-69.8	56.3	-53.4	-1.1	49.2
A. allosaccharophila	**M8**	105.5	-65.7	100.1	61.6	82.9	92
	M92	98	-32.8	84.2	34	91.8	76.5
A. salmonicida	**M76**	95.6	99.4	71.3	51.3	29.2	88.2
	M77	65.1	10.8	81.1	-29.7	62.2	84.3
A. jandaei	**M28**	51.6	104.4	91.8	78	101.1	79.1
A. sobria	**M49**	57.4	92.7	95.3	21	99.5	78.5
P. shigelloides	**M9**	37.1	28	76.1	46.2	92.1	88.2
	M45	85.3	-8.4	30.3	-53.2	122.8	71.9
	M46	82.4	45.3	87.2	93.2	67.6	88
	M47	80.4	10.8	90.8	39.1	68.2	60.1
	M66	100.5	68.3	101	82.2	87	100.2
	M67	-1.3	-19.6	71	88.4	60.1	93.8

*Percent reduction=Percentage reduction = $[\ ((C - B) - (T - B))/(C - B)\ ] \times 100$, where B=average absorbance per well for blank wells, C=average absorbance per well for control wells, T=average absorbance per well for treated wells(Pitts *et al*., 2003),

[#]CCCP = carbonyl cyanide 3-chlorophenylhydrazone, [#]NMP = 1-(1-naphthylmethyl)-piperazine, [#]PAβN = phenylalanine arginine β-naphthylamide.

3.3.5. Effect of DNase I on initial attachment and biofilm detachment

DNase I significantly inhibited attachment of 59.2% (32/54) of isolates (p= 0.004). It was observed that in a pre-formed biofilm assays, DNase I significantly increased detachment of 64.8% (35/54) of isolates ($p < 0.001$). In the initial attachment assays, DNase I increased attachment of isolates M8, M17, M25, M26, M39, M41, M45, M50, M58, M59, M60, M63, M64, M66, M68, M90, M94, M99, as well as *A. hydrophila* ATCC 7966^T and *A. caviae* ATCC 15468^T type strains. Forisolates M9, M18 and M62,DNase I had no effect on their initial attachment (Fig. 3.7).

DNase I was more effective in detaching biofilms than in inhibiting attachment of isolates. DNase I was observed to increase attachment of isolates M5, M13, M17, M22, M26, M34, M88, M90, M92, M94 and M96 in the pre-formed biofilm assays (Fig. 3.8). It had no effect on isolates M25, M32, M46, M51, M50, M65, M95 and M99. DNase I effectively inhibited biofilm formation of isolates M17, M94, M90 and M26 in both initial attachment and pre-formed biofilm assays. With *A. hydrophila* ATCC 7966^T and *A. caviae* ATCC 15468^T, DNase I increased detachment of both type strains.

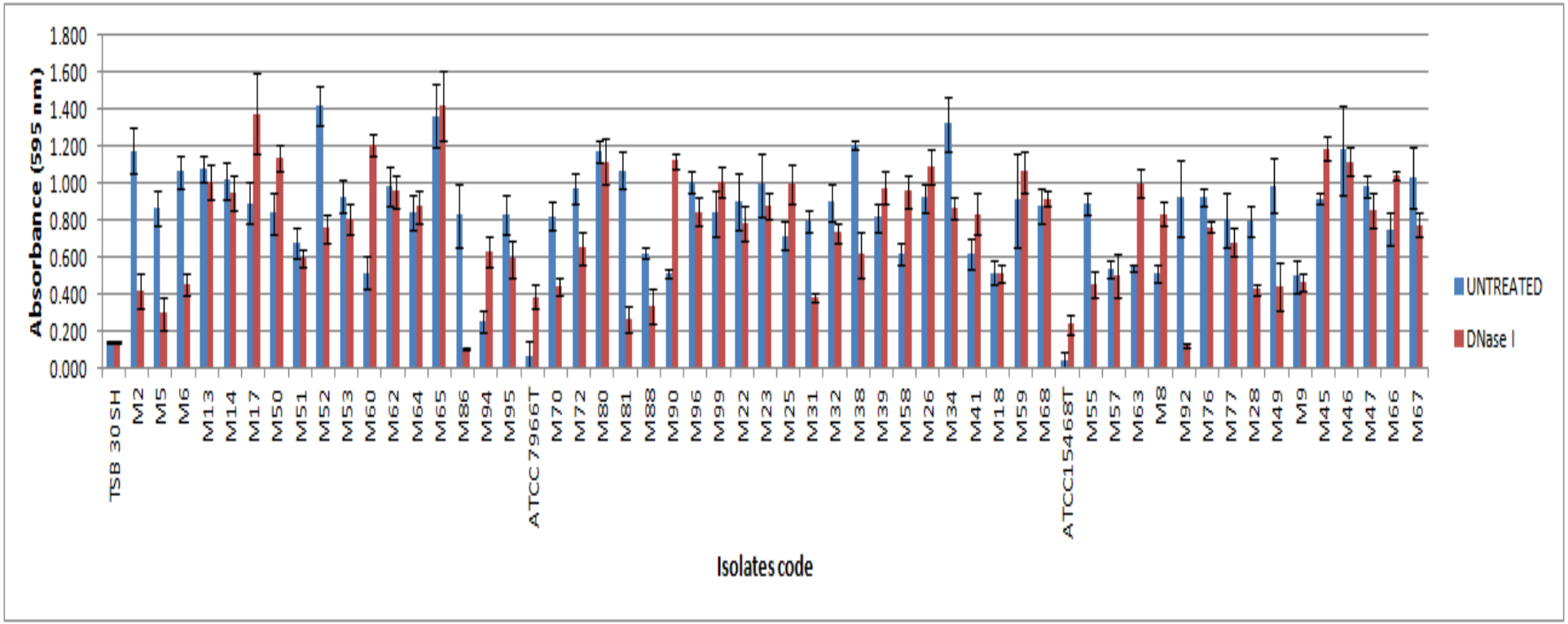

Figure 3.7: Effect of 1 mg/ml DNase I on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

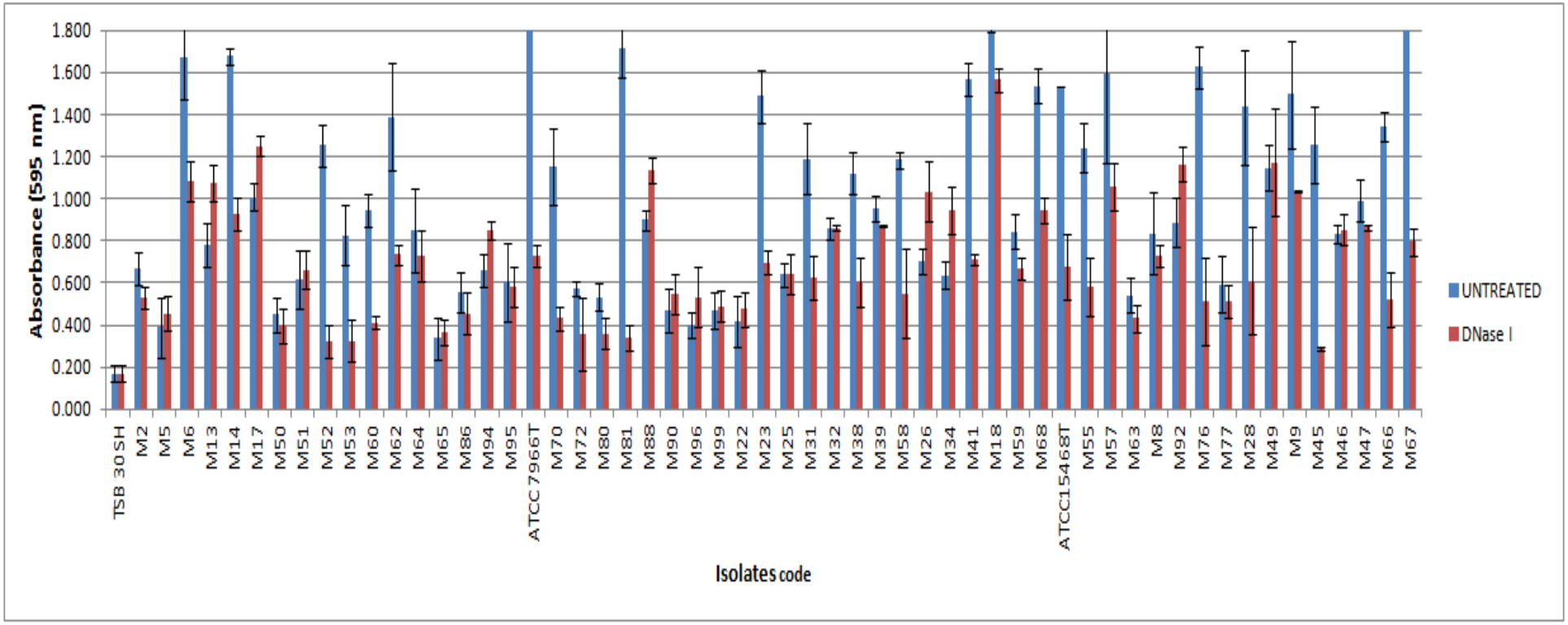

Figure 3.8: Effect of 1mg/mlDNase I on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates using microtiter plate assays.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63); *A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77); *A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

3.3.6. Species-specific effect of DNase I on biofilm formation

The species-specific effect of DNase I in both initial attachment and pre-formed assays is given in Table 3.9. DNase I increased detachment of 100% of *A. caviae* and *A. veronii*isolates in the pre-formed biofilm assays (Table 3.9). While DNase I in the initial attachment assay did not inhibit any of the *A. caviae* isolates, it inhibited 66.7% (2/3) of *A. veronii* isolates (Table 3.9). DNase I inhibited 58.8% (10/17) and 53% (9/17) of *A. hydrophila* isolates in both initial attachment and pre-formed biofilm asssays (Table 3.9). With *A. culicicola*, DNase I inhibited 62.5% (5/8) of isolates in the initial attachment assays and detached 75% (6/8) of isolates.

Table 3.9: Species-specific effect of DNase I on initial attachment and pre-formed biofilm

Species designation	**% Inhibition**	
	Initial attachment	**Preformed biofilm**
***A. hydrophila* (n=17)**	58.8 (10/17)	53 (9/17)
***A. culicicola* (n=8)**	62.5 (5/8)	75 (6/8)
***A. bestiarum* (n=8)**	75 (6/8)	50 (4/8)
***Aeromonas* spp. (n=3)**	33.3 (1/3)	33.3 (1/3)
***A. caviae* (n=3)**	0	100 (3/3)
***A. veronii* (n=3)**	66.7 (2/3)	100 (3/3)
***A. allosaccharophila* (n=2)**	50 (1/2)	50 (1/2)
***A. salmonicida* (n=2)**	100 (2/2)	100 (2/2)
***A. jandaei* (n=1)**	100 (1/1)	100 (1/1)
***A. sobria* (n=1)**	100 (1/1)	100 (1/1)
***P. shigelloides* (n=6)**	50 (3/6)	83.3 (5/6)

3.3.7. Determination of percent reduction following DNase I treatment

After determining the percent reduction, it was observed that DNase I reduced biofilms of 64.8% (35/54) of isolates at the time of inoculation (Table 3.10). In the initial attachment assays, percent reduction ranged from 1.3 to 1644.5% and percent induction ranged from 4.5 to 334.8%. In the pre-formed biofilm assays, DNase I reduced biofilms of 66.7% (63/54) of isolates (Table 3.10). It was observed that percent reduction ranged from 4.3 to 88.9% and percent induction ranged from 0.2 to 114.4%.

Table 3.10: Percent reduction of DNase I on initial attachment and pre-formed biofilm of *Aeromonas* spp. and *P. shigelloides* isolates

Species designation	Isolates	% Reduction	
		Initial attachment	Pre-formed biofilm
A. hydrophila	**M2**	73.4	27.5
	M5	78.9	-30.9
	M6	66.3	39.3
	M13	7.4	-48.3
	M14	7.3	49.6
	M17	-64.2	-29.4
	M50	-42.7	18.7
	M51	14.8	-10
	M52	-34.6	88.9
	M53	15.5	76.5
	M60	-184.7	68.8
	M62	86.6	88.9
	M64	-4.7	17.6
	M65	-4.5	-13.4
	M86	105.7	31.5
	M94	-334.8	-39.8
	M95	34.5	4.3
	ATCC 7966^T	13.8	73.1
A. bestiarum	**M70**	56.1	73.3
	M72	38.9	53.4
	M80	5.2	-114.4
	M81	52	85.8
	M90	-167.4	-25.8
	M96	18.7	-23.3
	M99	-24.6	-6.4

A. culicicola	**M22**	15.4	-23.4
	M23	13	60
	M25	-48.1	-0.8
	M31	63.7	55.3
	M32	21.6	-0.2
	M38	55.8	54.2
	M39	-23.6	10.8
	M58	-68.9	62.7
Aeromonas spp.	**M26**	2.7	53.6
	M34	38.9	22.1
	M41	-45.2	61.1
A. caviae	**M18**	1.3	16.9
	M59	-19.9	26.3
	M68	-5.6	43.5
	ATCC 15468^{T}	1644.5	62.6
A. veronii	**M55**	58.5	61.3
	M57	9.5	37.8
	M63	-113.4	29.1
A. allosaccharophila	**M8**	-84.6	16
	M92	102.4	-38.5
A. salmonicida	**M76**	20.8	76.3
	M77	18.3	19.6
A. jandaei	**M28**	56.7	65.2
A. sobria	**M49**	64.9	-2.6
P. shigelloides	**M9**	9.2	34.8
	M45	-21.2	-62.2
	M46	6.3	-3.1
	M47	15.6	15.7
	M66	29	66.9
	M67	-47.8	70.3
	M88	59.9	-32.4

*Percent reduction=Percentage reduction = $[\ ((C - B) - (T - B))/(C - B)\ ] \times 100$, where B=average absorbance per well for blank wells, C=average absorbance per well for control wells, T=average absorbance per well for treated wells(Pitts *et al.*, 2003).

3.4. Discussion

Aeromonas spp. isolates have been suggested to be rapidly developing resistance mechanisms against different antimicrobial agentsdue to their widespread use (Igbinosa *et al.*, 2012). The presence of EPIs restores the activity of antimicrobial agents by blocking the efflux pumps from pumping them out of the cell (Kvist *et al*., 2008). This study used EPIs in combination with antimicrobial agents to identify efflux pump-associated antimicrobial resistance in *Aeromonas* spp and closely related *P. shigelloides* species. It was observed that *Aeromonas* spp. isolates in the present study were more susceptible to quinolones (ofloxacin, norfloxacin and ciprofloxacin) and aminoglycosides (amikacin and gentamicin). In contrast to the results obtained in the current study, *Aeromonas* spp. (*A. media* and *A. punctata* subsp. *punctata*) were observed to be highly resistant to quinolones which was due to mutations in type II topoisomerase genes (Cattoir *et al*., 2008). However, Blasco *et al*. (2008) observed that *Aeromonas* spp. isolates isolated from water reservoirs and cooling systems were moderately susceptible to quinolones. *Aeromonas* spp. isolates together with *V. cholerae* and *P. shigelloides* isolated from Cambe Stream were observed to be susceptible to norfloxacin (Gibotti *et al*., 2000). The isolates were more resistant to metabolic inhibitors (trimethoprim and sulphamethoxazole), penicillins (ampicillin) and tetracyclines (tetracycline). Ribeiro *et al*. (2010) suggested that eventhough *Aeromonas* spp. are resistant to penicillins, they are also resistant to aminoglycosides. Thus, *Aeromonas* spp. isolates showed resistance to amikacin and gentamicin (aminoglycosides) together with ampicillin and trimethopim–sulphamethoxazole (Gibotti *et al*., 2000). In agreement with this study, *Aeromonas* spp. isolates isolated from shrimp hatcheries and ponds were shown to be highly resistant to ampicillin (Vaseeharan *et al*., 2005). *Aeromonas* and *Plesiomonas* spp. from tilapia in Trinidad were highly resistant to ampicillin, followed by trimethoprim-sulphamethoxazole (Newaj-Fyzul *et al*., 2008). Pérez-Valdespino *et al*. (2009) reported that *Aeromonas* spp. isolates from human stool samples from case of diarrhoea in Mexico were highly resistant to tetracycline and trimethoprim-sulphamethoxazole. *A. salmonicida* was also suggested to be more resistant to tetracycline and trimethoprim-sulphamethoxazole (Bello-Lopez *et al*., 2009).

While CCCP inhibited efflux of 11 antimicrobial agents, NMP and PAβN inhibited efflux of 6 and 10 antimicrobial agents, respectively (Tables 3.2 - 3.4). The efflux of cefpodoximein 14.8% of isolates was completely inhibited with CCCP. This was judged based on the fact that if

the inhibitor was effective, the isolates would be resistant in the absence and susceptible in the presence of the inhibitor. CCCP blocks the energy required by efflux pumps in order to function (Kvist *et al*., 2008). Thus, inhibiting the efflux pumps indirectly by depriving them of energy provides a promising control strategy. NMP followed CCCP and it completely inhibited efflux of cefpodoxime in 7.4% of isolates, while the least effective, PAβN completely inhibited efflux of cefpodoximein 5.6% of isolates. NMP and PAβN are the substrates of RND pumps and act as competitive inhibitor of antimicrobial agents, as a result they have been shown to increase susceptibility to different antimicrobial agents (Bina *et al*., 2009). The action of these EPIs is limited to certain classes of antibiotics (Bina *et al*., 2009). The effectiveness of EPIs has also been suggested to be dependent on their mechanisms (Pannek *et al*., 2006). Bina *et al*. (2009) compared the RND-deficient strain of *V. cholerae* and test isolates to see if the NMP and PAβN were effective. They observed that NMP and PAβN reduced the MICs of deoxycholate, cholate and erythromycin. PAβN when combined with either levofloxacin (Marquez, 2007; Pagès and Amaral, 2009) or fluoroqinolone was shown to increase the susceptibility of *P.aeruginosa* (Pages and Amaral, 2009). PAβN increased the susceptibility of *Acinetobacter baumannii* to clarithromycin, rifampicin or linezolid (Pannek *et al*., 2006) which correlates with finding of the current study, although different antimicrobial agents were investigated.

NMP was most effective in preventing initial attachment and reducing biofilm formation by *Aeromonas* species isolates. NMP decreased initial adherence of 98.1% of isolates and increased biofilm detachment of 100% of isolates, respectively. This suggests that it is possible to eradicate biofilm formed by *Aeromonas* spp. and *P. shigelloides* isolates by blocking the RND pumps, which is a target of both NMP and PAβN. It is not clear why PAβN was only effective in treating pre-formed biofilm and not initial attachment. It is possible that the mechanism of action or the target sites of these inhibitors played a role in their respective efficacies. Since efflux proteins are up-regulated in the mature biofilm, both NMP and PAβN reduced biofilm formation by *E. coli* and *K.pneumoniae* (Kvist *et al*., 2008). These inhibitors had not yet been tested previously against *Aeromonas* spp. isolates, however, Mahamoud *et al*. (2007) observed that NMP was effective in inhibiting biofilm formation of *A.baumannii*.

Percent reduction which measures the efficacy of treatments (Pitts *et al*., 2003) was also used to further confirm the effectiveness of EPIs against *Aeromonas* spp. and *P. shigelloides*

isolates. NMP proved to be the most effective EPI in inhibiting the two major stages of biofilm development which are initial attachment and mature biofilm. NMP inhibited initial attachment of isolates and detached all isolates from biofilms.In the current study, NMP was also more effective in inhibiting individual species when compared to CCCP and PAβN. Of the 11 different species examined, NMP completely inhibited initial attachment of isolates in 10 species and caused detachment of the biofilms of all species. All three EPIs used in the current studywere effective in inhibiting attachment of cells to form biofilms and also in detaching biofilms of both *Aeromonas* spp. and *P. shigelloides*, however, NMP proved to be the best candidate. PAβN was more effective than CCCP in the pre-formed biofilm assays and in the initial attachment assays it was *vice versa*. More detailed studies on the use of EPIs against *Aeromonas* spp. and the mechanisms by which these inhibitors affect this species are required.

The effect of DNase I which digests eDNAwas also examined in the present study and it was more effective in inhibiting the mature biofilm than initial attachment. Tetz and Tetz (2010) suggested that in *S.aureus*, DNase I was more effective on a matured biofilm where eDNA is constantly produced. In the mature biofilm, eDNA joins together with other components to make the extracellular polymeric substance (Das *et al.*, 2010). Treatment of *P. aeruginosa* biofilm with DNase I was observed to reduce biofilm formation over the growth period (Andrews *et al*., 2010; Whitchurch *et al*., 2002). As observed in the present study, Lappann *et al*. (2010) observed that biofilm formed by *Neisseria meningitidis* was highly sensitive to DNase I treatment. DNase I proved to be effective in inhibiting initial attachment of *A. bestiarum* followed by *A. culicicola*, however, for pre-formed biofilms, DNase I proved to be more effective in inhibiting detachment of the same isolates. The same was obtained with *P. shigelloides* where members of this genus were detached from the biofilm rather than their initial attachment being inhibited. The effectiveness of DNase I in pre-formed biofilm assays shows that many species were more susceptible to DNase I in a mature biofilm where eDNA is highly produced and incorporated in the EPS. Using the percent reduction, it was further confirmed that DNase I was more effective in detaching biofilms than in inhibiting initial attachment.The results obtained in the current study indicate that DNase I ismore useful in treating biofilm thathave already been formed rather than treating the one that is developing.

CHAPTER 4

Inhibition of biofilm formation by aquatic *Aeromonas* spp. isolates using quorum sensing inhibitors

4.1. Introduction

During the QS process, bacteria communicate with each other *via* production of auto-inducers molecules that are only produced when a certain cell density is reached (Ponnusamy *et al.*, 2009). Acyl homoserine lactones (AHLs) in *Vibrio fischeri* are produced by the LuxI synthase and they diffuse out of the cell until the required cell density is achieved (Kirke *et al*., 2004; Chan *et al*., 2011). AHLs then diffuse inside the cell and bind to their cognate proteins (LuxR) followed by induction of gene expression after the complex binds to these genes. In *Aeromonas* spp.,the signal generator and signal receptor are AhyI and AhyR,respectively (Chan *et al*., 2011). The diversity of AHLs have been suggested to result from the N-acyl chains with carbons that range from 4-14 and C-3 position on the side chain of the AHL which can either be substituted by 3-oxo, 3 hydroxyl or a fully methylene group (Cataldi *et al*., 2007). The diversity of these molecules aid bacteria of the same species to recognize each other rather than different species that are also present within the same community (Taga and Bassler, 2003).

A.tumefaciens A136 which detects long-chain AHLs (Zhu andWinans, 1998) and*C.violaceum* CV026 which detects short- and medium-chain AHLs (McClean *et al*., 1997) are the two commonly used biosensors. The main signalling molecules that are produced by *A. hydrophila* were observed to be C-4 AHL and C-6 AHL (Chan *et al*., 2011; Medina-Martínez *et al*., 2006; Morgan-Sagastume *et al*., 2005). *A. hydrophila* together with *A. salmonicida* were reportedto produce N-butyryl homoserine lactone as their main signalling molecule (Swift *et al*., 1997). Other members of *Aeromonas* species such as *A. salmonicida* have been shown to produceOHL, d-DHL, t-DHL and N-decanoylhomoserine lactone (Cataldi *et al.*, 2007), while C-4 AHL has been identified in *A. sobria*, and C-4 AHL and 3-oxo-C-6 AHL have been identified in *A. caviae*as its major AHLs (Medina-Martínez *et al*., 2006).

The produced AHLs have been observed toinfluence biofilm formation (Lynch *et al*., 2002), C-4 AHL and C-6 AHL in *A. hydrophila* appeared to be important in biofilm formation and for its development after an AHL-mutant strain was compared with its corresponding parent strain (Lynch *et al*.,2002). The formation of micro-colonies was also associated with AHL

production (Labbate*et al*., 2004). Since AHLs influence biofilm formation (Khajanchi *et al.*, 2009) and induce production of virulence factors (Khajanchi *et al*., 2010), targeting QS with QSIs provides a promising control strategy.

The (2-)(-4-)bromomethylene-2(SH)-furanone was shown to inhibit biofilm formation of *P. aeruginosa*, *A. hydrophila* (Ponnusamy *et al.*, 2010) and *Hafnia alvei* (Viana *et al.*, 2009). Ponnusamy *et al*. (2010) observed that halogenated furanones which act as competitive inhibitors of AHLs, inhibited the growth of *A. hydrophila.* This inhibitor has also been shown to inhibit the swarming motility of*Serratia liquefaciens* by binding to the *swrA* gene, which is controlled by QS (Rasmussen *et al*., 2000). *S*-adenosylhomocysteine (SAHC) was shown to have an inhibitory effect against *P. aeruginosa*. This *S*-adenosyl methionine analog is believed to inhibit AHL synthesis, however, the mechanism of action is not fully understood (Hentzer and Givskov, 2003). Vanillin interacts with AHL receptors and interferes with the binding of AHLs to their cognate receptors.Vanillin was shown to inhibit both short and long chain AHLs in *A. hydrophila* resultinginbiofilm formation inhibition of this species (Ponnusamy *et al*., 2009). Cinnamaldehyde reduced the biofilm-forming ability of *Burkholderia* species by targeting QS with an unknown mechanism of action (Brackman *et al*., 2009). Targeting quorum sensing by use of these four quorum sensing inhibitor (QSIs) provides a promising control strategy to treat resistant biofilm-associated infections. The aim of this study was thus to detect aeromonad AHL production using biosensors and to determine the effect of four QSIs [(2-)(-4-)bromomethylene-2(SH)-furanone, SAHC, trans-cinnamaldehyde and vanillin] on aeromonad initial attachment and mature biofilms.

4.2. Materials and Methods

4.2.1. Detection of acyl homoserine lactoneproduction using biosensors

In order to detect AHL production by study isolates, 24 h TSA cultures were cross-streaked against the 24 h-grown *C. violaceum* CV026 biosensor grown on LB agar plates, or against the *A. tumefaciens*A136 biosensor grown on LB agar plates [with 50 µg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)being spread on the plate prior to inoculation]. *C. violaceum* ATCC 31532 was used as a positive control in the *C. violaceum* CV026 bioassay, while *A. tumefaciens* strain KYC6 was used as a positive control in the *A. tumefaciens*A136

bioassay. Plates were incubated at 30 ºC for 24 h. Positive assays were due to the production of the purple pigment, violacein by the *C. violaceum* CV026 reporter (McClean *et al.*, 1997)and AHL induction of β-galactosidase breaking down X-gal by *A. tumefaciens*A136 resulting in a blue color (Swift*et al*., 1997).

4.2.2. Effect of quorum sensing inhibitors on biofilm formation

Quorum sensing inhibitors [(2-)(-4-)bromomethylene-2(SH)-furanone, S-adenosylhomocysteine (SAHC), trans-cinnamaldehyde and vanillin] were used to determine their effect on initial attachment and biofilm detachment using modified microtiter plate assays (Basson *et al*., 2008). Isolates were grown overnight in TSB, washed three times with sterile distilled water and the turbidity of the cell suspensions adjusted to that equivalent to a 0.5 McFarland standard. The first assay investigated the effect of QSIs on initial attachment of aeromonad isolates. QSIs at a final concentration of 5 µg/ml(2-)(-4-)bromomethylene-2(SH)-furanone, 5 µg/ml SAHC, 100 µM trans-cinnamaldehyde and 5 µg/ml vanillin were added to 90 µl TSB and 10 µl of respective cell suspensions and incubated for 24 h at 30 ºC with agitation. For the second assay, biofilms were grown for 24 h without treatment at 30 ºC, following which pre-formed biofilms were exposed to 5 µg/ml(2-)(-4-)bromomethylene-2(SH)-furanone, 5 µg/ml SAHC, 100 µM trans-cinnamaldehyde and 5 µg/ml vanillin in TSB (90 µl) and incubated for a further 24 h at 30 ºC with agitation. The negative control contained only broth, while the positive controls contained the respective cell suspensions in TSB with no QSIs added.

Contents of each well were aspirated, washed three times with 250 µl of sterile distilled water and the remaining cells were fixed with 200 µl of methanol for 15 min. After air-drying, wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Excess crystal violet was removed by gently rinsing plates under running tap water and air dried. Dye bound to the adherent cells was resolubilized with 150 µl of 33% (*v*/*v*) glacial acetic acid, and the optical density (OD) of each well was obtained at 595 nm using the Multiskan RC (Ascent F1, Thermolabsystems). Tests were done in triplicate, on two separate occasions and the results averaged (Basson *et al*., 2008). The $OD_{595\ nm}$of the control wells without QSIs were compared to wells with QSIs to determine the effect of these QSIs on biofilm formation. The percentage

reduction was calculated from the blank, control, and treated absorbance values as described previously in section 2.2.4.

4.2.3. Statistical analysis

Differences in adhesion between untreated and treated samples were determined by Paired *t*-tests or Wilcoxon signed rank tests if the homogeneity of variances test failed (SigmaStat V3.5, Systat Software, Inc; San Jose, CA, USA). Differences were considered significant if $p < 0.05$.

4.3. Results

4.3.1. Detection of acyl homoserine lactones using biosensors

Of the 48 *Aeromonas* and six *Plesiomonas* spp. isolates that were examined, only a single *A. hydrophila*isolate (M13) induced the production of the pigment violacein by the *C. violaceum* CV026 biosensor (Fig. 4.1) while all isolates induced the utilization of X-gal to produce a blue color when using the *A. tumefaciens* A136 biosensor.

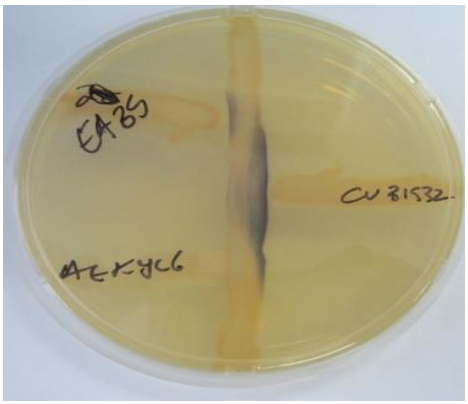

Figure 4.1: Induction of *C.violaceum*CV026 by an*A. hydrophila* isolate (M13) isolate to produce the purple violacein pigment.

4.3.2. Effect of quorum sensing inhibitorsin the initial attachment

Cinnamaldehyde inhibited initial attachment of 64.8% (35/54) of isolates including *A. caviae* ATCC 15468^T, it increased attachment of isolates M31, M32, M38, M41, M49, M65, M92, M77, M95 and had no effect against isolates M2, M5, M23, M39, M46, M50, M96, M80, and *A. hydrophila* ATCC 7966^T (Fig. 4.2). Cinnamaldehyde treatments were statistically significant ($p \leq$

0.013). Furanone inhibited initial attachment of 63% (34/54) of isolates and *A. caviae* ATCC 15468^T, increased attachment of isolates M5, M8, M14, M17, M31, M47, M38, M76, M77, M80, M94, M95, M96and *A.hydrophila* ATCC 7966^T and had no effect against isolates M13, M41, M46, M49, M57, M66 and M88 (Fig. 4.3). Furanone treatments were not statistically significant (p = 0.104). SAHC inhibited initial attachmentof 72.2% (39/54) of isolates and increased attachment of isolates M8, M13, M25, M32, M47, M77, M66, M94, M95 and *A.hydrophila* ATCC 7966^T (Fig. 4.4). SAHC had no effect on isolates M5, M38,M47, M52, M64, M65, M76 and *A. caviae* ATCC 15468^T (Fig. 4.4). SAHC treatments were not statistically significant (p = 0.254). Vanillin was observed to inhibit initial attachment of 59.3% (22/54) of isolates and *A. caviae* ATCC 15468^T (Fig. 4.5). This QSI increased attachment of isolates M8,M50, M53, M62, M72, M76, M77, M88, M94 and *A. hydrophila* ATCC 7966^T. Vanillin was also observed to have no effect on isolatesM5, M13, M17, M32, M38, M41, M46, M47 M65, M80, M90 and M95 (Fig. 4.5). Vanillin treatments were not statistically significant (p = 0.195). Initial attachment inhibition was observed to decrease in the following order: SAHC > cinnamaldehyde > furanone > vanillin (Figs 4.2-4.5, Table 4.1).

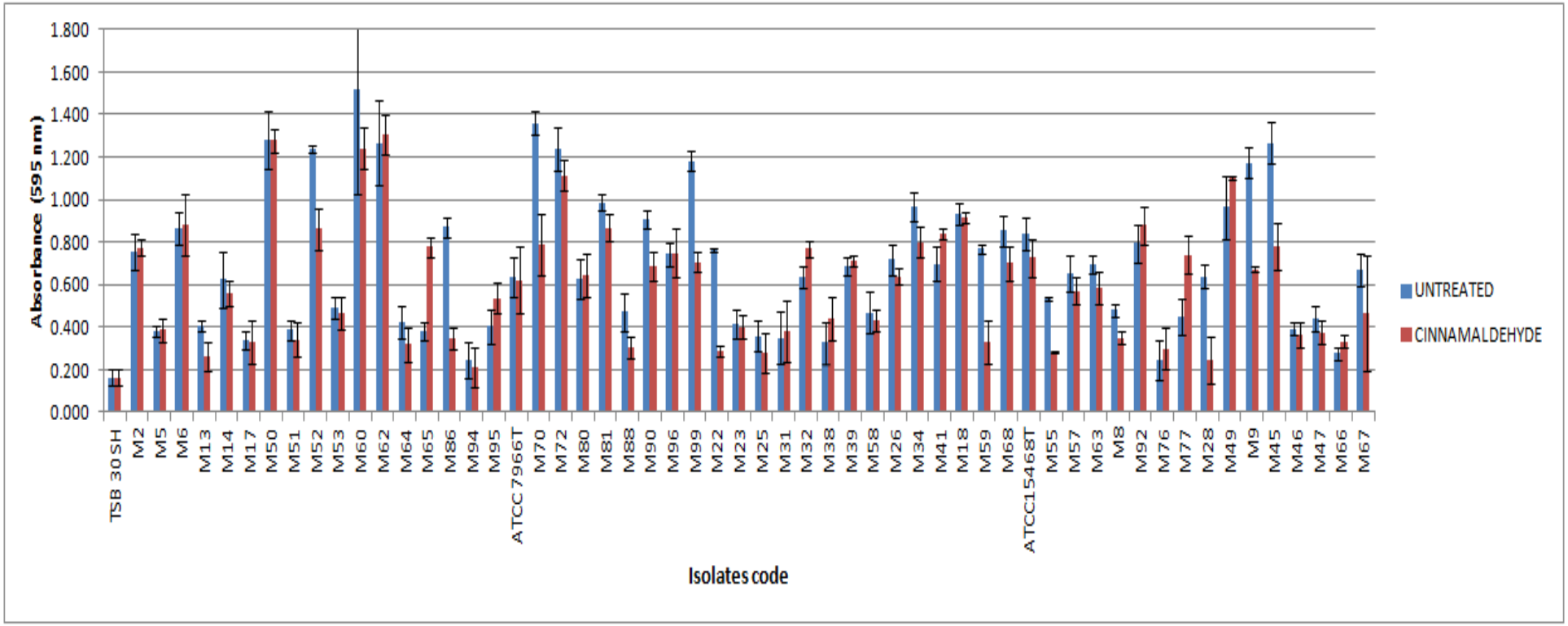

Figure 4.2: Effect of 100 µM cinnamaldehyde on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates following addition at the time of inoculation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

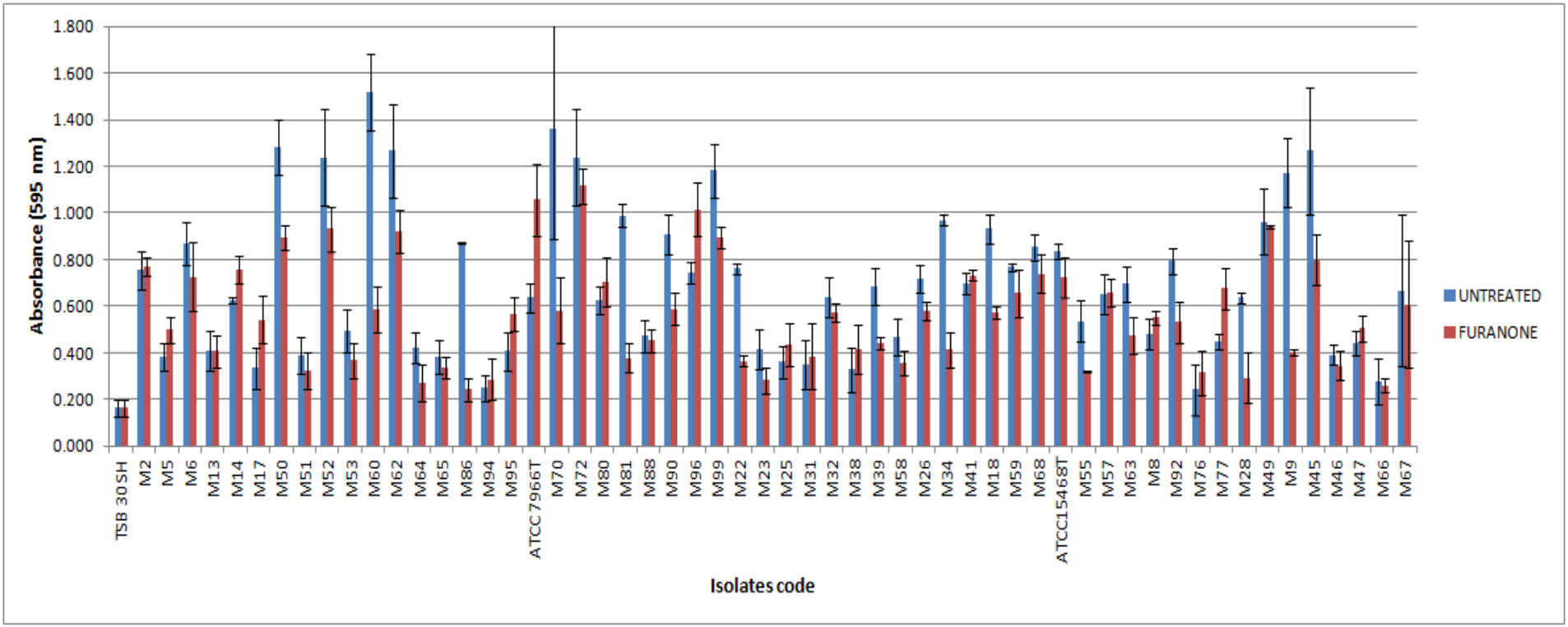

Figure 4.3: Effect of 5 µg/ml (2-)(-4-)bromomethylene-2(SH)-furanone on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates following addition at the time of inoculation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

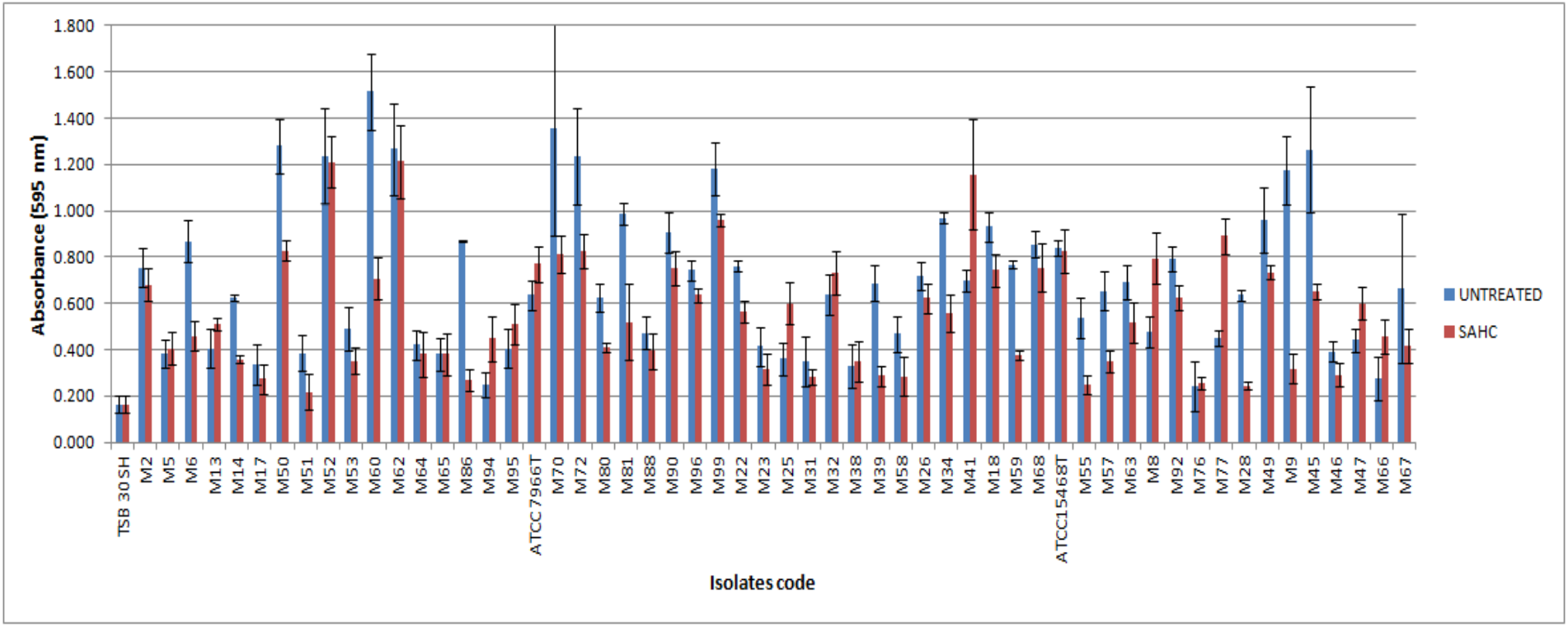

Figure 4.4: Effect of 5 µg/ml*S*-adenosylhomocysteine (SAHC) on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates following addition at the time of inoculation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

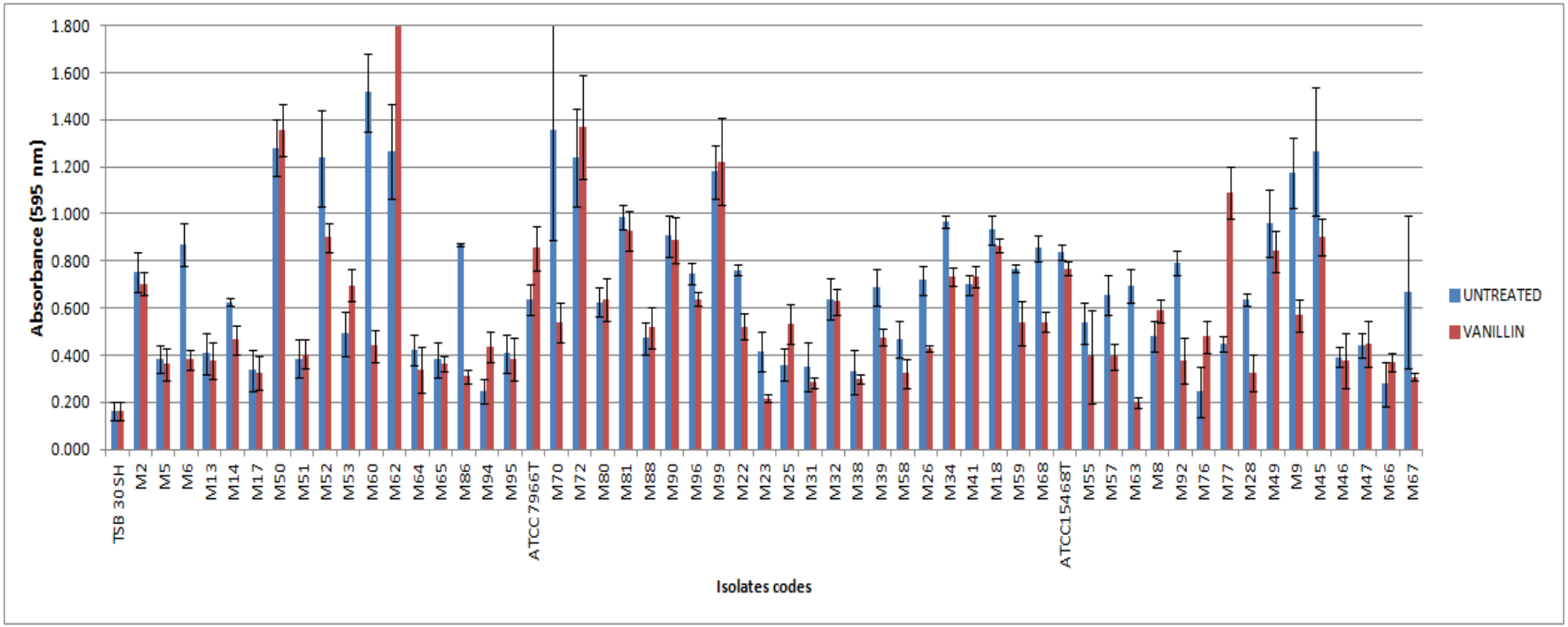

Figure 4.5: Effect of 5 µg/ml vanillin on initial attachment of *Aeromonas* and *Plesiomonas* spp. isolates following addition at the time of inoculation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

SAHC followed by cinnamaldehyde was the most effective QSI in inhibiting initial attachment. While furanone was the third most effective in QSI in the initial attachment, vanillin was the least effective QSI (Table 4.1).

Table 4.1: Effect of QSIson initial attachment and pre-formed biofilm of *Aeromonas* spp. and *Plesiomonas* spp. isolates

QSIs*	Initial attachment			Pre-formed biofilm		
	% Decrease	% Increase	% No effect	% Decrease	% Increase	% No effect
Cinnamaldehyde (100 µM)	64.8 (35/54)	22.2 (12/54)	13 (7/54)	64.8 (35/54)	20.4 (11/54)	14.8 (8/54)
Furanone (5 µg/ml)	63 (34/54)	24.1 (13/54)	13 (7/54)	64.8 (35/54)	20.4 (11/54)	14.8 (8/54)
SAHC# (5 µg/ml)	72.2 (39/54)	18.5 (10/54)	9.3 (5/54)	74.1 (40/54)	18.5 (10/54)	7.4 (4/54)
Vanillin (5 µg/ml)	59.3 (22/54)	18.5 (10/54)	22.2 (12/54)	61.1 (33/54)	22.2 (12/54)	16.7 (9/54)

*QSIs = quorum sensing inhibitors, #SAHC=S-adenosylhomocysteine

4.3.2.1. Species-specific effect of QSIson initialattachment

The species-specific effects of all four QSIs on initial attachment are given in Table 4.2. In the initial attachment assays, furanone proved to be more effective against *A. hydrophila*isolates by inhibiting initial attachment of 64.7% (11/17) of these isolates (Table 4.2). Cinnamaldehyde inhibited initial attachment of 75% (6/8) of *A. bestiarum*isolates (Table 4.2). Furanone and SAHC inhibited 100% of unspecified *Aeromonas* spp. isolates. Cinnamaldehyde, vanillin and SAHC inhibited 66.7% of *A. caviae* isolates (Table 4.2). SAHC was the best candidate against *P. shigelloides*since it inhibited initial attachment of 66.7% (4/6) of isolates.

Table 4.2: Species-specific effect of QSIson initial attachment

Species Designation	% Inhibition			
	Cinnamaldehyde 100 µM	**Furanone 5 µg/ml**	**SAHC* 5 µg/ml**	**Vanillin 5 µg/ml**
***A. hydrophila* (n=17)**	58.8 (10/17)	64.7 (11/17)	47.1 (8/17)	41.2 (7/17)
***A. culicicola* (n=8)**	25 (2/8)	37.5 (3/8)	37.5 (3/8)	50 (4/8)
***A. bestiarum* (n=8)**	75 (6/8)	37.5 (3/8)	25 (2/8)	50 (4/8)
***Aeromonas* spp. (n=3)**	66.7 (2/3)	100 (3/3)	100 (3/3)	66.7 (2/3)
***A. caviae* (n=3)**	66.7 (2/3)	33.3 (1/3)	66.7 (2/3)	66.7 (2/3)
***A. veronii* (n=3)**	66.7 (2/3)	33.3 (1/3)	66.7 (2/3)	66.7 (2/3)
***A. allosaccharophila* (n=2)**	50 (1/2)	50 (1/2)	50 (1/2)	50 (1/2)
***A. salmonicida* (n=2)**	100 (2/2)	50 (1/2)	50 (1/2)	0
***A. jandaei* (n=1)**	0	0	0	0
***A. sobria* (n=1)**	100 (1/1)	0	0	0
***Plesiomonas shigelloides* (n=6)**	50 (3/6)	33.3 (2/6)	66.7 (4/6)	33.3 (2/6)

*SAHC = S-adenosylhomocysteine

4.3.3. Effect of QSIs on pre-formed biofilm

In the pre-formed biofilm assays, cinnamaldehyde induced detachment of 64.8% (35/54) of isolates and *A. caviae* ATCC 15468^T, as well as increasing attachment of isolates M32, M38, M39, M41, M49, M65, M67, M76, M77, M92, M95 and it had no effect against isolates M2, M5, M17, M31, M50, M62, M80, M96 and *A. hydrophila* ATCC 7966^T (Fig. 4.6). Cinnamaldehyde treatments were statistically significant (p = 0.001). Furanone induced detachment of 64.8% (35/54) of isolates and *A. caviae* ATCC 15468^T (Fig. 4.7). It increased attachment of isolates M5, M8, M14 ,M17, M38, M47, M80, M76, M77, M95, M96, and *A. hydrophila* ATCC 7966^T and had no effect against isolates M2, M13, M31, M41, M57, M67, M88, M94(Fig. 4.7). Furanone treatments were statistically significant (p < 0.001). SAHC induced detachmentof 74.1% (40/54) of isolates and increased attachment of isolates M8, M13, M25, M32, M41, M47, M67, M77, M94, M95and *A. hydrophila* ATCC 7966^T (Fig. 4.8). SAHC had no effect against isolates M5, M65, M18, M76 and *A. caviae* ATCC15468^T(Fig. 4.8). SAHC treatments were statistically significant (p < 0.001). Vanillin induced detachmentof 61.1% (33/54)of isolates and increased attachment of isolates M8, M25, M41, M50, M53, M62, M72,

M76, M77, M88, M94, M99 and *A. hydrophila* ATCC 7966^{T} (Fig. 4.9). Vanillin also induced detachment of *A. caviae* ATCC 15468^{T} and had no effect against isolates M5, M17, M38, M46, M47 M51, M65, M80, M95 (Fig. 4.9). Vanillintreatments were statistically significant(*p* value = 0.006). An increase in biofilm detachment was observed in the following order: SAHC > cinnamaldehyde = furanone > vanillin (Figs 4.6-4.9, Table 4.1).

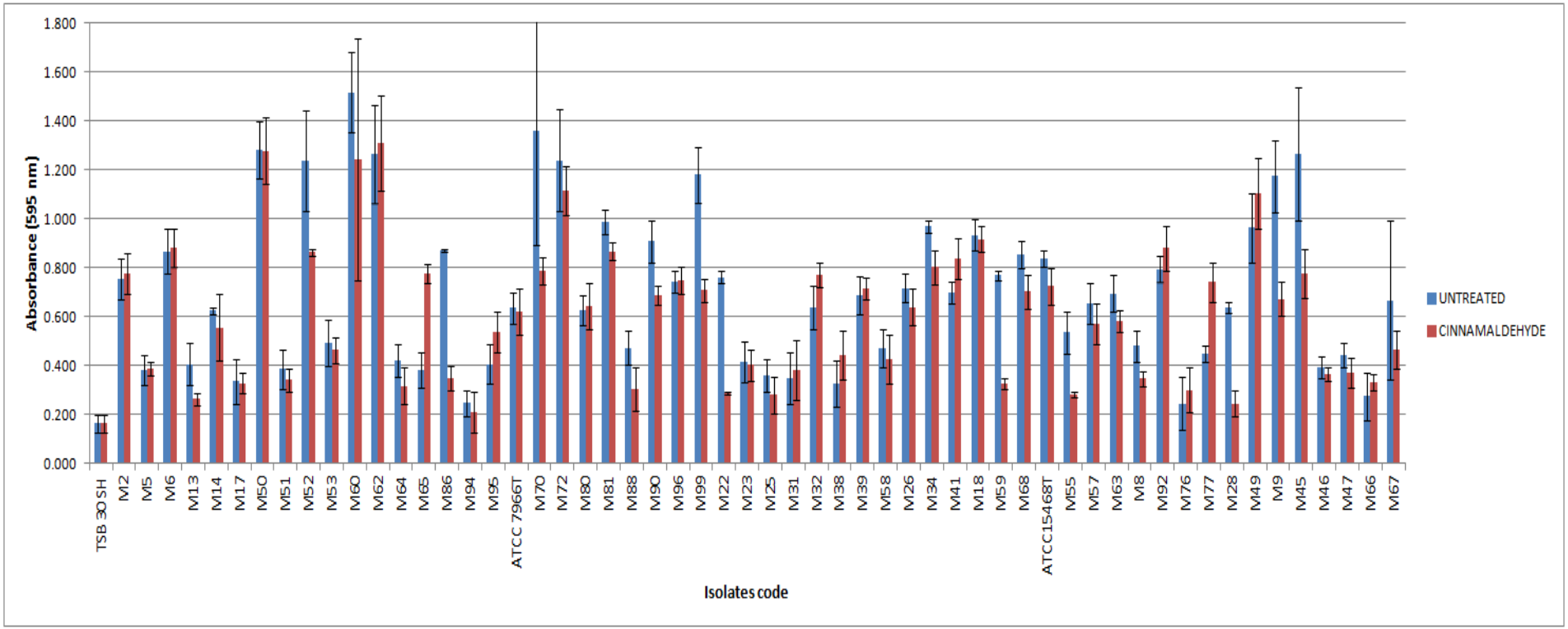

Figure 4.6: Effect of 100 µM cinnamaldehyde on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates following addition after 24 h biofilm formation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

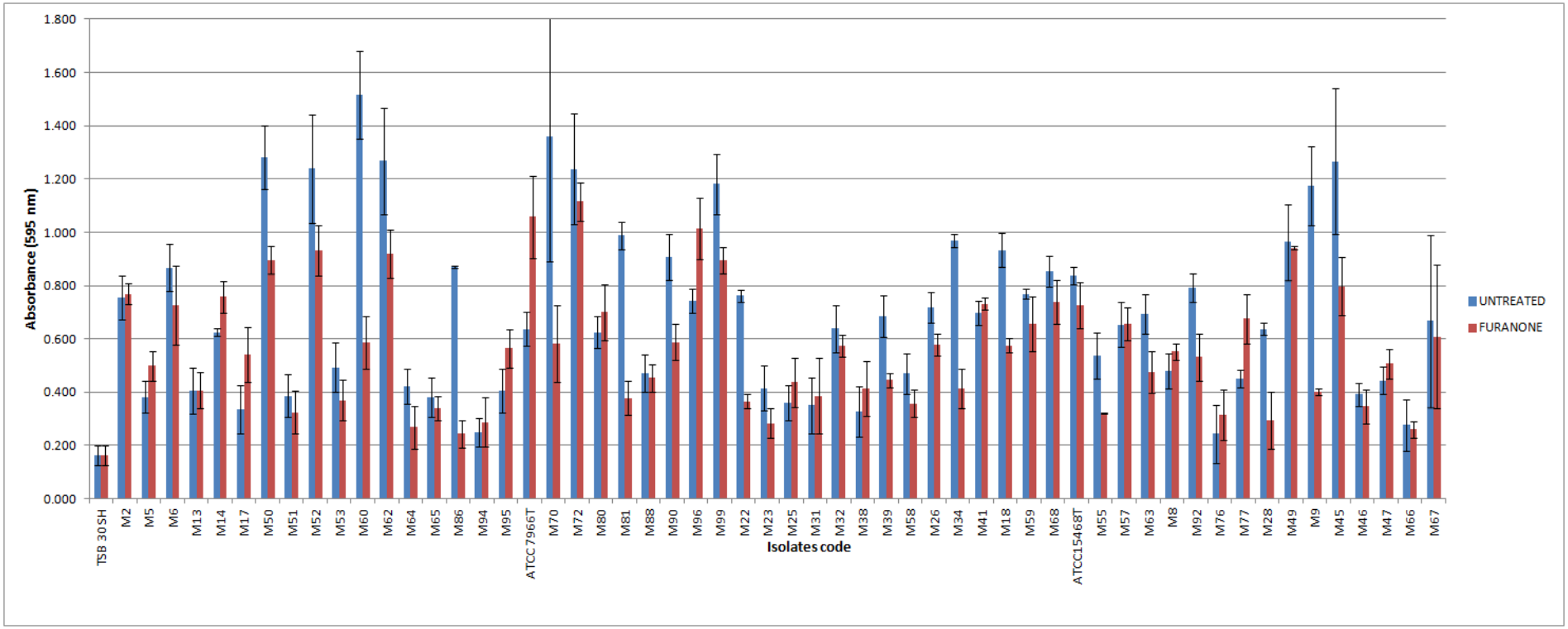

Figure 4.7: Effect of 5 µg/ml(2-)(-4-)bromomethylene-2(SH)-furanone on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates following addition after 24 h biofilm formation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

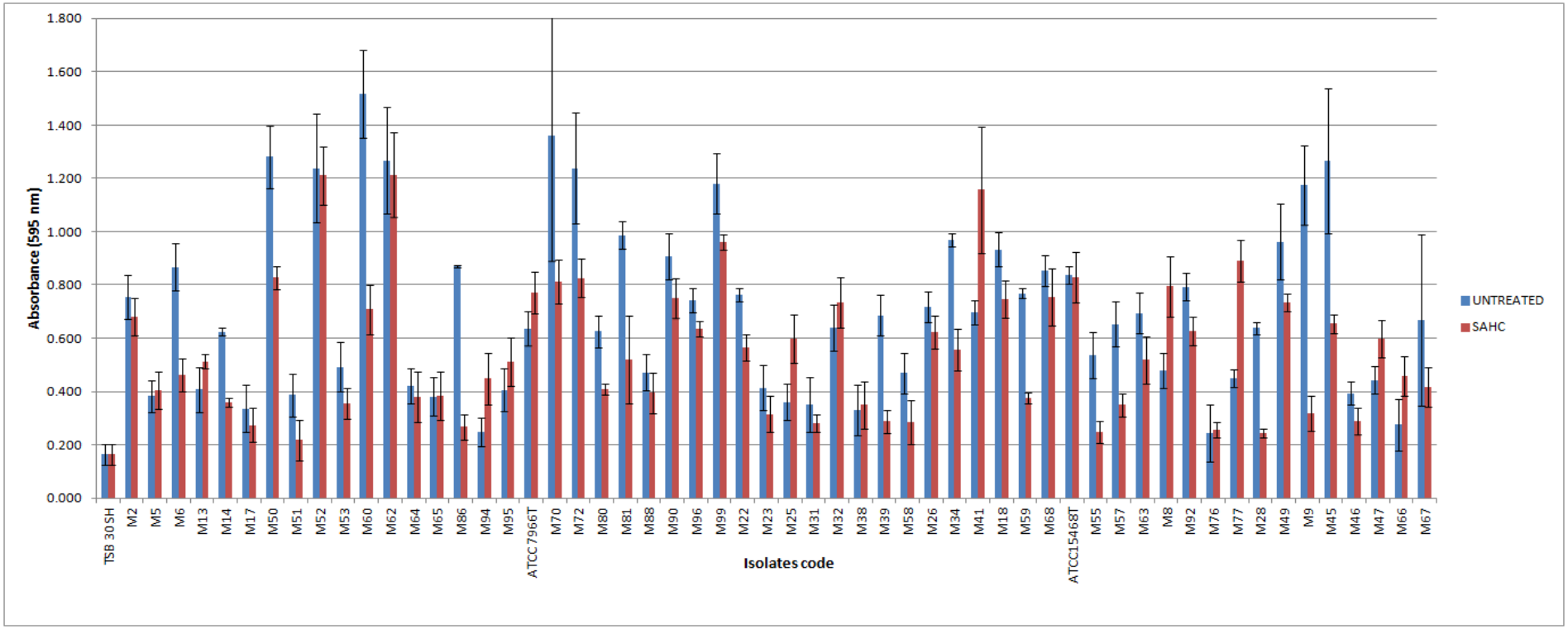

Figure 4.8: Effect of 5 µg/ml *S*-adenosylhomocysteine(SAHC) on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates following addition after 24 h biofilm formation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

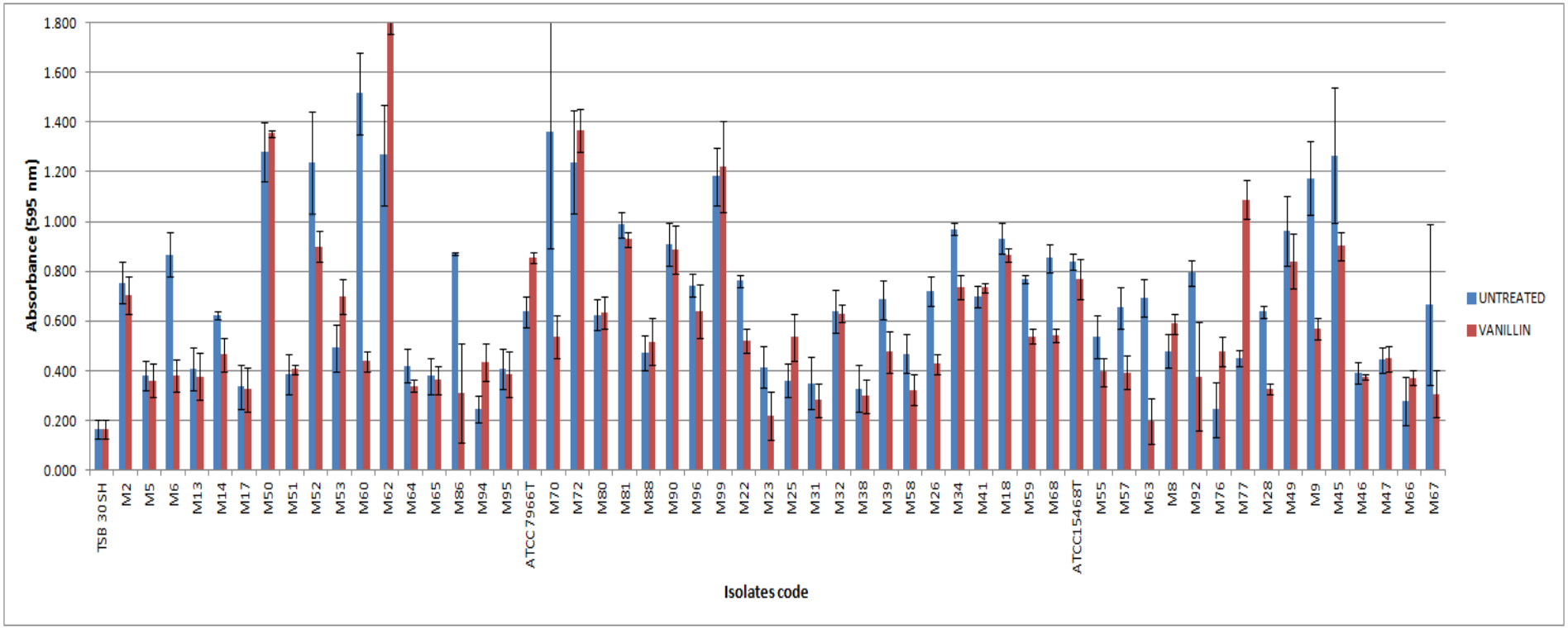

Figure 4.9: Effect of 5 µg/ml vanillin on pre-formed biofilm of *Aeromonas* and *Plesiomonas* spp. isolates following addition after 24 h biofilm formation.*A. hydrophila* (M2, M5, M6, M13, M14, M17, M50, M51, M52, M53, M60, M62, M64, M65, M86, M94, M95, ATCC 7966^{T}); *A. bestiarum* (M70, M72, M80, M81, M88, M90, M96, M99); *A. culicicola* (M22, M23, M25, M31, M32, M38, M39, M58); *Aeromonas* spp. (M26, M34, M41); *A. caviae* (M18, M59, M68, ATCC 15468^{T}); *A. veronii* (M55, M57, M63);*A. allosaccharophila* (M8, M92); *A. salmonicida* (M76, M77);*A. jandaei* (M28); *A. sobria* (M49); *Plesiomonas shigelloides* (M9, M45, M46, M47, M66, M67).

SAHC was also the most effective inhibitor in increasing detachment of the biofilm. While cinnamaldehyde and furanone were both the second most effective QSIs, vanillin was the least effective (Table 4.1).

4.3.3.1. Species-specific effect of QSIson mature biofilm

The species-specific effects of all four QSIs on mature biofilm are given in Table 4.3. In the pre-formed biofilm assays, SAHC was more effective in increasing detachment of 64.7% (11/54) of *A. hydrophila*isolates (Table 4.3). Vanillin and SAHC were the best candidates against *A. culicicola* isolates with both increasing biofilm detachment of 62.5% (5/8) of *A. culicicola* isolates (Table 4.3). SAHC was observed to detach biofilm of 100% of *A. bestiarum* isolates. Furanone, SAHC and vanillin increased biofilm detachment of66.7% (2/3) of *Aeromonas* spp. isolates. While furanone and SAHC detached 100% of *A. caviae*biofilms, cinnamaldehyde, SAHC and vanillin detached 100% of *A. veronii*biofilms. *P. shigelloides* biofilms were effectively detached by cinnamaldehyde and SAHC, with both inhibitors increasing biofilm detachment of 83.3% (5/6) of isolates (Table 4.3).

Table 4.3: Species-specific effect of QSIs on pre-formed biofilm

Species designation	**% Inhibition**			
	Cinnamaldehyde 100 µM	**Furanone 5 µg/ml**	**SAHC* 5 µg/ml**	**Vanillin 5 µg/ml**
***A. hydrophila* (n=17)**	58.8 (10/17)	58.8 (10/17)	64.7 (11/17)	29.4 (5/17)
***A. culicicola* (n=8)**	37.5 (3/8)	50 (4/8)	62.5 (5/8)	62.5 (5/8)
***A. bestiarum* (n=8)**	87.5 (7/8)	87.5 (7/8)	100 (8/8)	50 (4/8)
***Aeromonas* spp. (n=3)**	33.3 (1/3)	66.7 (2/3)	66.7 (2/3)	66.7 (2/3)
***A. caviae* (n=3)**	66.7 (2/3)	100 (3/3)	100 (3/3)	66.7 (2/3)
***A. veronii* (n=3)**	100 (3/3)	66.7 (2/3)	100 (3/3)	100 (3/3)
***A. allosaccharophila* (n=2)**	100 (2/2)	50 (1/2)	50 (1/2)	100 (2/2)
***A. salmonicida* (n=2)**	0	0	0	0
***A. jandaei* (n=1)**	100 (1/1)	100 (1/1)	100 (1/1)	100 (1/1)
***A. sobria* (n=1)**	0	0	100 (1/1)	100 (1/1)
***Plesiomonas shigelloides* (n=6)**	83.3 (5/6)	66.7 (4/6)	50 (3/6)	83.3 (5/6)

*SAHC = S-adenosylhomocysteine

4.3.4. Percentage reduction following QSIs treatments

The percent reduction following QSI treatments at time of inoculation and on pre-formed biofilm, respectively are shown in Table 4.4. For initial attachment assays, cinnamaldehyde treatment inhibited initial attachment of 63% (34/54) of isolates. Percent reduction for cinnamaldehyde ranged from 2.4 to 101.6% and percent induction ranged from 0.3 to 118.5%(Table 4.4). Furanone inhibited initial attachment of 59.3% (32/54) of isolates. Its percent reduction ranged from 3.0 to 74.8% and percent induction ranged from 3.3 to 158.3%. SAHC inhibited intial attachment of 55.6% (30/54) of isolates. Percent reduction for SAHC ranged from 7.3 to 78.1% and percent induction ranged from 1.6 to 152.8%. Vanillin treatment inhibited initial attachment of 63% (34/54) of isolates.Percent reduction for vanillin ranged from 2.4 to 72.5% and percent induction ranged from 3.1 to 151.5%.

In the pre-formed biofilm assays, SAHCinhibited the biofilm of 74.1% (40/54) of isolates. Percent reduction for SAHC ranged from 1.5 to 85.4% and percent induction ranged from 0.8 to 235.4%. Vanillin inhibited the biofilm of 70.4% (38/54) of isolates. Percent reduction for vanillin ranged from 2.6 to 93.4% and percent induction ranged from 1.8 to 291.1%. Furanone and trans-cinnamaldehyde inhibited the biofilm of 68.5% (37/54) and 66.7% (36/54) of isolates, respectively. For furanone, percent reduction ranged from 0.3 to 88.8% and percent induction ranged from 1.0 to 120.2%. Percent reduction for cinnamaldehyde ranged from 0.4 to 82.6% and percent induction ranged from 1.0 to 101.6%.

Table 4.4: Percent reduction of vanillin, cinnamaldehyde, furanone and *S*-adenosylhomocysteine on initial attachment and pre-formed biofilm of *Aeromonas*spp.and*P. shigelloides*isolates

Species designation	Isolates	% Reduction							
		Initial attachment				Pre-formed biofilm			
		Cinnamaldehyde	Furanone	SAHC[#]	Vanillin	SAHC[#]	Cinnamaldehyde	Furanone	Vanillin
A. hydrophila	**M2**	58	61.4	65.5	70.9	12.5	-3.3	-2.2	8.6
	M5	24	35.7	15.2	19.7	-10	-1.8	-53.5	9.4
	M6	30.4	50.5	25.3	-14.6	57.9	-2	20.2	69.1
	M13	23.4	7.4	27.7	-12.7	-43.2	58.9	0.3	12.5
	M14	-50.1	-54.7	-119.9	-114.7	57.6	14.6	-28.8	34.5
	M17	-2.5	-37.5	-19.9	-10.9	35.8	3.5	-120.2	6.6
	M50	-32.8	-25.4	-37.4	-3.1	40.6	0.4	34.5	-6.5
	M51	29	4.6	21	38.7	76	20.3	27.9	-8.5
	M52	-14.2	-139.3	-65.7	8.7	2.6	35	28.5	31.5
	M53	-118.5	-100.1	-49.1	-52.1	42.4	8.9	37.6	-62.3
	M60	-20.5	42.4	54	31.6	59.7	20.3	68.8	79.7
	M62	10	65.7	55.5	58	4.9	-3.7	31.5	-54.8
	M64	-5.2	15.7	8.8	-66.4	15.8	39.9	59	32.1
	M65	-4.7	4.4	-6.6	-11.9	-0.8	-183.3	19.6	7.6
	M86	-3.6	3	-27.1	-16.8	85.4	73.6	88.8	79.2
	M94	8.1	16.1	7.3	23.4	-235.4	44.3	-46	-219.7
	M95	9.9	-79.5	-48.1	-74.5	-43	-54.3	-65.2	8.4
	ATCC 7966[T]	-60.2	-3.4	20.5	43.3	-28.4	3.1	-88.8	-45.8
A. bestiarum	**M70**	18.1	53.8	34.9	72.5	45.8	47.8	65	68.8
	M72	23.9	29	11.8	14.9	38.3	11.5	11.4	-12.2
	M80	47.7	56.1	52	4.8	46.9	-4.2	-16.4	-1.9
	M81	18.8	16.2	-3.6	19.4	56.7	14.6	74	7.1
	M88	85.4	27.6	-11.9	-8.3	25.1	54.4	6.2	-14.5
	M90	-89.4	-3.3	-43.6	-62.2	21.1	29.3	42.9	2.6
	M96	21.3	25.7	-65.2	18.4	18.6	-1	-46.7	18
	M99	27.2	-29.4	16.8	2.4	21.7	46.5	28.1	-4.1
A. culicicola	**M22**	2.8	-8	-22.6	25.1	32.8	79	66.2	40.2
	M23	2.4	21	25.6	-44.2	39.4	4.8	52.5	78.5
	M25	-16	-8.9	-5.9	22.5	-121.4	40.4	-39.2	-89.1
	M31	-23.7	-89.9	22.4	12.9	37.3	-17.7	-18.8	36.2
	M32	9.1	12.9	39	-8.3	-19.9	-27.8	13.4	1.9

	M38	14.9	-17.4	40	22.8	-12.4	-69.6	-51.9	18.3
	M39	33.6	23.2	43.5	24.1	76.4	-5.3	46.2	40
	M58	-13.5	17.9	-26	-48.2	60.5	13.2	36.6	47.9
Aeromonas spp.	**M26**	58.8	-10.4	-1.6	3.5	17	14.2	25.2	52.2
	M34	-5.3	9.2	14.5	24.1	51	20.8	69	28.8
	M41	39.9	68.5	10.8	42.1	-86	-26.2	-6.4	-6.7
A. caviae	**M18**	12.6	55.7	53.8	28.6	24.4	2	46.4	8.7
	M59	96	59.1	22.8	66	64.8	72.9	18.5	38.2
	M68	13.9	33.5	-28.5	13.4	14.3	22.1	16.8	45.2
	ATCC 15468^{T}	-47.3	27.3	42.1	2.4	1.5	16.7	16.7	10.3
A. veronii	**M55**	101.6	74.8	39.2	29.3	77.3	68.3	57.7	38
	M57	-57	39	20.5	23.3	62.1	16.6	-1	52.9
	M63	83.7	-36	-52.8	50.3	33.3	21	41.1	93.4
A. allosaccharophila	**M8**	16	58.6	11.8	23.6	-99.6	41.5	-22.7	-34.4
	M92	-9	-16.9	-83.2	13.8	26.6	-13.8	41.7	66
A. salmonicida	**M76**	28.9	39.1	32.3	-3.9	-14.4	-70.5	-90.2	-291.1
	M77	42	-21.1	-22.9	68.2	-154.3	-101.6	-78.7	-223.2
A. jandaei	**M28**	101.3	-28.5	-97.4	-151.5	83.2	82.6	72.8	65.5
A. sobria	**M49**	-26.3	-158.3	-132.8	8.3	28.5	-17.6	2.6	15.3
P. shigelloides	**M9**	85.7	-29	78.1	28.3	84.6	49.6	76.4	59.9
	M45	23.6	-10.1	36.1	25.4	55.5	44.3	42.5	33
	M46	20.6	-11.5	-16.7	-8.2	44.5	11.8	20.2	8.1
	M47	-0.3	-11.4	18.5	-26	-56	24.8	-22.5	-1.8
	M66	-17.5	10.6	-58.6	-109.3	-159.7	-48.7	15.1	-83.9
	M67	-6.1	13.4	16.8	31.7	49.4	40.1	11.7	71.5

*Percent reduction=Percentage reduction = $[\ ((C - B) - (T - B))/(C - B)\ ] \times 100$, where B=average absorbance per well for blank wells, C=average absorbance per well for control wells, T=average absorbance per well for treated wells(Pitts *et al*., 2003),

[#]SAHC = S-adenosylhomocysteine.

4.4. Discussion

Aeromonas spp. are known to produce a diversity of AHLs for bacterial communication (Chan *et al*., 2011). Based on biosensor responses, the *Aeromonas* spp. isolates in the this study produce long-chain AHLs as their major QS molecules. All 54 isolates tested induced the utilization of X-gal to produce a blue color by *A. tumefaciens* A136, which detects a wide range of AHLs, including long chain AHLs. As observed in the present study *Aeromonas* spp. have been documentedto produce long-chain AHLs such as N-octanoylhomoserine lactone (C-8 AHL), N-dodecanoylhomoserine lactone (C-12 AHL) and N-tetradecanoylhomoserine lactone (C-14 AHL) and N-decanoylhomoserine lactone (DHL) (Cataldi *et al.,* 2007) which is in agreement with findings of this study. Only isolate M13, which is an *A. hydrophila* isolate, was observed to induce production of the pigment violacein by the *C. violaceum* CV026 biosensor which detects short and medium (C-4 to C-8) AHLs. *A. hydrophila* have been observed to produce C4-AHL and C6-AHL as their two major types of AHLs (Chan *et al*., 2011; Medina-Martínez *et al*., 2006).The majority of *Aeromonas* spp. isolates appeared to be producing long chain AHLs enabling their detection by *A. tumefaciens* A136.

QSIs used in the present study proved to be important candidates to control biofilm formation since they inhibited both initial attachment and pre-formed biofilm. However, these inhibitors were more effective in treating pre-formed biofilms than initial attachment. SAHC was most effective in treating both initial attachment and pre-formed biofilm (Table 4.1). Hentzer and Givskov (2003) observed that SAHC displayed activity against *P. aeruginosa* biofilm. The use of the S-adenosylmethionine analogue such as SAHC is suggested since these molecules inhibit the synthesis of the signal molecule (Defoirdt*et al*., 2004). In *P. aeruginosa,* this *S*-adenosylmethionine analogue was shown to inhibit the LuxI homologue, RhlI, by up to 97% (Defoirdt*et al.*, 2004). As confirmed by percent reduction, SAHC was the best QSI candidate, in inhibiting initial adhesion and detaching mature biofilm.

Cinnamaldehyde was the second most effective QSI when treating initial attachment as well as mature biofilms. Brackman *et al*. (2009) observed that cinnamaldehyde inhibited biofilm formation of *Burkholderia* spp. by binding to short chain AHLs, as did Niu and Gilbert (2004) who observed that cinnamaldehyde inhibited biofilm formation by *E. coli*.Amalaradjou *et al*. (2010) also showed that cinnamaldehyde eradicated 24 h biofilmsformed by uropathogenic *E.*

coli.

Other QSIs such as furanones, which are suggested to interfere with AHL synthesis, have been shown to interfere with mature biofilm rather than with initial attachment (Rasmussen *et al.,* 2000) and this corresponds with results obtained in the this study. Halogenated furanones are antagonists to AHLsand by inhibiting both short and long chain AHLs they affect biofilm formation (Ponnusamy *et al*., 2010). Ponnusamy *et al*. (2010) observed that 0.2 mg/ml of furanone reduced the biofilmmass of *A. hydrophila* to 17% and when used at 1 mg/ml it reduced the mass to 32%. In the current study, furanone reduced the biofilm mass of *Aeromonas* and *Plesiomonas* spp. isolates to 63 and 64.8% in the initial attachment and pre-formed biofilm assays (Table 4.1), respectively.

Vanillin has been shown to inhibit *Aeromonas* spp. biofilm formation by inhibiting long chain AHLs rather than short chain AHLs. Kappachery *et al*. (2010) demonstrated that by interfering with QS,vanillin reduces biofilm formation of *A. hydrophila* without inhibiting growth of cells within the biofilm. Low concentrations of vanillin such as 0.25 mg/ml reduced biofilm formation of *A. hydrophila* by 43% (Ponnusamy *et al*., 2009).In present study 5 μg/ml of vanillin reduced biofilm formation of *Aeromonas* and *Plesiomonas* spp. by 59.3 and 61.1% in the initial attachment and pre-formed biofilm assays biofilms (Table 4.1), respectively.

When the different species were examined individually, it was foundthat some *Aeromonas* species (*A. hydrophila, A. culicicola* and *A. bestiarum*) and *P. shigelloides* were more resistant to the action of QSIs in the initial attachment assay, however, in the pre-formed biofilmassays majority of the QSIs were more effective againstthe same species. QSIs are effective against *Aeromonas* and *Plesiomonas* spp. isolates in the pre-formed biofilm assays rather than the initial attachment stage assays and SAHC is the most suitable. This raises questions such as: Is the activity of QSIs species-related or is a variable mechanism of inhibition being exerted on the different species. QSIs are effective in treating mature biofilm but further studies need to be conducted to determine how these molecules work and also to whether they can be effective if applied in fish farm environments.

CHAPTER 5

General Discussion and Conclusions

Aeromonas spp. are one of the major fish pathogens in the aquatic environment. Members of this species have been isolated in diverse places such as sewage, water systems, food products and vegetables(Farmer *et al*., 2006). These then serve as a source of diseases for humans and other different animals. Considering the pathogenicity of *Aeromonas* spp. in the aquatic environments, the current study investigated diverse control strategies to limit biofilm formation and/or quorum sensing by *Aeromonas* spp. isolates.

The MICs of different antimicrobial agents (azithromycin, ciprofloxacin, ceftazidime, gentamicin, and tetracycline) were observed to range between 0.064-64 μg/ml. Gentamicin displayed the lowest MIC range (MIC ranged from 0.0048-32 μg/ml) when compared to other antimicrobial agents. As expected, the MBICs were higher than MICs. The MBICs of antimicrobial agents against most *Aeromonas* spp. isolates were observed to be 4096 μg/ml. The most effectiveconcentration of antimicrobial agents between sub-MIC, MIC and supra-MIC when targeted against biofilms formed by *Aeromonas* spp. isolates were the MIC exposures.MIC exposures of gentamicin inhibited initial attachment of 100% (28/28) of isolates andMIC exposures of azithromycin detached biofilms of 82.1% (23/28) of isolates.

The combination of EPIs with antimicrobial agents is suspected to provide a synergestic effect and to inhibit biofilm formation(Pagès and Amaral, 2009). In the current study, CCCP completely inhibited efflux of cefpodoxime in 14.8% of isolatesand proved to be the best candidate to be used in combination with antimicrobial agents. However, when EPIs were used on their own, NMP proved to be the best candidate. NMP inhibited attachment of 98.1% of isolates and detached biofilms of 100% of isolates. DNase I was observed to be more effective in the pre-formed biofilm assay where it detached 64.8% (35/54) of isolates rather than in the intial attachment where it inhibited initial attachment of 59.2% (32/54) of isolates.

The production of AHLs by *Aeromonas* spp. was detected prior to investigating the effect of QSIs against these isolates. While all 54 isolates were observed to produce long chain AHLs, only a single *A. hydrophila*isolate M13 was observed to produce short chain AHLs. SAHC was observed to be the most effectiveQSI as it inhibited initial attachment of 72.2% (39/54) of isolates and increased detachment of 74.1% (40/54) of isolates.

Future studies may focus on applying combinations of antimicrobial agents, EPIs, lytic enzymes and QSIs used in the current study in aquatic settings where *Aeromonas* spp. and other related aquatic pathogens cause diseases. In addition, HPLC could be used to identify different AHLs that are produced by these South African *Aeromonas* spp. isolates. The mechanism by which EPIs and QSIs inhibit*Aeromonas* spp. isolates is not fully understood, thus, future studies might focus on understanding how these inhibitors behave within *Aeromonas*spp. planktonic cells and sessile cells.

REFERENCES

Alcaide, E., M. D. Blasco, and C. Esteve. 2010. Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. Research in Microbiology **161**:40-45.

Allesen-Holm, M., K. B. Barken, L. Yang, M. Klausen, J. S. Webb, and S. Kjelleberg. 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Molecular Microbiology **59**:1114–1128.

Amalaradjou, M. A. R., A. Narayanan, S. A. Baskaran, and K. Venkitanarayanan. 2010. Antibiofilm effect of trans-cinnamaldehyde on uropathogenic *Escherichia coli*. Journal of Urology **184**:358-363.

Anderl, J.N, M. J. Franklin, and P.S. Stewart. 2002. Role of antibiotic penetration limitation in *Klebsiella pneumonia* biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial Agents and Chemotherapy **44**:1818-1824.

Andrews, J. M. 2001. Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy **48**:5-16.

Andrews, J. S., S. A. Rolfe, W. E. Huang, J. D. Scholes and S. A. Banwart. 2010. Biofilm formation in environmental bacteria is influenced by different macromolecules depending on genus and species. Journal of Environmental Microbiology **12**:2496-2507.

Asahi, Y., Y. Noiri, J. Igarashi, H. Asai, H. Suga, and S. Ebisu. 2010. Effects of N-acylhomoserine lactone analogues on *Porphyromonas gingivalis* biofilm formation. Journal of Periodontal Research **45**:255–261.

Basson, A., L.A. Flemming, and H.Y.Chenia. 2008. Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacteriumjohnsoniae*-like isolates. Microbial Ecology **55:**1-14.

Bechet, M., and R. Blondeau. 2003. Factors associated with the adherence and biofilm formation by *Aeromonas caviae* on glass surfaces. Journal of Applied Microbiology **94**:1072-1078.

Behlau, I.,and M.S.Gilmore. 2008. Microbial biofilms in ophthalmology and infectious disease. Archives of Ophthalmology **126**:1572-1581.

Belem-Costa, A., and J.E.P. Cyrino. 2006. Antibiotic resistence of *Aeromonas hydrophila* isolated from *Piaractus mesopotamicus* (Holmberg, 1887) and *Oreochromis niloticus* (Linnaeus, 1758). Journal of Agricultural Science **63**:281-284.

Bello-Lopez, J.M., E. Fernandez-Rendon and E. Curiel-Quesada. 2009. In vivo transfer of plasmid pRAS1 between *Aeromonas salmonicida* and *Aeromonas hydrophila* in artificially infected *Cyprinus carpio L*. Journal of Fish Diseases **33**:251–259.

Bina, X. R., J. Philippart, and J. E. Bina. 2009. Effect of the efflux inhibitors 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-beta-naphthylamide on antimicrobial susceptibility and virulence factor production in *Vibrio cholerae*. Journal of Antimicrobial Chemotherapy **63**:103-108.

Bjarnsholt, T., T. Tolker-Nielsen, N. Høiby, and M. Givskov. 2010. Interference of *Pseudomonas aeruginosa* signalling and biofilm formation for infection control. Expert Reviews in Molecular Medicine **12**:1-19.

Blair, J. M. and L. J. V. Piddock. 2009. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Current Opinion in Microbiology **12**:512-519.

Blasco, M.D., C.Esteve, E. Alcaide. 2008. Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. Journal of Applied Microbiology. **105**:469-475.

Böckelmann, U., A. Janke, R. Kuhn, T. R. Neu, J. Wecke, J. R. Lawrence, and U. Szewzyk. 2006. Bacterial extracellular DNA forming a defined network-like structure. FEMS Microbiology Letters **262**:31-38.

Bomo, M., M. V. Storey, and N. J. Ashbolt. 2004. Detection, integration and persistence of aeromonads in water distribution pipe biofilms. Journal of Water and Health **2**:83-96.

Boyen, F., V. Eeckhaut, F. Van Immerseel, F. Pasmans, R. Ducatelle, and F. Haesebrouck. 2009. Quorum sensing in veterinary pathogens:mechanisms, clinical importance and future perspectives. Veterinary Microbiology **135**:187–195.

Brackman, G., U. Hillaert, S. Van Calenbergh, H. J. Nelis, and T. Coenye. 2009. Use of quorum sensing inhibitors to interfere with biofilm formation and development in *Burkholderia multivorans* and *Burkholderia cenocepacia*. Research in Microbiology **160**:144-151.

Butler, M. T.,Q. Wang, and R. M. Harshey. 2010. Cell density and mobility protect swarming bacteria against antibiotics. Proceedings of the National Academy of Sciences USA **107**:3776-3781.

Canals, R., S. Vilches, M. Wilhelms, J. G. Shaw, S. Merino, and J. M. Tomás. 2007. Non-structural flagella genes affecting both polar and lateral flagella-mediated motility in *Aeromonas hydrophila.*Microbiology **153**:1165-1175.

Cargill, J. S and M. Upton. 2009.Low concentrations of vancomycin stimulatebiofilm formation in some clinical isolates of *Staphylococcus epidermidis*. Journal of Clinical Pathology **62**:1112-1116.

Carmen, J.C. B.L. Roeder, J.L. Nelson, R.L. Robison Ogilvie, R.A. Robison, G. B Schaalje and W.G. Pitt. 2005. Treatment of biofilm infections on implants with low-frequency ultrasound and antibiotics. American Journal of Infection Control **33**:78–82.

Cars, O., and I. Odenbok-Toraqrist. 1993. The post-antibiotic sub-MIC effect *in vitro* and *in vivo*. Journal of Antimicrobial Chemotherapy **31**:159-166.

Castro-Escarpulli, G., M.J. Figueras, G. Aguilera-Arreola, L. Soler, E. Fernandez-Rendon, G.O. Aparicio, J. Guarro, and M.R. Chacon. 2003. Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. International Journal of Food Microbiology **84**:41–49.

Cataldi, T.R.I., G. Blanco, L. Palazzo, and V. Quaranta. 2007. Occurrence of N-acyl-L-homoserine lactones in extracts of some Gram-negative bacteria evaluated by gas chromatography-mass spectrometry. Analytical Biochemistry **36**:226-235.

Cattoir, V., L. Poirel, C. Aubert, C.-J.Soussy, and P. Nordmann. 2008. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. Emerging Infectious Diseases **14**:231-237.

Cerca, B., S. Martins, G. Pier, R. Oliveira, and J. Azevedo. 2005. The relationship between inhibition of bacterial adhesion to a solid surface by sub-MICs of antibiotics and subsequent development of a biofilm.Research in Microbiology **156**:650-655.

Chaignon, P., I. Sadovskaya, C. Ragunah, N. Ramasubbu, J. B. Kapla andS. Jabbouri. 2007. Susceptibility of staphylococcal biofilms to enzymatic treatmentsdepends on their chemical composition. Applied Microbiology and Biotechnology **75**:125–132.

Chan, K.G., S. D. Puthucheary, X.-Y.Chan, W.-F.Yin, C.-S.Wong, W.-S. S. Too, and K.-H. Chua. 2011. Quorum sensing in *Aeromonas* species isolated from patients in Malaysia. Current Microbiology **62**:167-172.

Chmielewski, R.A.N., and J.F. Frank. 2003. Biofilm formation and control in food processing facilities. Comprehensive Reviews in Food Science and Food Safety **2**:22-32.

Clinical and Laboratory Standards Institute.2007. Performance standards for antimicrobial susceptibility testing; 17th informational supplement. CLSI M100-S17.CLSI, Wayne, PA.

Corral F.D., E.E. Shotts, and J. Brown. 1990. Adherence, haemagglutination and cell surface characteristics of motile aeromonads virulent for fish. Journal of Fish Diseases **13**:255-268.

Couce, A., and J. Blazquez. 2009. Side effects of antibiotics on genetic variability.FEMS Microbiology Review **33**:531–538.

Curtin, J., and M. Cormican. 2003. Measuring antimicrobial activity against biofilm bacteria.Reviews in Environmental Science and Bio/Technology **2:**285–291.

Dal Sasso, M., M. Culici, C. Bovio, and P.C. Braga. 2003. Gemifloxacin: effects of sub-inhibitory concentrations on various factors affecting bacterial virulence. International Journal of Antimicrobial Agents **21**:325-333.

Das, T., P. K. Sharma, H. J. Busscher, H. C. van der Mei, and B. P. Krom. 2010. Role of extracellular DNA in initial bacterial adhesion and surface aggregation. Applied and environmental microbiology **76**:3405-3408.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**:295-298.

de Carvalho C.C.C.R. 2007. Biofilms: recent developments on an old battle. Recent Patents on Biotechnology **1**:49-57.

Declerck, P., J. Behets, A. Margineanu, V. van Hoef, B. De Keersmaecker, and F. Ollevier. 2009. Replication of *Legionella pneumophila* in biofilms of water distribution pipes. Research in Microbiology **164**:593-603.

Defoirdt, T., N. Boon, P. Bossier, and W. Verstraete. 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fightinfections in aquaculture. Aquaculture **240**:6419–6423

del Pozo J.L., and R. Patel. 2007. The challenge of treating biofilm-associated bacterial infection. Clinical Pharmacology and Therapeutics **82**:204-209.

Dhar, N., and J. D. McKinney. 2007. Microbial phenotypic heterogeneity and antibiotic tolerance. Current Opinion in Microbiology **10**:30–38.

Dogruoz, N., D. Goksay, E. IIhan-Sungun, and A. Cotuk. 2009. Pioneer colonizer microorganisms in biofilm formation on galvanized steel in a stimulated recirculating cooling-water system. Journal of Basic Microbiology **49**:S5-S12
.

Duma, S. T. 2012.Characterization of factors involved in and affecting biofilm formation by *Aeromonas* spp. isolates. University of KwaZulu Natal Masters Dissertation.

Dynes, J.J., J.R. Lawrence, D.R. Korber, G.D.W. Swerhone, G.G. Leppard, and A.P. Hitchcock. 2009. Morphological and biochemical changes in *Pseudomonas fluorescens* biofilms induced by sub-inhibitory exposure to antimicrobial agents. Canadian Journal of Microbiology **55**:163-178.

Elhariry, H.M. 2011. Biofilm formation by *Aeromonas hydrophila* on green-leafy vegetables: cabbage and lettuce. Food borne Pathogens and Disease **8**:125-131.

Estrela A.B., M.G Heck, and W.R. Abraham. 2009. Novel approaches to control biofilm infections. Current Medicinal Chemistry **16**:1512-1530.

Farmer, J.J., III, M.J. Arduino, and F.W. Hickman-Brenner. 2006. The Genera *Aeromonas* and *Plesiomonas*. The Prokaryotes **6**:564-596.

Francolini, I., and G. Donelli. 2010. Prevention and control of biofilm-based medical-device-related infections. Fems Immunology and Medical Microbiology **59**:227-238.

Garcıa-Castillo, M., M.I. Morosini, A. Valverde, and F. Almaraz, F. Baquero, R. Canton, and R. del Campo. 2007. Differences in biofilm development and antibiotic susceptibility among *Streptococcus pneumoniae* isolates from cystic fibrosis samples and blood cultures. Journal of Antimicrobial Chemotherapy **59**:301-304.

Gavin, R., A.A. Rabaan, S. Merino, J.M. Tomas, I. Gryllos, and J.G. Shaw. 2002. Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. Molecular Microbiology **43**:383-397.

Gefen, O., and N. Q. Balaban. 2009. The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiology Letters **33**:704-717.

Gibotti, A., H.O. Saridakis, J.S. Pelayo, K.C. Tagliari and D.P. Falcao. 2000. Prevalence and virulence properties of *Vibrio cholerae* non-O1, *Aeromonas* spp. and *Plesiomonas shigelloides* isolated from Cambe Stream (State of Parana, Brazil). Journal of Applied Microbiology **89**:70-75.

Gould, I.M., and F. M. Mackenzie. 2002**.** Antibiotic exposure as a risk factor for emergence of resistance: the influence of concentration. Journal of Applied Microbiology **31**:78–84.

Gudesblat, G.E., P.S. Torres, and A.A. Vojnov. 2009.*Xanthomonas campestris* overcomes Arabidopsis stomatal innate immunity through a DSF cell-cell signal-regulated virulence factor. Plant Physiology **149**:1017–1027.

Haddadin, R.N.S., S. Saleh, I.S.I. Al-Adham, T.E.J. Buultjens, and P.J. Collier. 2009. The effect of sub-minimal inhibitory concentration on virulence factors expressed by *Staphylococcus aureus* biofilms. Journal of Applied Microbiology **108**:1281-1291.

Hannula, M., and M. Hanninen. 2008. Effect of putative efflux pump inhibitors and inducers on the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli*. Journal of Medical Microbiology **57:**851–855.

Hentzer, M., and M. Givskov. 2003. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. Journal of Clinical Investigation **112**:1300-1307.

Hentzer, M, K. Riedel, T.B. Rasmussen,A.Heydorn, J. B Andersen, M. R. Parsek, S.A. Rice, L. Eberl, S. Molin, N. Høiby, S. Kjelleberg, and M. Givskov. 2003. Inhibition of quorum sensing in *Pseudomonasaeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology **148**:87–102.

Hernould, M., S. Gagne, M. Fournier, C. Quentin, and C. Arpin. 2008. Role of the AheABC efflux pump in *Aeromonas hydrophila* intrinsic multidrug resistance. Antimicrobial Agents and Chemotherapy **52**:1559-1563.

Hirakata, Y., A. Kondo, K. Hoshino, H. Yano, K. Arai, A. Hirotani, H. Kunishima, N. Yamamoto, M. Hatta, M. Kitagawa, S. Kohno, and M. Kaku. 2009. Efflux pump inhibitors reduce the invasiveness of *Pseudomonas aeruginosa*. International Journal of Antimicrobial Agents **34**:343-346.

Høiby, N., T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu. 2010. Antibacterial biofilms in bacterial biofilms. International Journal of Antimicrobial Agents **35**:322-332.

Igbinosa, I. H., E. Igumbor, F. Aghdasi, T. Mvuyo, and A. I. Okoh. 2012. Emerging *Aeromonas* species infections and their significance in public health. The Scientific World Journal **doi:10.1100/2012/625023**:1-13.

Igbinosa, I. H., and A. I. Okoh. 2012. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. The Scientific World Journal **doi:10.1100/2012/764563**:1-6.

Ikonomidis, A., A. Tsakris, M. Kanellopoulou, A. N. Maniatis, and S. Pournaras. 2008. Effect of the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on *Pseudomonas aeruginosa* biofilm development. Letters in Applied Microbiology **47**:298–302.

Izano, E.A., S.M. Shah, and J.B. Kaplan. 2009. Intercellular adhesion and biocide resistance in nontypeable *Haemophilus influenzae* biofilms. Microbial Pathogenesis **46**:207–213.

Jacobs, L., and H. Y. Chenia. 2007. Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. International Journal of Food Microbiology **114**:295-306.

Janda, J.M., and S.L.Abbott. 2010. The Genus *Aeromonas*: Taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews **23**:35-73.

Kaplan, J.B. 2010. Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. Journal of Dental Research **89**:205-218.

Kappachery, S., P. Diby, Y. Jeyong, and H. K. Ji. 2010. Vanillin, a potential agent to prevent biofouling of reverse osmosismembrane.Biofouling **26**:667-672.

Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiology Letters **230**:13–18.

Khajanchi, B. K., J. Sha, E. V. Kozlova, T. E. Erova, G. Suarez, J. C. Sierra, V. L. Popov, A. J. Horneman, and A. K. Chopra. 2009. N-acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and *in vivo* virulence in a clinical isolate of *Aeromonas hydrophila*. Microbiology **155**:3518-31.

Khajanchi, B. K., A. Fadl, M. Borchardt, R. L. Berg, A. J. Horneman, M. E. Stemper, S. W. Joseph, N. P. Moyer, J. Sha, and A. K. Chopra. 2010. Distribution of virulence factors and molecular fingerprinting of *Aeromonas* species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. Applied and Environmental Microbiology **76**:2313-2325.

Kim, J., J. S. Hahn. M.J. Franklin. P.S. Stewart, and J. Yoon. 2009. Tolerance of dormant and active cells in *Pseudomonas aeruginosa* PA01 biofilm to antimicrobial agents. Journal of Antimicrobial Chemotherapy **63**:129-135.

Kirke, D. F., S. Swift, M. J. Lynch, and P. Williams. 2004. The *Aeromonas hydrophila* LuxR homologue AhyR regulates the N-acyl homoserine lactone synthase, AhyI positively and negatively in a growth phase-dependent manner. FEMS Microbiology Letters **241**:109-117.

Kirov, S.M., M. Castrisios, and J.G. Shaw. 2004. *Aeromonas* flagella (polar) are enterocyte adhesins that contribute to biofilm formation on surfaces. Infection and Immunity **72**:1939-1945.

Kociolek M. G. 2009. Quorum-sensing inhibitors and biofilms.Anti-Infective Agents in Medicinal Chemistry **8**:315-326.

Kozlova, E.V., V.L. Popov, J. Sha, S.M. Foltz, T.E. Erova, S.L. Agar, A.J. Horneman, and A.K. Chopra. 2008. Mutation in the S-ribosylhomocysteinase (luxS) gene involved in quorum sensing affects biofilm formation and virulence in a clinical isolate of *Aeromonas hydrophila*. Microbial Pathogenesis **45**:343-354.

Kvist, M., V. Hancock, and P. Klemm. 2008. Inactivation of efflux pumps abolishes bacterial biofilm formation. Applied and Environmental Microbiology **74**:7376-7382.

Labbate, M., S.Y. Queck, K.S. Koh, S.A. Rice, M. Givskov, and S. Kjelleberg. 2004. Quorum-sensing controlled biofilm development in *Serratia liquefaciens* MG1. Journal of Bacteriology **186**:692–698.

Landini P., D. Antoniani, J.G. Burgess, and R. Nijland. 2010. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. Applied Microbiology and Biotechnology **86**:813-823.

Lappann, M., H. Claus, T. van Alen, M. Harmsen, J. Elias, S. Molin, and U. Vogel. 2010. A dual role of extracellular DNA during biofilm formation of *Neisseria meningitidis*. Molecular Microbiology **75**:1355-1371.

Lequette, Y., G. Boels, M. Clarisse, and C. Faille. 2010. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling **26**:421–431.

Li, Y., Y. Xie, R. Chen, H. Xu, G. Zhang, J. Li, and X. Li. 2009. Effects of combined treatment with sansanmycin and macrolides on*Pseudomonas aeruginosa* and formation of biofilm.Biomedical and Environmental Sciences **22**:170-177.

Liaqat, I., F. Sumbal and A.N. Sabri.2009**.** Tetracycline and chloramphenicol efficiency against selected biofilm forming bacteria.Current Microbiology**59**:212–220.

Lim, J.-H., and H.-I.Yun. 2001**.** Postantibiotic effects and postantibiotic sub-MIC effects of erythromycin, roxithromycin, tilmicosin, and tylosin on *Pasteurella multocida*. International Journal of Antimicrobial Agents **17**:471-476.

Lomovskaya, O., and W. Watkins. 2001. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. Journal of Molecular Microbiology Biotechnology**3**:225-236.

Lukkana, M., J. Wongtavatchai, and R. Chuanchuen. 2011. Expression of AheABC efflux system and plasmid profile of *A. hydrophila* isolatesfrom farmed Nile tilapia. Thai Journal of Veterinary Medicine **41**:529-533.

Lupo, A., S. Cogne, and T. U. Berendonk. 2012. Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. Frontiers in Microbiology **3**: 1-13.

Lynch, M.J., S. Swift, D.F. Kirke, W. Keevil, C.E. R. Dodd, and P. Williams. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environmental Microbiology **4**:18–28.

Magnet, S., P. Courvalin, and T. Lambert. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrobial Agents and Chemotherapy **45**:3375-3380.

Mah, T.F.C., and G.A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology **9**:34-39.

Mahamoud, A., J. Chevalier, S. Alibert-Franco, W. V. Kern, and J.-M. Pagès. 2007. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. Journal of Antimicrobial Chemotherapy **59**:1223-1229.

Majtan, J., L. Majtanova, M. Xu, and V. Majtan. 2007. In vitro effect of subinhibitory concentrations of antibiotics on biofilm formation by clinical strains of *Salmonella enterica* serovar Typhimurium isolated in Slovakia. Journal of Applied Microbiology **104**:1294–1301.

Marquez, M. 2007. Bacterial efflux systems and efflux pumps inhibitors.Biochimie **87**:1137–1147.

McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. Stewart, and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones. Microbiology **143:**3703-3711**.**

Medina-Martinez, M.S., M. Uyttendaele, and V. Demolder. 2006. Influence of food system conditions on N-acyl homoserine lactones production by *Aeromonas* spp. International Journal of Food Microbiology **112**:244–252.

Molin S., and T. Tolker-Nielsen. 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Current Opinion in Biotechnology **14**:255-261.

Morgan-Sagastume, F., N. Boon, S. Dobbelaere, and T. Defoirdt. 2005. Production of acylated homoserine lactones by *Aeromonas* and *Pseudomonas* strains isolated from municipal activated sludge. Canadian Journal of Microbiology **11**:924-933.

Morohoshi, T., M. Kato, K. Fukamachi, N. Kato, and T. Ikeda. 2008. *N*-Acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472. FEMS Microbiology Letters **279**:124-130.

Nadell C.D., J.B. Xavier, S.A. Levin, and K.R. Foster. 2008. The evolution of quorum sensing in bacterial biofilms. Plos Biology **6**:171-179.

Newaj-Fyzul, A., A. Mutani, A. Ramsubhag and A. Adesiyun. 2008. Prevalence of bacterial pathogens and their anti-microbial resistance in tilapia and their pond water in Trinidad. Zoonoses and Public Health **55**:206–213

Nishikawa, Y., A. Hase, J. Ogawasara, S.M. Scotland, H.R. Smith, and T.Kimura. 1994. Adhesion to and invasion of human colon-carcinoma caco-2 cells by *Aeromonas* strains. Journal of Medical Microbiology **40**:55-61.

Niu, C., and E.S. Gilbert. 2004. Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied and Environmental Microbiology **70**:6951–6956.

Pagès, J.-M., and L. Amaral. 2009. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. Biochimica et Biophysica Acta **1794**:826-833.

Pan, J., and D. Ren. 2009. Quorum sensing inhibitors: a patent overview. Expert Opinion on Therapeutic Patents **19**:1581-1601.

Pankey, G. A., and L.D. Sabath. 2004. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clinical Infectious Disease**38**:864–70.

Pannek, S., P.G. Higgins, P.Steinke, G. Jonas, M.Akova, J.A.Bohnert, H.Seifert, and W.V.Kern. 2006. Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-ß-naphthylamide. Journal of Antimicrobial Chemotherapy **57**:970–974.

Parker, J.L., and J.G. Shaw. 2011. *Aeromonas* spp. clinical microbiology and disease. Journal of Infection **62**:109-118.

Peeters, E., H.J. Nelis and T. Coenye. 2009. *In vitro* activity of ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and trimethoprim/sulfamethoxazole against planktonic and sessile *Burkholderia cepacia* complex bacteria. Journal of Antimicrobial Chemotherapy **64**: 801–809.

Perez-Giraldo, C., G. Cruz-Villaloin, R. Sanchez-Silos, R. Martinez-Rubio, M.T. Blanco, and A.C Gomez-Garcia. 2003. *In vitro* activity of allicin against *Staphylococcus epidermidis* and influence of sub-inhibitory concentrations on biofilm formation. Journal of Applied Microbiology **95**:709-711.

Pérez-Valdespino, A., E. Fernández-Rendón, and E. Curiel-Quesada. 2009. Detection and characterization of class 1 integronsin *Aeromonas* spp. isolated from human diarrheic stool in Mexico. Journal of Basic Microbiology **49**:572–578.

Pettit, R.K., C.A. Weber, M.J. Kean, H Hoffmann, G.R. Pettit, R. Tan, K.S. Franks, and **M.L**. Horton. 2005. Microplate alamar blue assay for *Staphylococcus epidermidis*biofilm susceptibility testing.Journal of Antimicrobial Agents and Chemotherapy **49**:2612–2617.

Picao, R.C., L. Poirel, A. Demarta, C.S.F. Silva, A.R. Corvaglia, O. Petrini, and P. Nordmann. 2008. Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. Journal of Antimicrobial Chemotherapy **62**:948-950.

Piddock, L.J.V. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews **19**:382-402.

Pitts, B., M.A. Hamilton, N. Zelver, and **P.S. Stewart.** 2003. A microtiter-plate screening method for biofilm disinfection and removal. Journal of Microbiological Methods **54**:269-276.

Pompilio, A., C. Catavitello, C. Picciani, P. Confalone, R. Piccolomini, V. Savini, E. Fiscarelli, D. D'Antonio, and G. Di Bonaventura. 2010. Sub-inhibitory concentrations of moxifloxacin decrease adhesion and biofilm formation of *Stenotrophomonas maltophila* from cystic fibrosis. Journal of Medical Microbiology **59**:76-81.

Ponnusamy, K., D. Paul, and **J.H. Kweon**. 2009. Inhibition of quorum sensing mechanism and *Aeromonas hydrophila* biofilm formation by vanillin. Environmental Engineering Science **26**:1359-1363.

Ponnusamy, K., D. Paul, Y. Sam Kim, and **J.H. Kweon.** 2010. 2(5H)-furanone: a prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition. Brazilian Journal of Microbiology **41**:227-234.

Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. Journal of Molecular Microbiology and Biotechnology **3**: 255-264.

Poole, K., and O. Lomovskay. 2006. Can efflux inhibitors really counter resistance? Drug Discovery Today: Therapeutic Strategies **3**:145-152.

Presterl, E, S. Hajdu, A.M. Lassnigg, A.M. Hirsch, J. Holinka, and W. Graninger. 2009. Effects of azithromycin in combination with vancomycin,daptomycin, fosfomycin, tigecycline, and ceftriaxone on *Staphylococcus epidermidis* biofilms. Antimicrobial Agents and Chemotherapy **53**:3205–3210

Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin and D. Qu. 2007**.** Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis.* Microbiology **153**:2083–2092.

Raad I., H. Hanna, Y. Jiang, T. Dvorak, R. Reitzel, G. Chaiban, R. Sherertz, and R. Hachem. 2007. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus*bacteremic isolates embedded in biofilm. Antimicrobial Agents and Chemotherapy **51**:1656-1660.

Raina, S., D. De Vizio, M. Odell, M. Clements, S. Vanhulle, and T. Keshavarz. 2009. Microbial quorum sensing: a tool or a target for antimicrobial therapy? Biotechnology and Applied Biochemistry **54:**65-84.

Ramalivhana, J.N., C.L. Obi, and S.R. Moyo. 2009. Antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa using VITEK 2 system, micro scan walk away, disk diffusion and E-test method. African Journal of Microbiology Research **3**:903-913.

Ramón-García, S., C. Martín, J. a Aínsa, and E. De Rossi. 2006. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. The Journal of Antimicrobial Chemotherapy **57**:252-259.

Rasmussen, T.B., and M. Givskov.2006. Quorum sensing inhibitors: a bargain of effects. Microbiology **152**:895-904.

Rasmussen, T. B., M. Manefield, J. B. Andersen, L. Eberl, U. Anthoni, C. Christophersen, P. Steinberg, S. Kjelleberg, and M. Givskov. 2000. How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratialiquefaciens* MG1. Microbiology **146**:3237-3244.

Reiter, K. C., B. Villa, T. da Silva Paim, C. F. de Oliveira, and P. A. d'Azevedo. 2013. Inhibition of biofilm maturation by linezolid inmeticillin-resistant *Staphylococcus epidermidis*clinical isolates: comparison with other drugs.Journal of Medical Microbiology **62**:394–399.

Rice, S. A., and K.S. Koh. 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. Journal of Bacteriology **187**:3477-3485.

Ribeiro, N. F. F., C. H. Heath, J. Kierath, S. Rea. M. Duncan-Smith, and F. M. Wood. 2010. Burn wounds infected by contaminated water: Case reports, review of the literature and recommendations for treatment. BURNS **36**:9-22

Ryan, R. P., and J. M. Dow.2008.Diffusible signals and interspecies communication in bacteria. Microbiology **154**:1845-1858.

Santos, P.G., P.A. Santos, A.R. Bello, and A.C. Freitas-Almeida. 2010. Association of *Aeromonas caviae* polar and lateral flagella with biofilm formation. Letters in Applied Microbiology **52**:49–55.

Sandoe, J.A.T., J. Wysome, A.P. West, J. Heritage, and M.H. Wilcox. 2006. Measurement of ampicillin, vancomycin, linezolid and gentamicin activity against enterococcal biofilms. Journal of Antimicrobial Chemotherapy **44**:1818–1824

Senderovich, Y., Y. Gershtein, E. Halewa, and M. Halpern. 2008. *Vibrio cholerae* and *Aeromonas*: do they share a mutual host? ISME Journal **2**:276-283.

Shaw, P.D., G. Ping, S.L. Daly, C. Cha, J.E. Cronan, K.L. Rinehart, and S.K. Farrand. 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin layer chromatography. BiochemicalJournal **94**:6036–6041.

Shi, W., J. Jian-ping, X. Ning, H. Zhi-mi, and W. Yu-yue. 2005. Inhibitory effects of reserpine and carbonyl cyanide m-chloro-phenylhydrazone on fluoroquinolone resistance of *Acinetobacter baumannii*. Chinese Medical Journal **4**:340-343.

Simoes, M., L.C.Simoes, and M.J.**Vieira**. 2010. A review of current and emergent biofilm control strategies. Lwt-Food Science and Technology **43**:573-583.

Starner, T.D., J.D. Shrout, M. R. Parsek, P.C. Appelbaum, and G. Kim. 2008. Subinhibitory concentrations of azithromycin decrease nontypeable *Haemophilus influenzae*biofilm formation and diminish established biofilms. Antimicrobial Agents and Chemotherapy **52**:137–145.

Steindler, L., and V. Venturi.2007**.**Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. FEMS Microbiology Letters **266**:1-9.

Swift, S., A.V. Karlyshev, L. Fish, E.L. Durant, M.K. Winson, S.R. Chhabra, P. Williams, S. Macintyre, and G.S. Stewart. 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. Journal of Bacteriology **179**:5271-5281.

Taga, M. E. and B. L. Bassler. 2003.Chemical communication among bacteria. Proceedings of the National Academy of Sciences USA **100**:14549-14554.

Takahashi, N., K. Ishihara, T. Kato, and K.Okuda. 2007. Susceptibility of *Actinobacillus actinomycetemcomitans* to six antibiotics decreases as biofilm matures. Journal of Antimicrobial Chemotherapy **59**:59-65.

Tarighi, S., and P. Taheri. 2011. Different aspects of bacterial communications signals. Journal of Microbiology and Biotechnology **27**:1267-1280.

Tetz, G. V., N. K. Artemenko, and V. V. Tetz. 2009. Effect of DNase and antibiotics on biofilm characteristics. Antimicrobial Agents and Chemotherapy **53**:1204-1209.

Tetz, V. V., and G. V. Tetz. 2010. Effect of extracellular DNA destruction by DNase I on characteristics of forming biofilms. DNA and Cell Biology **29**:399-405.

Vaseeharan, B., P. Ramasamya, T. Muruganc and J.C. Chen. 2005.In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. International Journal of Antimicrobial Agents **26**:285–291.

Viana, E.S., M.E.M. Campos, A.R. Ponce, H.C. Mantovani, and M.C.D Vanetti. 2009. Biofilm formation and acyl homoserine lactone production in *Hafnia alvei* isolated from raw milk. Biological Research **42**:427-436.

Vital, M., D. Stucki, T. Egli, and F. Hammes. 2010. Evaluating the growth potential of pathogenic bacteria in Water.Applied and Environmental Microbiology **76**:6477–6484.

Wang, J.H., C. Chushan, X. Wang, P. Zhao, and S. Fan. 2010. Extraction, purification and identification of bacterial signal molecules based on N-acyl homoserine lactones. Microbial Biotechnology **4**:479-490.

Whitchurch, C.B., T. Tolker-Nielsen, P.C. Ragars, and J.S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. Science **295**:1487.

Wilhelms, M., S.Vilches, R. Molero, J. G. Shaw, J. M. Tomas, and S. Merino. 2009. Two redundant sodium-driven stator motor proteins are involved in *Aeromonas hydrophila* polar flagellum rotation. Journal of Bacteriolology **191**:2206–2217.

Wojnicz, D., and S. Jankowski. 2007. Effects of subinhibitoryconcentrations of amikacin and ciprofloxacin on the hydrophobicityand adherence to epithelial cells of uropathogenic*Escherichia coli*strains. International Journal of Antimicrobial Agents **29**:700–704.

Zhang, L., A.J. Hinz, J. Nadeau and T. Mah. 2011**.** *Pseudomonas aeruginosa* tssC1 links type VI secretion and biofilm-specific antibiotic resistance. Journal of Bacteriolology **193**:5510–5513.

Zhu, J., and S. C. Winans. 1998. Activity of the quorum-sensing regulator TraR of *Agrobacterium tumefaciens* is inhibited by a truncated, dominant defective TraR-like protein. MolecularMicrobiology **27**:289-297.

APPENDIX A: DATA FOR CHAPTER 2

Table 1A: MBICs absorbance readings

M18													**M55**											
MH	AZM	StDev	CAZ	StDev	CIP	StDev	GN	StDev	TET	StDev	Untreated	StDev	MH	AZM	StDev	CAZ	StDev	CIP	StDev	GN	StDev	TET	StDev	Untreated	
		0.021		0.021		0.021		0.021		0.021					0.014		0.014		0.014		0.014		0.014		
0.150	1.148	0.099	1.080	0.109	1.363	0.097	1.469	0.056	1.225	0.107	1.110	0.068	0.118	1.089	0.046	1.054	0.090	1.042	0.017	1.042	0.014	1.054	0.067	1.072	0.091
0.150	1.194	0.078	0.906	0.081	1.130	0.018	1.139	0.017	1.334	0.085	1.110	0.068	0.118	1.417	0.142	1.590	0.045	1.044	0.127	1.316	0.077	2.155	0.035	1.072	0.091
0.150	0.984	0.054	0.712	0.021	1.097	0.062	1.093	0.088	1.308	0.083	1.110	0.068	0.118	1.422	0.083	1.492	0.087	1.635	0.068	1.155	0.053	0.922	0.053	1.072	0.091
0.150	0.973	0.067	0.931	0.040	0.672	0.052	0.763	0.045	1.012	0.062	1.110	0.068	0.118	0.737	0.051	1.112	0.089	1.018	0.086	1.023	0.100	0.822	0.077	1.072	0.091
0.150	0.671	0.065	0.716	0.029	0.685	0.074	0.648	0.077	0.708	0.035	1.110	0.068	0.118	0.689	0.093	1.360	0.097	1.403	0.063	0.842	0.049	0.810	0.010	1.072	0.091
0.150	0.548	0.089	0.672	0.064	0.549	0.061	0.496	0.056	0.695	0.069	1.110	0.068	0.118	0.752	0.061	0.688	0.056	1.047	0.095	0.629	0.059	0.737	0.048	1.072	0.091
0.150	0.502	0.062	0.606	0.065	0.415	0.084	0.340	0.066	0.549	0.091	1.110	0.068	0.118	0.730	0.051	0.810	0.054	0.655	0.057	0.492	0.063	0.605	0.039	1.072	0.091
0.150	0.343	0.041	0.392	0.072	0.326	0.077	0.340	0.043	0.370	0.082	1.110	0.068	0.118	0.707	0.064	0.564	0.040	0.607	0.059	0.482	0.078	0.336	0.024	1.072	0.091
M67		StDev		StDev		StDev		StDev		StDev	Untreated		**M17**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.027	CAZ	0.027	CIP	0.027	GN	0.027	TET	0.027	1.023	0.095	MH	AZM	0.027	CAZ	0.027	CIP	0.027	GN	0.027	TET	0.027	Untreated	
0.116	1.035	0.020	0.872	0.080	0.766	0.053	0.732	0.055	0.729	0.021	1.023	0.095	0.148	1.697	0.085	0.917	0.067	1.252	0.076	2.245	0.010	1.555	0.012	1.741	0.122
0.116	0.689	0.016	0.763	0.087	0.451	0.041	0.640	0.042	1.047	0.019	1.023	0.095	0.148	1.311	0.065	1.091	0.096	0.950	0.030	1.213	0.096	1.428	0.050	1.741	0.122
0.116	0.750	0.076	0.761	0.070	0.620	0.048	0.629	0.043	0.757	0.066	1.023	0.095	0.148	1.328	0.092	0.744	0.049	1.480	0.081	1.127	0.060	1.590	0.053	1.741	0.122
0.116	0.709	0.055	0.724	0.033	0.792	0.061	0.646	0.048	1.047	0.052	1.023	0.095	0.148	1.017	0.081	0.914	0.090	0.977	0.052	1.119	0.073	0.814	0.044	1.741	0.122
0.116	1.616	0.050	0.708	0.044	0.684	0.067	0.773	0.072	0.808	0.054	1.023	0.095	0.148	1.563	0.019	0.958	0.074	1.035	0.090	0.993	0.084	1.578	0.022	1.741	0.122
0.116	0.942	0.057	0.650	0.053	0.734	0.044	0.939	0.069	0.900	0.061	1.023	0.095	0.148	1.522	0.028	0.990	0.040	0.963	0.086	0.938	0.029	0.757	0.040	1.741	0.122
0.116	0.689	0.038	0.680	0.064	0.750	0.028	0.846	0.084	0.713	0.084	1.023	0.095	0.148	1.034	0.028	0.747	0.062	1.016	0.049	0.935	0.028	0.649	0.074	1.741	0.122
0.116	1.114	0.085	0.667	0.015	0.485	0.075	0.765	0.026	1.654	0.026	1.023	0.095	0.148	1.004	0.061	0.638	0.021	0.808	0.045	0.660	0.035	0.439	0.037	1.741	0.122
M92													**M2**		StDev		StDev		StDev		StDev		StDev	
MH	AZM	StDev	CAZ	StDev	CIP	StDev	GN	StDev	TET	StDev	Untreated		MH	AZM	0.014	CAZ	0.014	CIP	0.014	GN	0.014	TET	0.014	Untreated	
0.155	1.081	0.032	1.691	0.032	0.793	0.032	1.065	0.032	1.551	0.032	1.085	0.077	0.126	1.289	0.068	1.173	0.062	1.200	0.102	2.072	0.091	1.056	0.083	1.449	0.058
0.155	1.054	0.104	1.281	0.045	2.448	0.039	0.845	0.089	1.056	0.033	1.085	0.077	0.126	1.585	0.079	1.488	0.057	1.041	0.102	1.504	0.073	1.896	0.031	1.449	0.058
0.155	1.161	0.034	1.686	0.064	1.286	0.062	1.171	0.035	1.080	0.076	1.085	0.077	0.126	2.043	0.071	1.252	0.127	1.174	0.096	1.652	0.061	1.217	0.108	1.449	0.058
0.155	2.391	0.046	0.992	0.056	0.976	0.046	0.772	0.025	1.819	0.026	1.085	0.077	0.126	1.115	0.059	1.144	0.056	1.086	0.087	1.144	0.030	0.948	0.048	1.449	0.058
0.155	0.730	0.019	1.084	0.017	1.205	0.009	0.697	0.045	0.644	0.032	1.085	0.077	0.126	1.263	0.174	1.078	0.090	1.263	0.180	0.909	0.078	0.815	0.038	1.449	0.058
0.155	0.487	0.026	0.669	0.020	0.964	0.052	1.005	0.025	0.745	0.073	1.085	0.077	0.126	0.794	0.048	0.886	0.078	0.979	0.062	0.557	0.077	0.741	0.033	1.449	0.058
0.155	0.566	0.038	0.622	0.063	0.834	0.026	0.994	0.022	0.521	0.042	1.085	0.077	0.126	0.630	0.110	0.700	0.054	0.607	0.045	0.476	0.044	0.620	0.020	1.449	0.058
0.155	0.756	0.063	0.520	0.045	0.633	0.053	0.359	0.062	0.466	0.034	1.085	0.077	0.126	0.501	0.059	0.520	0.028	0.476	0.089	0.599	0.080	0.548	0.052	1.449	0.058
M38		StDev		StDev		StDev		StDev		StDev			**M46**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.010	CAZ	0.010	CIP	0.010	GN	0.010	TET	0.010	Untreated		MH	AZM	0.009	CAZ	0.009	CIP	0.009	GN	0.009	TET	0.009	Untreated	
0.144	1.224	0.089	1.204	0.054	2.146	0.092	1.179	0.016	1.276	0.073	1.371	0.093	0.131	2.255	0.090	1.413	0.105	1.629	0.077	1.332	0.084	1.438	0.066	1.194	0.087
0.144	1.174	0.092	1.181	0.095	1.349	0.047	1.867	0.085	1.283	0.084	1.371	0.093	0.131	1.081	0.078	1.063	0.082	1.054	0.090	1.116	0.004	1.008	0.093	1.194	0.087
0.144	0.680	0.102	0.669	0.026	0.737	0.087	1.100	0.032	0.975	0.096	1.371	0.093	0.131	0.730	0.078	0.749	0.059	0.744	0.073	0.754	0.063	0.892	0.056	1.194	0.087
0.144	0.927	0.082	0.816	0.053	0.670	0.088	0.735	0.048	0.991	0.051	1.371	0.093	0.131	0.600	0.048	0.740	0.056	0.712	0.039	0.659	0.027	0.605	0.078	1.194	0.087
0.144	0.937	0.013	0.442	0.048	0.501	0.044	0.634	0.041	0.649	0.087	1.371	0.093	0.131	0.596	0.074	0.661	0.153	0.651	0.078	0.606	0.088	0.549	0.065	1.194	0.087
0.144	0.410	0.064	0.492	0.043	0.338	0.026	0.624	0.064	0.566	0.025	1.371	0.093	0.131	0.543	0.049	0.544	0.083	0.415	0.036	0.548	0.073	0.427	0.083	1.194	0.087
0.144	0.407	0.047	0.427	0.083	0.537	0.061	0.435	0.037	0.558	0.096	1.371	0.093	0.131	0.389	0.087	0.395	0.058	0.343	0.067	0.540	0.067	0.355	0.029	1.194	0.087
0.144	0.366	0.041	0.352	0.025	0.369	0.032	0.363	0.036	0.452	0.022	1.371	0.093	0.131	0.413	0.039	0.377	0.019	0.294	0.082	0.443	0.082	0.283	0.042	1.194	0.087
M90		StDev		StDev		StDev		StDev		StDev			**M41**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.014	CAZ	0.014	CIP	0.014	GN	0.014	TET	0.014	Untreated		MH	AZM	0.037	CAZ	0.037	CIP	0.037	GN	0.037	TET	0.037	Untreated	
0.142	0.434	0.042	0.723	0.007	0.760	0.056	0.515	0.062	1.065	0.040	1.104	0.051	0.195	1.214	0.053	1.630	0.336	1.234	0.125	1.390	0.198	1.589	0.109	1.484	0.101
0.142	1.110	0.059	1.139	0.062	0.738	0.052	0.664	0.050	1.229	0.016	1.104	0.051	0.195	1.014	0.082	0.961	0.255	0.971	0.048	0.976	0.063	1.078	0.110	1.484	0.101
0.142	0.528	0.061	0.993	0.059	0.337	0.051	0.486	0.028	0.351	0.037	1.104	0.051	0.195	0.691	0.143	0.876	0.203	1.114	0.096	0.921	0.077	1.099	0.059	1.484	0.101
0.142	0.404	0.053	1.148	0.036	0.494	0.013	0.323	0.025	0.734	0.033	1.104	0.051	0.195	0.544	0.063	0.676	0.180	0.652	0.127	0.970	0.054	1.115	0.008	1.484	0.101
0.142	0.524	0.092	0.266	0.008	0.393	0.020	0.369	0.044	0.562	0.040	1.104	0.051	0.195	0.539	0.038	0.676	0.219	0.545	0.104	0.860	0.044	0.676	0.094	1.484	0.101
0.142	0.456	0.071	0.607	0.048	0.393	0.046	0.680	0.049	0.645	0.081	1.104	0.051	0.195	0.477	0.102	0.576	0.242	0.704	0.032	0.621	0.082	0.491	0.055	1.484	0.101
0.142	0.696	0.034	1.017	0.082	0.521	0.039	0.670	0.052	0.772	0.048	1.104	0.051	0.195	0.620	0.059	0.563	0.126	0.613	0.122	0.653	0.062	0.575	0.090	1.484	0.101
0.142	0.426	0.051	0.486	0.025	0.534	0.037	0.649	0.062	0.772	0.082	1.104	0.051	0.195	0.331	0.081	0.460	0.076	0.630	0.068	0.346	0.079	0.361	0.057	1.484	0.101

M63		StDev		StDev		StDev		StDev		StDev			**M76**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.023	CAZ	0.023	CIP	0.023	GN	0.023	TET	0.023	Untreated		MH	AZM	0.010	CAZ	0.010	CIP	0.010	GN	0.088504	TET	0.010	Untreated	
0.119	1.546	0.035	1.546	0.012	1.607	0.059	1.607	0.080	1.546	0.052	1.577	0.091	0.145	1.210	0.127	1.210	0.139	1.416	0.036	1.5454	0.058312	1.210	0.093	1.313	0.076
0.119	0.890	0.040	2.163	0.116	1.079	0.072	2.037	0.112	0.830	0.045	1.577	0.091	0.145	1.022	0.091	0.981	0.069	1.060	0.103	1.673667	0.055506	1.080	0.098	1.313	0.076
0.119	0.705	0.035	0.972	0.015	0.979	0.048	1.136	0.033	0.756	0.044	1.577	0.091	0.145	1.045	0.064	0.998	0.029	0.700	0.059	1.14875	0.084795	0.668	0.073	1.313	0.076
0.119	0.732	0.029	0.749	0.026	0.868	0.054	0.886	0.026	0.843	0.038	1.577	0.091	0.145	0.982	0.102	0.649	0.087	0.656	0.082	1.06625	0.046548	0.677	0.073	1.313	0.076
0.119	0.745	0.029	0.800	0.046	0.925	0.071	0.776	0.037	0.748	0.051	1.577	0.091	0.145	0.642	0.092	0.447	0.024	0.465	0.054	0.7212	0.064821	0.731	0.082	1.313	0.076
0.119	0.706	0.054	0.937	0.049	0.560	0.050	1.009	0.100	0.875	0.057	1.577	0.091	0.145	0.634	0.067	0.710	0.106	0.415	0.065	0.6668	0.083098	0.563	0.123	1.313	0.076
0.119	0.648	0.049	0.667	0.049	0.563	0.078	0.815	0.048	0.764	0.022	1.577	0.091	0.145	0.606	0.053	0.552	0.126	0.381	0.056	0.5308	0.065937	0.471	0.111	1.313	0.076
0.119	0.481	0.065	0.644	0.039	0.429	0.054	0.285	0.066	0.433	0.028	1.577	0.091	0.145	0.252	0.054	0.359	0.076	0.312	0.001	0.3475	0.094675	0.320	0.021	1.313	0.076
M64		StDev		StDev		StDev		StDev		StDev			**M94**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.014	CAZ	0.014	CIP	0.014	GN	0.014	TET	0.014	Untreated		MH	AZM	0.024	CAZ	0.024	CIP	0.024	GN	0.024	TET	0.024	Untreated	
0.124	1.519	0.106	1.519	0.053	1.668	0.052	1.668	0.051	1.519	0.100	1.594	0.076	0.147	1.088	0.064	1.038	0.086	0.746	0.116	1.143	0.100	0.998	0.069	1.529	0.084
0.124	1.308	0.035	0.994	0.096	2.027	0.034	1.036	0.079	1.965	0.095	1.594	0.076	0.147	1.116	0.075	0.697	0.042	1.033	0.101	1.055	0.084	0.963	0.021	1.529	0.084
0.124	0.979	0.044	1.581	0.099	1.136	0.080	2.033	0.117	0.665	0.068	1.594	0.076	0.147	0.685	0.118	0.603	0.050	0.881	0.085	0.782	0.103	0.814	0.096	1.529	0.084
0.124	1.093	0.061	1.096	0.056	1.310	0.070	1.318	0.071	0.666	0.046	1.594	0.076	0.147	0.622	0.075	0.539	0.120	0.709	0.074	0.777	0.089	0.624	0.071	1.529	0.084
0.124	0.983	0.055	1.132	0.096	1.134	0.067	1.340	0.122	0.824	0.042	1.594	0.076	0.147	0.865	0.048	0.665	0.056	0.749	0.026	0.721	0.039	0.824	0.018	1.529	0.084
0.124	0.707	0.067	1.057	0.087	0.822	0.047	0.919	0.065	0.645	0.032	1.594	0.076	0.147	0.602	0.077	0.650	0.042	0.610	0.051	0.716	0.040	0.595	0.071	1.529	0.084
0.124	0.662	0.054	0.681	0.067	0.593	0.037	0.889	0.092	0.516	0.020	1.594	0.076	0.147	0.570	0.037	0.610	0.037	0.586	0.049	0.503	0.021	0.591	0.058	1.529	0.084
0.124	0.447	0.057	0.570	0.069	0.501	0.060	0.478	0.084	0.377	0.023	1.594	0.076	0.147	0.302	0.050	0.315	0.057	0.311	0.064	0.468	0.031	0.371	0.058	1.529	0.084
M57		StDev		StDev		StDev		StDev		StDev			**M59**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.022	CAZ	0.022	CIP	0.022	GN	0.022	TET	0.022	Untreated		MH	AZM	0.019	CAZ	0.019	CIP	0.019	GN	0.019	TET	0.019	Untreated	
0.141	1.546	0.081	1.130	0.034	1.106	0.062	1.077	0.065	1.674	0.052	1.406	0.050	0.127	1.458	0.100	1.117	0.129	1.459	0.096	1.120	0.075	1.712	0.165	1.475	0.068
0.141	1.033	0.107	0.838	0.031	0.944	0.048	1.120	0.092	1.500	0.118	1.406	0.050	0.127	1.162	0.061	0.991	0.061	0.846	0.111	1.037	0.069	1.321	0.112	1.475	0.068
0.141	0.784	0.065	0.674	0.034	0.776	0.061	0.656	0.033	0.846	0.034	1.406	0.050	0.127	1.017	0.101	0.694	0.041	0.964	0.088	0.911	0.094	1.187	0.115	1.475	0.068
0.141	0.805	0.061	0.742	0.018	1.066	0.080	0.965	0.033	0.755	0.043	1.406	0.050	0.127	0.624	0.083	0.607	0.041	0.957	0.065	0.973	0.085	1.053	0.076	1.475	0.068
0.141	0.753	0.017	0.762	0.019	0.706	0.053	0.707	0.026	0.769	0.061	1.406	0.050	0.127	0.619	0.049	0.677	0.039	0.657	0.053	0.878	0.082	0.698	0.066	1.475	0.068
0.141	0.857	0.079	0.718	0.068	0.767	0.042	0.669	0.055	0.664	0.061	1.406	0.050	0.127	0.557	0.104	0.615	0.090	0.508	0.135	0.638	0.074	0.656	0.080	1.475	0.068
0.141	0.752	0.073	0.652	0.048	0.658	0.033	0.638	0.048	0.666	0.048	1.406	0.050	0.127	0.490	0.047	0.461	0.114	0.540	0.024	0.423	0.076	0.577	0.079	1.475	0.068
0.141	0.476	0.026	0.442	0.052	0.465	0.071	0.541	0.086	0.586	0.037	1.406	0.050	0.127	0.345	0.029	0.342	0.074	0.371	0.030	0.424	0.038	0.428	0.080	1.475	0.068
M95		StDev		StDev		StDev		StDev		StDev			**M9**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.013	CAZ	0.013	CIP	0.013	GN	0.013	TET	0.013	Untreated		MH	AZM	0.021	CAZ	0.021	CIP	0.021	GN	0.021	TET	0.021	Untreated	
0.148	2.309	0.055	2.498	0.048	1.516	0.051	1.495	0.059	2.117	0.043	1.703	0.081	0.155	3.327	0.136	2.442	0.040	1.387	0.094	1.014	0.036	1.377	0.050	1.283	0.085
0.148	1.920	0.066	1.062	0.141	1.903	0.027	1.415	0.192	2.032	0.060	1.703	0.081	0.155	1.543	0.152	1.111	0.096	0.984	0.050	0.908	0.029	1.144	0.152	1.283	0.085
0.148	1.540	0.063	1.334	0.043	1.147	0.072	1.463	0.075	2.089	0.067	1.703	0.081	0.155	1.007	0.037	1.051	0.063	0.663	0.062	0.486	0.057	0.814	0.054	1.283	0.085
0.148	0.990	0.072	0.789	0.085	1.072	0.120	0.883	0.057	1.444	0.072	1.703	0.081	0.155	0.675	0.030	0.993	0.039	0.613	0.077	0.527	0.083	0.677	0.113	1.283	0.085
0.148	0.795	0.057	0.954	0.069	0.756	0.016	0.748	0.056	1.094	0.027	1.703	0.081	0.155	0.378	0.075	0.687	0.066	0.724	0.125	0.471	0.055	0.640	0.026	1.283	0.085
0.148	0.699	0.057	0.767	0.029	0.701	0.024	0.659	0.049	0.980	0.067	1.703	0.081	0.155	0.427	0.146	0.667	0.027	0.504	0.077	0.433	0.087	0.371	0.049	1.283	0.085
0.148	0.631	0.042	0.699	0.049	0.704	0.029	0.626	0.030	0.756	0.055	1.703	0.081	0.155	0.416	0.042	0.591	0.059	0.447	0.100	0.320	0.071	0.351	0.017	1.283	0.085
0.148	0.515	0.065	0.460	0.074	0.476	0.047	0.470	0.040	0.732	0.042	1.703	0.081	0.155	0.293	0.075	0.294	0.018	0.346	0.082	0.273	0.066	0.334	0.059	1.283	0.085
M70		StDev		StDev		StDev		StDev		StDev			**M88**		StDev		StDev		StDev		StDev		StDev		
MH	AZM	0.018	CAZ	0.018	CIP	0.018	GN	0.018	TET	0.018	Untreated		MH	AZM	0.015	CAZ	0.015	CIP	0.015	GN	0.015	TET	0.015	Untreated	
0.141	1.641	0.092	1.875	0.081	1.245	0.078	1.197	0.111	1.081	0.048	1.489	0.088	0.140	1.528	0.084	1.949	0.057	1.617	0.024	0.992	0.107	1.354	0.040	1.415	0.112
0.141	1.144	0.116	1.513	0.119	1.151	0.081	1.102	0.081	1.087	0.090	1.489	0.088	0.140	1.070	0.077	1.087	0.076	1.534	0.108	1.369	0.038	1.095	0.085	1.415	0.112
0.141	0.918	0.099	1.051	0.068	1.025	0.050	1.117	0.145	0.884	0.028	1.489	0.088	0.140	1.018	0.094	0.924	0.080	1.084	0.071	1.001	0.153	0.971	0.089	1.415	0.112
0.141	0.985	0.091	1.072	0.103	1.027	0.163	0.937	0.093	0.864	0.104	1.489	0.088	0.140	0.727	0.092	0.924	0.092	1.058	0.064	0.927	0.087	0.869	0.059	1.415	0.112
0.141	0.917	0.079	0.691	0.064	1.022	0.132	0.833	0.100	0.924	0.044	1.489	0.088	0.140	0.963	0.067	0.734	0.036	1.053	0.158	0.688	0.099	0.974	0.084	1.415	0.112
0.141	0.646	0.069	0.635	0.094	0.689	0.075	0.805	0.087	0.692	0.042	1.489	0.088	0.140	0.759	0.088	0.664	0.083	0.746	0.063	0.723	0.097	0.673	0.064	1.415	0.112
0.141	0.646	0.091	0.457	0.077	0.541	0.093	0.562	0.075	0.525	0.094	1.489	0.088	0.140	0.628	0.075	0.609	0.038	0.657	0.047	0.661	0.039	0.655	0.096	1.415	0.112
0.141	0.451	0.095	0.407	0.055	0.357	0.092	0.319	0.102	0.299	0.059	1.489	0.088	0.140	0.485	0.074	0.520	0.090	0.534	0.022	0.347	0.071	0.449	0.069	1.415	0.112

M51		StDev		StDev		StDev		StDev		StDev			**M58**		StDev		StDev		StDev		StDev			
MH	AZM	0.018	CAZ	0.018	CIP	0.018	GN	0.018	TET	0.018	Untreated		MH	AZM	0.028	CAZ	0.028	CIP	0.028	GN	0.028	TET	0.028	1.631	0.111
0.157	1.764	0.097	1.437	0.077	1.066	0.058	1.439	0.175	1.439	0.116	1.344	0.070	0.133	1.774	0.096	1.774	0.075	1.488	0.057	1.488	0.046	1.774	0.008	1.631	0.111
0.157	1.144	0.058	1.186	0.038	1.151	0.071	1.567	0.087	1.342	0.107	1.344	0.070	0.133	0.759	0.120	0.976	0.126	1.377	0.046	1.157	0.049	0.937	0.091	1.631	0.111
0.157	0.917	0.098	0.967	0.097	1.056	0.071	0.943	0.059	1.097	0.067	1.344	0.070	0.133	0.745	0.076	0.902	0.128	0.955	0.082	1.074	0.059	0.934	0.058	1.631	0.111
0.157	0.939	0.061	0.956	0.276	0.790	0.070	0.644	0.084	0.826	0.056	1.344	0.070	0.133	0.732	0.091	0.968	0.113	1.054	0.124	1.081	0.109	0.705	0.054	1.631	0.111
0.157	0.685	0.086	0.942	0.067	0.830	0.056	0.597	0.083	0.657	0.076	1.344	0.070	0.133	0.669	0.087	0.987	0.090	0.448	0.032	0.442	0.097	0.554	0.098	1.631	0.111
0.157	0.663	0.021	0.455	0.035	0.800	0.055	0.593	0.111	0.625	0.050	1.344	0.070	0.133	0.432	0.103	0.798	0.088	0.492	0.077	0.318	0.004	0.562	0.041	1.631	0.111
0.157	0.595	0.084	0.377	0.088	0.547	0.073	0.497	0.077	0.332	0.111	1.344	0.070	0.133	0.377	0.069	0.375	0.081	0.449	0.091	0.325	0.015	0.441	0.078	1.631	0.111
0.157	0.394	0.062	0.263	0.085	0.321	0.009	0.401	0.125	0.268	0.049	1.344	0.070	0.133	0.304	0.046	0.263	0.036	0.381	0.057	0.319	0.047	0.326	0.047	1.631	0.111
M23		StDev		StDev		StDev		StDev		StDev			**M28**		StDev		StDev		StDev		StDev			
MH	AZM	0.021	CAZ	0.021	CIP	0.021	GN	0.021	TET	0.021	Untreated	StDev	MH	AZM	0.026	CAZ	0.026	CIP	0.026	GN	0.026	TET	0.026	Untreated	
0.153	1.708	0.097	1.708	0.113	1.522	0.070	1.522	0.093	1.708	0.067	1.615	0.079	0.169	1.306	0.089	1.612	0.045	2.562	0.068	1.264	0.069	1.947	0.068	1.529	0.102
0.153	0.584	0.080	0.666	0.103	0.865	0.046	1.073	0.058	1.580	0.069	1.615	0.079	0.169	1.201	0.076	1.143	0.061	1.594	0.036	1.222	0.093	1.605	0.069	1.529	0.102
0.153	0.630	0.081	0.620	0.079	0.654	0.086	0.975	0.016	1.049	0.065	1.615	0.079	0.169	0.916	0.065	1.045	0.082	1.121	0.062	1.080	0.079	1.287	0.059	1.529	0.102
0.153	0.510	0.056	0.660	0.055	0.428	0.039	0.919	0.047	0.734	0.053	1.615	0.079	0.169	0.801	0.078	0.782	0.049	0.924	0.034	0.937	0.032	1.052	0.087	1.529	0.102
0.153	0.450	0.080	0.680	0.072	0.390	0.095	0.462	0.037	0.704	0.108	1.615	0.079	0.169	0.751	0.083	0.731	0.063	0.716	0.031	0.774	0.085	0.980	0.032	1.529	0.102
0.153	0.408	0.054	0.776	0.071	0.382	0.099	0.548	0.089	0.641	0.071	1.615	0.079	0.169	0.626	0.075	0.673	0.103	0.554	0.021	0.689	0.084	0.787	0.094	1.529	0.102
0.153	0.370	0.043	0.614	0.083	0.365	0.074	0.468	0.079	0.352	0.116	1.615	0.079	0.169	0.579	0.058	0.619	0.076	0.621	0.037	0.541	0.088	0.670	0.092	1.529	0.102
0.153	0.369	0.065	0.596	0.112	0.320	0.017	0.455	0.076	0.227	0.016	1.615	0.079	0.169	0.333	0.033	0.436	0.078	0.552	0.042	0.481	0.051	0.624	0.088	1.529	0.102
M96		StDev		StDev		StDev		StDev		StDev			**M31**		StDev		StDev		StDev		StDev			
MH	AZM	0.021	CAZ	0.021	CIP	0.021	GN	0.021	TET	0.021	Untreated		MH	AZM	0.014	CAZ	0.014	CIP	0.014	GN	0.014	TET	0.014	Untreated	
0.154	1.576	0.057	1.000	0.068	1.720	0.108	1.514	0.112	1.421	0.049	1.442	0.074	0.147	1.889	0.074	1.321	0.082	1.533	0.085	1.447	0.105	1.637	0.065	1.307	0.094
0.154	1.109	0.107	0.880	0.102	1.128	0.079	1.139	0.066	1.345	0.102	1.442	0.074	0.147	1.250	0.050	1.111	0.094	1.177	0.070	1.348	0.054	1.131	0.046	1.307	0.094
0.154	0.969	0.032	0.932	0.103	0.883	0.096	0.794	0.093	1.004	0.093	1.442	0.074	0.147	1.067	0.071	0.934	0.077	0.670	0.080	1.182	0.053	1.075	0.092	1.307	0.094
0.154	0.705	0.046	0.722	0.027	0.741	0.094	0.640	0.052	0.643	0.076	1.442	0.074	0.147	0.865	0.089	0.768	0.069	0.928	0.096	1.032	0.047	0.828	0.083	1.307	0.094
0.154	0.647	0.065	0.805	0.066	0.619	0.102	0.943	0.009	0.553	0.108	1.442	0.074	0.147	0.630	0.085	0.552	0.047	0.794	0.073	0.754	0.056	0.702	0.080	1.307	0.094
0.154	0.506	0.080	0.550	0.094	0.486	0.032	0.585	0.117	0.544	0.049	1.442	0.074	0.147	0.616	0.030	0.463	0.068	0.704	0.076	0.736	0.076	0.567	0.074	1.307	0.094
0.154	0.627	0.089	0.519	0.102	0.373	0.035	0.447	0.103	0.480	0.105	1.442	0.074	0.147	0.598	0.076	0.368	0.072	0.500	0.076	0.639	0.077	0.601	0.077	1.307	0.094
0.154	0.457	0.066	0.357	0.057	0.361	0.083	0.368	0.119	0.331	0.053	1.442	0.074	0.147	0.188	0.030	0.297	0.096	0.364	0.061	0.539	0.087	0.536	0.064	1.307	0.094
M77		StDev		StDev		StDev		StDev		StDev			**M49**		StDev		StDev		StDev		StDev			
MH	AZM	0.042	CAZ	0.042	CIP	0.042	GN	0.042	TET	0.042	Untreated		MH	AZM	0.027	CAZ	0.027	CIP	0.027	GN	0.027	TET	0.027	Untreated	
0.168	1.229	0.006	1.210	0.105	1.053	0.050	1.318	0.071	2.019	0.100	1.602	0.104	0.141	1.987	0.058	1.514	0.101	1.616	0.143	1.907	0.051	1.277	0.021	1.376	0.096
0.168	1.082	0.023	1.159	0.033	0.659	0.109	0.990	0.075	1.098	0.049	1.602	0.104	0.141	1.112	0.092	1.117	0.084	1.155	0.074	0.940	0.090	1.387	0.063	1.376	0.096
0.168	0.863	0.083	0.911	0.042	0.764	0.092	1.186	0.098	1.120	0.107	1.602	0.104	0.141	1.030	0.095	0.923	0.085	1.078	0.095	0.630	0.057	1.052	0.104	1.376	0.096
0.168	0.946	0.088	0.966	0.113	0.738	0.105	0.733	0.072	0.620	0.115	1.602	0.104	0.141	0.943	0.111	0.986	0.065	0.904	0.083	0.662	0.053	0.704	0.052	1.376	0.096
0.168	0.855	0.090	0.581	0.026	0.582	0.069	0.687	0.049	0.721	0.087	1.602	0.104	0.141	0.733	0.062	0.917	0.082	0.718	0.041	0.673	0.037	0.740	0.065	1.376	0.096
0.168	0.722	0.075	0.704	0.067	0.630	0.082	0.688	0.105	0.779	0.053	1.602	0.104	0.141	0.544	0.098	0.675	0.064	0.680	0.048	0.613	0.091	0.674	0.032	1.376	0.096
0.168	0.703	0.123	0.647	0.063	0.611	0.081	0.643	0.098	0.708	0.054	1.602	0.104	0.141	0.429	0.076	0.545	0.079	0.574	0.034	0.518	0.090	0.557	0.067	1.376	0.096
0.168	0.349	0.087	0.320	0.050	0.416	0.077	0.493	0.125	0.536	0.073	1.602	0.104	0.141	0.394	0.081	0.420	0.078	0.285	0.053	0.490	0.080	0.435	0.075	1.376	0.096

*****AZM = azithromycin, *CAZ = ceftazidime, *CIP = ciprofloxacin, *GN = gentamicin, *TET = tetracycline

Table 2A: MICs absorbance readings after 24 h

Azithromycin									Ceftazidime								Ciprofloxacin								
	Average Untreated	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreate StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreate StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MI StDev
MH	0.154	0.006	0.154	0.006	0.154	0.006	0.154	0.006	MH	0.154	0.006	0.154	0.006	0.154	0.006	0.154	0.006	MH	0.154	0.006	0.154	0.006	0.154	0.006	0.154	0.006
M2	1.174	0.062	0.730	0.074	0.759	0.071	0.494	0.022	M2	1.174	0.062	0.644	0.065	1.079	0.099	0.743	0.056	M2	1.174	0.062	1.703	0.075	0.805	0.080	0.186	0.072
M17	0.984	0.122	0.351	0.030	0.323	0.053	0.150	0.025	M17	0.984	0.122	0.185	0.032	1.181	0.065	0.676	0.067	M17	0.984	0.122	0.212	0.022	0.115	0.011	0.142	0.019
M51	1.457	0.162	0.554	0.015	0.318	0.055	0.675	0.052	M51	1.457	0.162	0.429	0.073	1.153	0.246	0.506	0.087	M51	1.457	0.162	0.275	0.042	0.181	0.019	0.140	0.022
M64	0.987	0.112	0.184	0.038	0.162	0.039	0.160	0.015	M64	0.987	0.112	0.600	0.062	1.268	0.098	0.442	0.063	M64	0.987	0.112	0.180	0.027	0.179	0.026	0.177	0.020
M94	0.763	0.017	0.844	0.074	0.719	0.100	1.073	0.087	M94	0.763	0.017	0.180	0.058	0.191	0.076	0.181	0.074	M94	0.763	0.017	0.607	0.053	0.158	0.069	0.730	0.033
M95	1.077	0.080	1.064	0.087	0.862	0.090	0.871	0.074	M95	1.077	0.080	1.040	0.087	0.942	0.100	0.270	0.141	M95	1.077	0.080	1.163	0.110	0.129	0.005	0.167	0.015
ATCC 7966^T	0.982	0.083	0.843	0.095	0.770	0.045	0.632	0.072	ATCC 7966^T	1.060	0.083	0.640	0.074	0.746	0.091	0.083		ATCC 7966^T	0.982	0.083	1.158	0.068	1.150	0.045	0.962	0.095
M70	1.049	0.075	0.519	0.113	0.124	0.035	0.153	0.068	M70	1.049	0.075	0.122	0.019	0.135	0.040	0.140	0.018	M70	1.049	0.075	0.834	0.062	0.116	0.001	0.081	0.003
M88	0.619	0.129	0.152	0.009	0.109	0.006	0.105	0.002	M88	0.619	0.129	0.427	0.055	0.156	0.039	0.184	0.014	M88	0.619	0.129	1.241	0.072	0.531	0.043	0.586	0.039
M90	0.503	0.110	0.545	0.047	0.167	0.058	0.345	0.102	M90	0.503	0.110	0.250	0.089	0.261	0.092	0.301	0.099	M90	0.503	0.110	0.892	0.078	0.093	0.005	0.080	0.010
M96	0.639	0.094	0.560	0.102	0.383	0.073	0.341	0.106	M96	0.639	0.094	0.173	0.034	0.208	0.051	0.156	0.019	M96	0.639	0.094	1.002	0.047	0.172	0.091	0.151	0.040
M23	1.265	0.062	0.423	0.004	0.322	0.111	0.625	0.097	M23	1.265	0.062	0.449	0.088	1.205	0.132	0.711	0.087	M23	1.265	0.062	0.938	0.087	0.572	0.102	0.539	0.074
M31	0.761	0.079	0.637	0.052	0.749	0.052	0.539	0.061	M31	0.761	0.079	0.261	0.004	0.339	0.032	0.251	0.023	M31	0.761	0.079	0.791	0.069	0.631	0.069	0.380	0.048
M38	0.702	0.041	0.667	0.089	0.725	0.084	0.737	0.056	M38	0.702	0.041	0.890	0.074	0.891	0.039	0.574	0.049	M38	0.702	0.041	0.581	0.085	0.257	0.093	0.180	0.042
M58	1.597	0.101	0.775	0.089	0.505	0.086	0.400	0.028	M58	1.597	0.101	0.452	0.128	1.657	0.059	1.446	0.094	M58	1.597	0.101	0.569	0.052	0.818	0.078	0.371	0.039
M55	0.695	0.119	0.547	0.058	0.384	0.026	0.748	0.338	M55	0.695	0.119	0.617	0.099	0.174	0.010	0.235	0.048	M55	0.695	0.119	0.209	0.051	0.179	0.029	0.119	0.009
M57	0.624	0.133	0.570	0.116	0.504	0.103	0.584	0.052	M57	0.624	0.133	0.267	0.116	0.348	0.076	0.572	0.034	M57	0.624	0.133	1.868	0.096	1.151	0.064	1.090	0.042
M63	1.080	0.106	0.531	0.011	0.171	0.080	0.241	0.056	M63	1.080	0.106	0.174	0.091	0.151	0.038	0.187	0.037	M63	1.080	0.106	0.286	0.143	0.112	0.048	0.125	0.076
M18	1.303	0.059	0.739	0.037	0.290	0.102	0.372	0.060	M18	1.303	0.059	0.291	0.064	0.355	0.043	0.148	0.014	M18	1.303	0.059	0.187	0.017	0.132	0.029	0.149	0.048
M59	1.195	0.155	0.848	0.070	0.385	0.056	0.378	0.058	M59	1.195	0.155	0.616	0.024	0.209	0.029	0.270	0.086	M59	1.195	0.155	0.894	0.029	0.718	0.142	0.821	0.040
ATCC 15468^T	1.167	0.054	1.124	0.104	1.117	0.078	0.977	0.101	ATCC 15468^T	1.167	0.054	1.043	0.078	1.158	0.068	1.104	0.089	ATCC 15468^T	1.167	0.054	1.761	0.110	1.201	0.140	1.097	0.103
M76	1.039	0.074	0.448	0.076	0.333	0.090	0.564	0.068	M76	1.039	0.074	0.925	0.073	1.316	0.009	0.656	0.064	M76	1.039	0.074	0.868	0.098	0.189	0.017	0.151	0.007
M77	0.774	0.026	0.666	0.082	0.372	0.060	0.153	0.029	M77	0.774	0.026	0.168	0.023	0.247	0.024	0.161	0.049	M77	0.774	0.026	0.169	0.029	0.362	0.106	0.194	0.072
M41	1.401	0.151	0.471	0.081	0.580	0.059	0.465	0.072	M41	1.401	0.151	0.439	0.154	0.590	0.059	0.250	0.031	M41	1.401	0.151	1.326	0.204	1.129	0.094	0.119	0.010
M92	1.709	0.077	0.300	0.077	0.109	0.008	0.358	0.098	M92	1.709	0.077	0.156	0.073	0.176	0.101	0.165	0.014	M92	1.709	0.077	0.094	0.017	0.138	0.061	0.332	0.083
M28	1.551	0.109	0.527	0.085	0.587	0.071	0.490	0.080	M28	1.551	0.109	0.273	0.065	0.337	0.087	0.258	0.052	M28	1.551	0.109	0.459	0.069	0.247	0.086	0.290	0.062
M49	0.943	0.073	0.618	0.060	0.279	0.023	0.312	0.045	M49	0.943	0.073	0.489	0.104	0.468	0.137	0.452	0.103	M49	0.943	0.073	0.900	0.059	0.454	0.056	0.122	0.004
M9	2.475	0.204	0.423	0.066	0.345	0.021	0.451	0.125	M46	2.475	0.204	0.582	0.090	0.785	0.012	1.218	0.024	M46	2.475	0.204	0.254	0.020	0.183	0.019	0.168	0.034
M46	1.340	0.235	0.383	0.040	0.438	0.071	0.527	0.041	M9	1.340	0.235	0.176	0.052	0.175	0.028	0.167	0.045	M9	1.340	0.235	0.139	0.025	0.211	0.080	0.145	0.059
M67	1.053	0.051	0.605	0.098	0.332	0.025	0.399	0.005	M67	1.053	0.051	0.410	0.064	0.441	0.097	0.251	0.018	M67	1.053	0.051	0.368	0.062	0.705	0.106	0.286	0.072
Gentamicin									**Tetracycline**																	
	Average Untreated	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreate StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev									
MH	0.154	0.006	0.154	0.006	0.154	0.006	0.154	0.006	MH	0.154	0.006	0.154	0.006	0.154	0.006	0.154	0.006								
M2	1.174	0.062	1.917	0.100	0.668	0.036	1.849	0.115	M2	1.174	0.062	0.977	0.073	0.465	0.108	0.607	0.050								
M17	0.984	0.122	0.149	0.031	0.389	0.039	0.192	0.010	M17	0.984	0.122	0.581	0.081	1.127	0.073	0.157	0.011								
M51	1.457	0.162	0.186	0.064	0.139	0.018	0.158	0.027	M51	1.457	0.162	0.186	0.064	0.526	0.016	0.452	0.106								
M64	0.987	0.112	0.143	0.019	0.132	0.016	0.172	0.045	M64	0.987	0.112	0.235	0.121	0.611	0.101	0.561	0.095								
M94	0.763	0.017	0.661	0.071	0.146	0.005	0.207	0.051	M94	0.763	0.017	0.813	0.084	0.272	0.040	0.305	0.024								
M95	1.077	0.080	0.848	0.026	0.436	0.049	0.771	0.019	M95	1.077	0.080	1.091	0.083	0.501	0.155	0.495	0.063								
ATCC 7966^T	0.982	0.083	0.865	0.116	0.654	0.088	0.413	0.078	ATCC 7966^T	0.982	0.083	0.934	0.100	0.816	0.088	0.569	0.083								
M70	1.049	0.075	0.126	0.038	0.091	0.012	0.158	0.103	M70	1.049	0.075	0.802	0.055	0.654	0.093	0.299	0.065								
M88	0.619	0.129	0.124	0.043	0.101	0.020	0.134	0.027	M88	0.619	0.129	0.499	0.098	0.353	0.052	0.377	0.066								
M90	0.503	0.110	0.258	0.054	0.105	0.031	0.082	0.017	M90	0.503	0.110	0.479	0.145	0.762	0.114	0.500	0.088								
M96	0.639	0.094	0.193	0.066	0.228	0.089	0.190	0.077	M96	0.639	0.094	0.777	0.044	0.660	0.084	0.574	0.078								
M23	1.265	0.062	1.308	0.035	0.567	0.055	0.800	0.074	M23	1.265	0.062	0.684	0.076	0.430	0.060	0.539	0.038								
M31	0.761	0.079	0.895	0.061	0.381	0.056	0.334	0.099	M31	0.761	0.079	0.986	0.076	0.640	0.099	0.350	0.043								
M38	0.702	0.041	0.554	0.122	0.411	0.050	0.156	0.035	M38	0.702	0.041	0.779	0.088	0.603	0.139	0.606	0.081								
M58	1.597	0.101	0.879	0.089	0.531	0.081	0.697	0.023	M58	1.597	0.101	0.694	0.075	0.630	0.105	0.655	0.032								
M55	0.695	0.119	0.796	0.080	0.101	0.010	0.120	0.013	M55	0.695	0.119	0.992	0.100	0.404	0.023	0.373	0.036								
M57	0.624	0.133	0.999	0.063	0.114	0.017	0.117	0.004	M57	0.624	0.133	0.643	0.126	0.321	0.109	0.328	0.088								
M63	1.080	0.106	0.103	0.063	0.117	0.070	0.135	0.046	M63	1.080	0.106	0.328	0.040	0.354	0.082	0.267	0.075								
M18	1.303	0.059	0.490	0.107	0.170	0.060	0.173	0.041	M18	1.303	0.059	0.576	0.063	0.548	0.084	0.399	0.083								
M59	1.195	0.155	0.111	0.020	0.100	0.009	0.143	0.049	M59	1.195	0.155	0.648	0.100	0.534	0.091	0.428	0.083								
ATCC 15468^T	1.167	0.054	1.129	0.013	0.947	0.054	0.774	0.092	ATCC 15468^T	1.167	0.054	1.496	0.080	0.981	0.016	0.675	0.066								
M76	1.039	0.074	0.194	0.015	0.193	0.026	0.178	0.009	M76	1.039	0.074	0.858	0.099	0.371	0.066	0.291	0.055								
M77	0.774	0.026	0.189	0.016	0.145	0.023	0.148	0.006	M77	0.774	0.026	0.403	0.147	0.306	0.018	0.351	0.051								
M41	1.401	0.151	0.494	0.112	0.188	0.044	0.175	0.077	M41	1.401	0.151	0.448	0.073	0.329	0.030	0.739	0.034								
M92	1.709	0.077	0.250	0.049	0.156	0.084	0.122	0.073	M92	1.709	0.077	0.204	0.127	0.213	0.019	0.359	0.053								
M28	1.551	0.109	0.433	0.117	0.181	0.063	0.165	0.039	M28	1.551	0.109	0.746	0.045	0.677	0.067	0.835	0.091								
M49	0.943	0.073	0.331	0.045	0.521	0.100	0.161	0.036	M49	0.943	0.073	0.351	0.069	0.364	0.009	0.362	0.048								
M46	1.340	0.235	0.132	0.024	0.151	0.042	0.119	0.007	M46	2.475	0.204	1.252	0.101	0.588	0.098	0.677	0.048								
M9	2.475	0.204	0.139	0.047	0.216	0.047	0.166	0.040	M9	1.340	0.235	0.399	0.111	0.273	0.071	0.206	0.046								
M67	1.053	0.051	0.800	0.037	0.727	0.075	0.522	0.055	M67	1.053	0.051	0.881	0.078	0.856	0.100	0.837	0.043								

Table 3A: MICs absorbance readings after 48 h

Azithromycin									Ceftazidime										Ciprofloxacin									
	Average Untreate	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreate StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MI StDev	
MH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	MH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	MH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	
M2	0.990	0.056	0.854	0.025	0.778	0.111	1.815	0.062	M2	0.990	0.056	1.041	0.056	0.793	0.086	1.479	0.112	M2	0.990	0.056	0.955	0.028	1.182	0.065	0.422	0.089	
M17	0.891	0.086	0.416	0.079	0.257	0.048	0.589	0.051	M17	0.891	0.086	1.144	0.086	0.499	0.059	0.483	0.262	M17	0.891	0.086	1.639	0.103	0.866	0.015	0.848	0.043	
M51	0.938	0.103	0.613	0.062	0.615	0.058	0.840	0.066	M51	0.938	0.103	0.303	0.103	0.570	0.050	0.370	0.057	M51	0.938	0.103	0.721	0.099	0.934	0.058	0.543	0.085	
M64	0.590	0.087	0.495	0.037	0.338	0.101	0.576	0.097	M64	0.590	0.087	0.465	0.087	0.641	0.107	0.522	0.050	M64	0.590	0.087	0.484	0.069	0.686	0.044	0.545	0.075	
M94	0.537	0.092	0.743	0.062	0.270	0.002	0.335	0.046	M94	0.537	0.092	0.396	0.092	0.531	0.111	0.367	0.120	M94	0.537	0.092	0.530	0.075	0.449	0.056	0.477	0.070	
M95	1.186	0.058	0.468	0.052	0.511	0.012	1.435	0.053	M95	1.186	0.058	1.495	0.058	1.108	0.109	0.866	0.040	M95	1.186	0.058	1.677	0.086	1.007	0.093	0.767	0.022	
ATCC 7966^T	0.749	0.077	0.711	0.085	0.427	0.078	0.686	0.065	ATCC 7966^T	1.106	0.077	0.711	0.085	0.427	0.078	0.686	0.065	ATCC 7966^T	0.749	0.077	0.426	0.082	0.644	0.025	0.554	0.089	
M70	0.733	0.100	1.605	0.124	2.671	0.112	0.782	0.034	M70	0.733	0.100	0.907	0.100	0.697	0.083	0.637	0.043	M70	0.733	0.100	0.640	0.088	0.495	0.094	0.317	0.070	
M88	0.757	0.094	0.355	0.049	0.589	0.024	0.394	0.015	M88	0.757	0.094	0.619	0.094	0.415	0.047	0.459	0.102	M88	0.757	0.094	0.804	0.080	0.477	0.094	0.781	0.068	
M90	0.643	0.064	0.486	0.020	0.459	0.081	0.727	0.095	M90	0.643	0.064	0.452	0.064	0.396	0.073	0.329	0.105	M90	0.643	0.064	0.386	0.056	0.440	0.049	0.368	0.007	
M96	1.043	0.058	0.679	0.086	0.748	0.078	0.584	0.103	M96	1.043	0.058	0.732	0.058	0.807	0.049	0.741	0.042	M96	1.043	0.058	1.055	0.019	0.565	0.082	0.544	0.063	
M23	1.653	0.081	1.811	0.128	1.667	0.218	1.903	0.070	M23	1.653	0.081	2.034	0.081	1.809	0.149	2.559	0.346	M23	1.653	0.081	1.677	0.050	2.192	0.173	1.579	0.100	
M31	0.797	0.062	0.474	0.094	0.444	0.070	0.575	0.066	M31	0.797	0.062	0.292	0.062	0.422	0.106	0.223	0.032	M31	0.797	0.062	0.514	0.096	0.504	0.075	0.253	0.040	
M38	0.942	0.087	0.322	0.092	0.605	0.077	0.310	0.082	M38	0.942	0.087	0.582	0.087	0.464	0.028	0.334	0.047	M38	0.942	0.087	0.964	0.060	0.400	0.021	0.684	0.066	
M58	0.769	0.081	0.209	0.152	0.121	0.017	0.151	0.010	M58	0.769	0.081	0.309	0.081	0.281	0.058	0.349	0.054	M58	0.769	0.081	0.206	0.077	0.248	0.045	0.178	0.024	
M55	1.250	0.071	1.487	0.094	1.193	0.137	1.693	0.117	M55	1.250	0.071	1.304	0.071	2.341	0.325	1.630	0.023	M55	1.250	0.071	1.646	0.065	1.842	0.046	0.984	0.081	
M57	0.374	0.035	0.743	0.096	0.675	0.047	0.906	0.084	M57	0.374	0.035	0.627	0.035	1.267	0.082	0.691	0.052	M57	0.374	0.035	0.705	0.081	0.461	0.119	0.316	0.095	
M63	0.768	0.037	0.705	0.035	0.500	0.082	0.818	0.108	M63	0.768	0.037	0.786	0.037	0.506	0.065	0.806	0.086	M63	0.768	0.037	1.114	0.126	0.422	0.036	0.877	0.104	
M18	1.082	0.015	1.097	0.043	0.423	0.076	0.672	0.081	M18	1.082	0.015	0.656	0.015	1.204	0.054	1.033	0.002	M18	1.082	0.015	1.089	0.108	0.598	0.085	0.310	0.030	
M59	1.257	0.058	0.823	0.100	1.086	0.072	0.899	0.070	M59	1.257	0.058	1.113	0.058	0.831	0.034	0.872	0.016	M59	1.257	0.058	1.351	0.087	1.291	0.023	0.894	0.071	
ATCC 15468^T	1.106	0.083	0.718	0.085	0.693	0.060	0.659	0.093	ATCC 15468^T	1.106	0.083	0.718	0.085	0.693	0.060	0.659	0.093	ATCC 15468^T	1.218	0.063	1.138	0.052	0.876	0.042	0.083	0.067	
M76	0.965	0.108	0.365	0.077	0.558	0.085	0.665	0.060	M76	0.965	0.108	1.014	0.108	0.640	0.066	0.527	0.085	M76	0.965	0.108	0.946	0.066	0.765	0.078	0.405	0.055	
M77	0.845	0.045	1.086	0.063	0.766	0.041	0.406	0.055	M77	0.845	0.045	0.299	0.045	0.245	0.042	0.298	0.076	M77	0.845	0.045	0.546	0.012	0.594	0.069	0.620	0.045	
M41	0.728	0.057	1.641	0.099	0.628	0.013	0.473	0.036	M41	0.728	0.057	0.491	0.057	1.544	0.042	0.579	0.045	M41	0.728	0.057	0.917	0.060	0.598	0.102	0.722	0.059	
M92	0.591	0.070	0.577	0.081	0.405	0.129	0.582	0.066	M92	0.591	0.070	0.385	0.070	0.882	0.038	1.052	0.191	M92	0.591	0.070	0.563	0.105	0.507	0.071	0.602	0.139	
M28	0.953	0.082	0.569	0.075	0.545	0.029	0.477	0.106	M28	0.953	0.082	0.620	0.082	1.884	0.004	0.645	0.071	M28	0.953	0.082	0.933	0.102	0.625	0.077	0.645	0.066	
M49	0.779	0.076	0.882	0.088	0.761	0.109	0.387	0.073	M49	0.779	0.076	0.123	0.076	0.153	0.020	0.142	0.044	M49	0.779	0.076	0.302	0.041	0.747	0.058	0.429	0.015	
M9	1.037	0.046	0.477	0.088	0.511	0.072	0.641	0.069	M9	1.037	0.046	0.803	0.046	0.954	0.053	1.368	0.059	M9	1.037	0.046	0.697	0.134	0.750	0.063	0.516	0.110	
M46	0.968	0.118	1.059	0.102	0.803	0.053	0.563	0.111	M46	0.968	0.118	0.547	0.118	0.789	0.022	0.571	0.054	M46	0.968	0.118	1.070	0.056	0.305	0.042	0.583	0.063	
M67	1.008	0.076	0.490	0.089	0.525	0.085	0.462	0.066	M67	1.008	0.076	0.549	0.076	0.418	0.034	0.363	0.047	M67	1.008	0.076	0.925	0.072	0.581	0.036	0.977	0.117	
Gentamicin									**Tetracycline**																			
	Average Untreate	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev		Average Untreate	Untreated StDev	Average Sub-MIC	Sub MIC StDev	Average MIC	MIC StDev	Average Supra-MI	Supra MIC StDev											
MH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	MH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001										
M2	0.990	0.056	0.911	0.097	1.025	0.093	0.867	0.095	M2	0.990	0.056	0.523	0.147	1.718	0.062	0.931	0.094										
M17	0.891	0.086	0.670	0.072	1.015	0.067	1.248	0.017	M17	0.891	0.086	0.855	0.066	0.656	0.115	0.332	0.125										
M51	0.938	0.103	0.748	0.031	0.691	0.065	0.683	0.035	M51	0.938	0.103	0.982	0.085	0.402	0.045	0.650	0.084										
M64	0.590	0.087	0.709	0.096	0.548	0.097	0.676	0.093	M64	0.590	0.087	0.660	0.050	0.671	0.096	0.736	0.095										
M94	0.537	0.092	0.375	0.061	0.918	0.071	1.288	0.027	M94	0.537	0.092	0.683	0.061	0.444	0.115	0.757	0.108										
M95	1.186	0.058	1.302	0.132	0.824	0.084	0.736	0.023	M95	1.186	0.058	1.767	0.113	1.978	0.007	0.876	0.101										
ATCC 7966^T	0.749	0.077	0.410	0.086	0.331	0.094	0.302	0.087	ATCC 7966^T	1.106	0.077	0.763	0.077	0.659	0.047	0.623	0.070										
M70	0.733	0.100	0.426	0.173	0.483	0.106	0.422	0.151	M70	0.733	0.100	0.415	0.053	0.389	0.085	0.469	0.065										
M88	0.757	0.094	0.489	0.079	0.454	0.032	0.812	0.112	M88	0.757	0.094	0.857	0.109	0.809	0.065	1.474	0.371										
M90	0.643	0.064	0.783	0.104	0.728	0.066	1.128	0.081	M90	0.643	0.064	0.879	0.097	0.500	0.139	0.919	0.075										
M96	1.043	0.058	0.570	0.039	0.559	0.107	0.795	0.072	M96	1.043	0.058	0.713	0.085	0.701	0.073	0.798	0.035										
M23	1.653	0.081	2.466	0.084	1.658	0.105	2.215	0.070	M23	1.653	0.081	1.580	0.017	2.176	0.095	2.547	0.108										
M31	0.797	0.062	0.871	0.122	0.481	0.058	0.518	0.140	M31	0.797	0.062	0.490	0.231	0.410	0.059	0.547	0.095										
M38	0.942	0.087	0.928	0.060	0.329	0.069	0.422	0.073	M38	0.942	0.087	0.635	0.095	0.222	0.009	0.615	0.067										
M58	0.769	0.081	0.192	0.029	0.198	0.055	0.201	0.011	M58	0.769	0.081	0.220	0.035	0.190	0.067	0.156	0.050										
M55	1.250	0.071	1.451	0.018	1.026	0.039	0.995	0.053	M55	1.250	0.071	1.208	0.012	1.708	0.076	1.451	0.040										
M57	0.374	0.035	0.555	0.097	0.333	0.134	0.421	0.049	M57	0.374	0.035	0.622	0.079	0.576	0.083	0.582	0.095										
M63	0.768	0.037	0.589	0.048	0.725	0.029	1.235	0.091	M63	0.768	0.037	0.987	0.055	0.575	0.072	0.421	0.139										
M18	1.082	0.015	0.935	0.046	0.535	0.096	0.600	0.073	M18	1.082	0.015	0.766	0.076	0.750	0.084	0.705	0.079										
M59	1.257	0.058	0.934	0.066	0.903	0.080	1.248	0.051	M59	1.257	0.058	0.639	0.099	1.090	0.082	1.262	0.101										
ATCC 15468^T	1.106	0.083	0.845	0.096	0.678	0.056	0.942	0.065	ATCC 15468^T	1.115	0.083	0.859	0.081	0.910	0.061	0.929	0.073										
M76	0.965	0.108	0.675	0.090	0.507	0.087	0.583	0.021	M76	0.965	0.108	1.018	0.056	1.027	0.079	0.869	0.056										
M77	0.845	0.045	0.838	0.052	0.469	0.075	0.497	0.023	M77	0.845	0.045	0.380	0.064	0.407	0.057	1.067	0.139										
M41	0.728	0.057	0.809	0.100	0.926	0.090	0.847	0.073	M41	0.728	0.057	1.132	0.070	0.668	0.038	1.020	0.117										
M92	0.591	0.070	0.405	0.080	0.308	0.049	0.366	0.072	M92	0.591	0.070	0.629	0.078	0.672	0.014	0.961	0.044										
M28	0.953	0.082	0.734	0.031	0.960	0.079	0.686	0.058	M28	0.953	0.082	0.781	0.062	0.726	0.057	0.777	0.086										
M49	0.779	0.076	0.280	0.110	0.219	0.115	0.134	0.025	M49	0.779	0.076	0.374	0.110	0.299	0.034	0.351	0.110										
M9	1.037	0.046	1.052	0.068	0.833	0.076	0.552	0.040	M9	1.037	0.046	0.850	0.081	0.669	0.114	0.556	0.037										
M46	0.968	0.118	0.406	0.028	0.551	0.052	0.945	0.064	M46	0.968	0.118	0.670	0.080	0.892	0.057	1.142	0.069										
M67	1.008	0.076	0.728	0.025	1.474	0.117	0.493	0.068	M67	1.008	0.076	0.455	0.046	0.548	0.082	0.697	0.089										

APPENDIX B: DATA FOR CHAPTER 3

Table 1B: Resistance, Intermediate and susceptibility profile of *A. hydrophila* isolates

M6									M2								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	10	R	12	R	0	R	0	R	AMP10	0	R	12	R	0	R	0	R
CPD10	29	S	32	S	28	S	32	S	CPD10	29	S	29.5	S	29	S	25.5	S
C30	27	S	25.5	S	14	I	29.5	S	C30	25.5	S	29.5	S	14	I	30	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	34.5	S	36	S	35	S	37.5	S	NOR10	24	S	34.5	S	35	S	36	S
ENR5	28	S	25.5	S	21	I	27	S	ENR5	19	I	27	S	23	S	26	S
OFX5	28.5	S	27.5	S	20.5	S	26	S	OFX5	23	S	26	S	22	S	24	S
CIP5	33.5	S	34	S	28.5	S	41	S	CIP5	36	S	38	S	23.5	S	37	S
NA30	30	S	29	S	25.5	S	30	S	NA30	26	S	31	S	26	S	29	S
TE30	10	R	0	R	0	R	9.5	R	TE30	10	R	9	R	0	R	0	R
CN10	27.5	S	30	S	28.5	S	28	S	CN10	25	S	28.5	S	28	S	24	S
S10	26.5	S	26	S	22	S	29	S	S10	24.5	S	26.5	S	23.5	S	27.5	S
AK30	27.5	S	26.5	S	25	S	26.5	S	AK30	28.5	S	26.5	S	27	S	27.5	S
AZM15	17.5	I	19	S	11.5	R	22.5	S	AZM15	21.5	S	17.5	I	17.5	I	26	S
E15	11.5	R	11	R	10.5	R	18	I	E15	10	R	14.5	I	12	R	15.5	I

M95									M51								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	0	R	0	R	0	R
CPD10	26	S	25	S	23	S	23	S	CPD10	29	S	31	S	37.5	S	32.5	S
C30	15.5	I	16	I	10.5	R	15	I	C30	16	I	22.5	S	22.5	S	24.5	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	37	S	37	S	32.5	S	34	S	NOR10	37.5	S	39	S	34.5	S	31	S
ENR5	22	I	22.5	I	18	I	19	I	ENR5	30	S	29	S	28	S	28.5	S
OFX5	26	S	23.5	S	22.5	S	22.5	S	OFX5	27	S	27.5	S	27.5	S	29	S
CIP5	34	S	35	S	35	S	35.5	S	CIP5	32	S	38	S	35.5	S	32.5	S
NA30	27	S	23	S	23	S	25	S	NA30	36	S	37.5	S	26	S	36	S
TE30	0	R	0	R	0	R	0	R	TE30	0	R	9.5	R	0	R	0	R
CN10	25	S	23	S	23	S	25.5	S	CN10	27.5	S	31.5	S	33	S	27.5	S
S10	22	S	20.5	S	21	S	21	S	S10	21.5	S	25.5	S	21	S	20	S
AK30	23.5	S	23.5	S	23	S	22	S	AK30	29.5	S	32.5	S	27	S	29	S
AZM15	14.5	I	16	I	12.5	R	14.5	I	AZM15	17.5	I	21	S	16.5	I	17	I
E15	0	R	0	R	0	R	0	R	E15	12	R	13.5	R	11.5	R	15	I

M17									94								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	11	R	0	R	AMP10	0	R	0	R	0	R	10	R
CPD10	26	S	28	S	28	S	27	S	CPD10	30	S	29	S	27	S	29	S
C30	30	S	27	S	21	S	27	S	C30	22	S	24	S	12	R	28	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	36	S	40	S	37	S	35	S	NOR10	33	S	35	S	32	S	33	S
ENR5	27	S	29	S	24	S	29	S	ENR5	26	S	29	S	24	S	25	S
OFX5	25	S	29	S	24	S	28	S	OFX5	25	S	25	S	25	S	26	S
CIP5	37	S	42	S	39	S	40	S	CIP5	35	S	35	S	34	S	36	S
NA30	27	S	24	S	24	S	25	S	NA30	30	S	30	S	26	S	30	S
TE30	10	R	10	R	10	R	9	R	TE30	0	R	0	R	0	R	0	R
CN10	27	S	29	S	28	S	29	S	CN10	26	S	26	S	25	S	27	S
S10	23	S	27	S	25	S	25	S	S10	25	S	25	S	24	S	26	S
AK30	25	S	29	S	26	S	29	S	AK30	24	S	26	S	26	S	24	S
AZM15	22	S	23	S	20	S	27	S	AZM15	22	S	20	S	15	I	20	S
E15	12	R	14	I	11	R	16	R	E15	12	R	15	I	12	R	12	R

M13								
	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	14	I	0	R	0	R
CPD10	31	S	30.5	S	29.5	S	27	S
C30	29	S	14	I	14	I	32	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	40	S	45	S	18	S	10.5	R
ENR5	40	S	40	S	13	R	15	R
OFX5	41	S	41	S	15	I	15	R
CIP5	14.5	R	19.5	I	17.5	I	13.5	R
NA30	0	R	0	R	0	R	0	R
TE30	0	R	0	R	0	R	0	R
CN10	28.5	S	30	S	25.5	S	28.5	S
S10	25.5	S	21	S	22.5	S	24	S
AK30	28.5	S	27	S	27	S	27	S
AZM15	27.5	S	25	S	25	S	15	I
E15	12.5	R	16	I	16	I	15	I

M52								
	CONTROL		CCCP		NMP		PABN	
AMP10	11	R	9	R	10.5	R	11	R
CPD10	29	S	28	S	25.5	S	28.5	S
C30	28	S	25.5	S	16	I	26.5	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	32	S	32	S	32	S	32	S
ENR5	24	S	25	S	24	S	25.5	S
OFX5	25	S	25	S	21.5	S	24	S
CIP5	33	S	34.5	S	34.5	S	33.5	S
NA30	25	S	27	S	23.5	S	27.5	S
TE30	0	R	0	R	0	R	9	R
CN10	26	S	27	S	25	S	27	S
S10	25.5	S	23.5	S	20.5	S	24.5	S
AK30	26	S	24	S	24	S	24	S
AZM15	20.5	S	17	I	14	I	19.5	S
E15	13	R	13	R	0	R	12	R

M50								
	CONTROL		CCCP		NMP		PABN	
AMP10	10	R	0	R	0	R	0	R
CPD10	14.5	R	13.5	R	29	S	24.5	S
C30	14.5	I	9	I	26.5	S	22.5	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	22.5	S	23.5	S	20	S	18	S
ENR5	22	I	21.5	I	17	I	20	S
OFX5	26	S	23	S	24	S	23.5	S
CIP5	29	S	26.5	S	25	S	24	S
NA30	0	R	0	R	0	R	0	R
TE30	16.5	I	14.5	R	12	R	11	R
CN10	29.5	S	27.5	S	26	S	24.5	S
S10	25.5	S	20.5	S	21.5	S	22.5	S
AK30	29	S	29	S	25	S	23.5	S
AZM15	13	R	20	S	12	R	11	R
E15	0	R	0	R	0	R	0	R

M86								
	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	13	R	0	R	0	R
CPD10	35.5	S	34.5	S	19.5	I	32	S
C30	20	S	0	R	14	I	21	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	19	S	26	S	26	S	19	S
ENR5	27	S	25	S	25	S	26	S
OFX5	29	S	26	S	26.5	S	27.5	S
CIP5	40	S	39	S	36	S	31	S
NA30	37	S	31	S	29	S	25	S
TE30	10	R	0	R	0	R	10.5	R
CN10	28	S	30.5	S	29	S	28	S
S10	26.5	S	26	S	26	S	25.5	S
AK30	28.5	S	28.5	S	29.5	S	28	S
AZM15	28.5	S	15	I	30	S	26.5	S
E15	14	I	12.5	R	11	R	16.5	I

M64								
	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	9	R	9	R	10	R
CPD10	19	I	26	S	19	I	21	I
C30	20	S	27	S	12	R	20	R
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	27	S	33	S	29	S	31	S
ENR5	22	I	24	I	20	I	21	I
OFX5	20	S	23	S	20	S	21	S
CIP5	28	R	36	S	23	S	29	S
NA30	22	S	27	S	22	S	21	S
TE30	0	R	9	R	0	R	0	R
CN10	22	S	25	S	22	S	21	S
S10	23	S	24	S	18	S	19	S
AK30	22	S	24	S	23	S	20	S
AZM15	16	I	15	I	13	R	14	I
E15	11	R	11	R	11	R	10	R

M53								
	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R
CPD10	21	S	18.5	I	20.5	I	20	I
C30	20.5	S	17.5	I	0	R	22.5	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	31	S	27	S	30.5	S	32	S
ENR5	21	I	20	I	17.5	I	21.5	I
OFX5	20.5	S	21	S	17	S	23	S
CIP5	32	S	26.5	S	29	S	33	S
NA30	21.5	S	21.5	S	21	S	22	S
TE30	0	R	0	R	0	R	0	R
CN10	24	S	23	S	24	S	24	S
S10	22	S	21	S	20	S	22	S
AK30	22.5	S	21.5	S	20.5	S	22	S
AZM15	21	S	18	S	13	R	19	S
E15	11	R	12.5	R	9	R	11	R

M62									M60								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	12	R	11	R	0	R
CPD10	0	R	32	S	25.5	S	28.5	S	CPD10	22	S	35	S	26	S	21	S
C30	23	S	22.5	S	16	I	13	I	C30	22	S	26	S	13	I	19	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	20	S	25	S	23.5	S	26	S	NOR10	26	S	33	S	33	S	30	S
ENR5	23	S	25.5	S	25.5	S	25.5	S	ENR5	24	S	29	S	22	S	24	S
OFX5	23	S	25.5	S	24	S	22.5	S	OFX5	22	S	29	S	23	S	23	S
CIP5	27	S	28.5	S	27	S	28	S	CIP5	29	S	40	S	33	S	29	S
NA30	27	S	23.5	S	26.5	S	13	R	NA30	24	S	36	S	27	S	25	S
TE30	9	R	7	R	9.5	R	0	R	TE30	9	R	11	R	0	R	9	R
CN10	26	R	22	S	25.5	S	15	S	CN10	23	S	28	S	26	S	22	S
S10	23	S	20.5	S	24.5	S	22	S	S10	23	S	31	S	22	S	20	S
AK30	24.5	S	23	S	24.5	S	25	S	AK30	23	S	28	S	25	S	20	S
AZM15	24	S	20.5	S	26.5	S	21	S	AZM15	13	R	12	R	16	I	8	R
E15	9	R	10.5	R	0	R	13	R	E15	11	R	10	R	11	R	10	R

M5									M14								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R	AMP10	10	R	10.5	R	11.5	R	0	R
CPD10	28	S	30	S	25.5	S	28.5	S	CPD10	32	S	32.5	S	28.5	S	28	S
C30	0	R	24	S	11.5	R	0	R	C30	27	S	29.5	S	19	S	28	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	27.5	S	0	R	0	R	21.5	S	RL25	0	R	0	R	0	R	0	R
NOR10	21.5	S	25.5	S	25.5	S	23.5	S	NOR10	37.5	S	39	S	34	S	39	S
ENR5	23	S	27.5	S	27.5	S	25	S	ENR5	29	S	32	S	28	S	28.5	S
OFX5	25.5	S	25.5	S	25.5	S	25	S	OFX5	27.5	S	28	S	22	S	27	S
CIP5	27.5	S	29	S	27	S	27	S	CIP5	38.5	S	38	S	34.5	S	40	S
NA30	18.5	I	20.5	S	14.5	I	18	I	NA30	30	S	30	S	25.5	S	32	S
TE30	0	R	10	R	0	R	0	R	TE30	9	R	10	R	10	S	0	R
CN10	17.5	S	27.5	S	16.5	S	18.5	S	CN10	28	S	29	S	29	S	24	S
S10	19.5	S	26	S	18.5	S	21	S	S10	27.5	S	28.5	S	24	S	28	S
AK30	27	S	25	S	18	S	21.5	S	AK30	27	S	27	S	27	S	27	S
AZM15	23.5	S	17.5	I	13.5	R	13.5	R	AZM15	17	R	20.5	S	17	I	25	S
E15	10	R	10.5	R	0	R	0	R	E15	13	R	12.5	R	9	R	18	I

M65								
	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	9	R	0	R	0	R
CPD10	20	I	19	I	20	I	21	S
C30	15	I	21	S	20	S	17	I
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	35	S	37	S	32	S	35	S
ENR5	24	S	26	S	24	S	23	S
OFX5	24	S	25	S	23	S	23	S
CIP5	29	S	36	S	25	S	29	S
NA30	23	S	26	S	24	S	23	S
TE30	0	R	8	R	0	R	0	R
CN10	22	S	27	S	23	S	25	S
S10	22	S	26	S	23	S	21	S
AK30	23	S	24	S	24	S	22	S
AZM15	19	S	20	S	20	S	19	S
E15	11	R	12	R	12	R	11	R

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 2B: Resistance, Intermediate and susceptibility profile of *A. bestiarum* isolates

M88									M72									M96									M81								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	13	R	0	R	0	R	AMP10	0	R	0	R	10	R	0	R	AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	9	R	0	R	0	R
CPD10	35.5	S	34.5	S	19.5	I	32	S	CPD10	13	R	18	I	20	I	18	I	CPD10	23	S	23	S	22	S	22	S	CPD10	0	R	24	S	12	R	12	R
C30	20	S	0	R	14	I	21	S	C30	23	S	25	S	23	S	22	S	C30	22	S	20	S	18	S	13	I	C30	15	I	21.5	S	15	I	15	I
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	19	S	26	S	26	S	19	S	NOR10	24	S	25	S	19	S	25	S	NOR10	30	S	26	S	29	S	28	S	NOR10	30	S	30	R	25	S	25	S
ENR5	27	S	25	S	25	S	26	S	ENR5	23	S	25	S	27	S	25	S	ENR5	24	S	24	S	26	S	25	S	ENR5	23	S	23	R	19	I	21	I
OFX5	29	S	26	S	26.5	S	27.5	S	OFX5	22	S	23	S	18	S	23	S	OFX5	23	S	23	S	20	S	22	S	OFX5	21	S	21	R	20	S	19	S
CIP5	40	S	39	S	36	S	31	S	CIP5	27	S	32	S	31	S	30	S	CIP5	32	S	34	S	30	S	28	S	CIP5	27	S	40	R	24	S	29	S
NA30	37	S	31	S	29	S	25	S	NA30	25	S	28	S	19	S	25	S	NA30	27	S	26	S	24	S	23	S	NA30	23	S	36	R	19	S	25	S
TE30	10	R	0	R	0	R	10.5	R	TE30	0	R	0	R	0	R	10	R	TE30	0	R	0	R	0	R	0	R	TE30	0	R	11	S	0	R	9	R
CN10	28	S	30.5	S	29	S	28	S	CN10	23	S	21	S	23	S	25	S	CN10	25	S	23	S	22	S	23	S	CN10	22	S	28	R	20	S	22	S
S10	26.5	S	26	S	26	S	25.5	S	S10	16	S	17	S	21	S	18	S	S10	20.5	S	21	S	23	S	19	S	S10	21	S	21	R	12	R	16	S
AK30	28.5	S	28.5	S	29.5	S	28	S	AK30	23	S	24	S	24	S	25	S	AK30	23	S	24	S	23	S	20	S	AK30	21	S	21	R	20	S	19	S
AZM15	28.5	S	15	I	30	S	26.5	S	AZM15	14	I	17	I	15	I	20	S	AZM15	17	I	20	S	17	I	17	I	AZM15	17	I	17	I	13	R	12	R
E15	14	I	12.5	R	11	R	16.5	I	E15	0	R	13	R	0	R	9	R	E15	10	R	11	R	9	R	10	R	E15	10.5	R	10.5	R	0	R	11	R

M90									M99									M70									M80								
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	10	R	0	R	10	R	0	R	AMP10	0	R	13	R	9	R	10	R	AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	0	R	0	R	0	R
CPD10	27	S	25	S	24	S	25	S	CPD10	11	R	36	S	12	R	11	R	CPD10	25	S	28	S	20	I	26	S	CPD10	15	R	21	S	25	S	19	I
C30	23	S	23	S	14	I	23	S	C30	14	I	21	S	12	R	15	I	C30	21	S	24	S	20	S	19	S	C30	17	I	22	S	13	R	22	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	34	S	33	S	34	S	28	S	NOR10	22	S	36	S	**23**	S	20	S	NOR10	33	S	35	S	27	S	32	S	NOR10	25	S	35	S	30	S	26	S
ENR5	24	S	25	S	23	S	24	S	ENR5	23	S	33	S	23	S	25	S	ENR5	25	S	20	I	23	S	21	I	ENR5	22	I	26	S	22	I	22	I
OFX5	24	S	24	S	20	S	22	S	OFX5	25	S	31	S	22	S	24	S	OFX5	24	S	19	S	22	S	19	S	OFX5	20	S	24	S	21	S	20	S
CIP5	34	S	33	S	30	S	34	S	CIP5	30	S	38	S	**34**	S	**26**	S	CIP5	30	S	39	S	34	S	31	S	CIP5	25	S	34	S	29	S	21	S
NA30	12	R	20	S	20	S	15	I	NA30	27	S	34	S	26	S	25	S	NA30	27	S	30	S	27	S	22	S	NA30	22	S	26	S	20	S	22	S
TE30	10	R	10	R	0	R	8	R	TE30	0	R	0	R	0	R	**0**	R	TE30	0	R	0	R	0	R	0	R	TE30	0	R	9	R	0	R	0	R
CN10	24	S	26	S	24	S	25	S	CN10	26	S	27	S	**26**	S	24	S	CN10	25	S	25	S	25	S	23	S	CN10	21	S	25	S	23	S	21	S
S10	23	S	24	S	21	S	23	S	S10	23	S	23	S	23	S	21	S	S10	23	S	27	S	24	S	19	S	S10	19	S	23	S	22	S	19	S
AK30	24	S	23	S	22	S	24	S	AK30	24	S	25.5	S	25	S	25	S	AK30	23	S	27	S	23	S	23	S	AK30	20	S	18	S	23	S	21	S
AZM15	16	I	17	I	13	R	18	S	AZM15	14	I	18	S	14	I	14	I	AZM15	15	I	19	S	17	I	16	I	AZM15	15	I	19	S	14	I	14	I
E15	0	R	12	R	0	R	9	R	E15	12	R	11	R	**10**	R	12	R	E15	11	R	12	R	13	R	9	R	E15	10	R	13	R	0	R	9	R

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 3B: Resistance, Intermediate and susceptibility profile of *A. culicicola* isolates

M22									M23								M25								M31							
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN	
AMP10	11	R	9	R	11	R	0	R	AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	9	R	0	R	0	R	AMP10	8.5	R	**0**	R	**9**	R	0	R
CPD10	29	S	24.5	S	26	S	21.5	S	CPD10	14.5	R	26.5	S	33.5	S	26	S	CPD10	12	R	27	S	10	R	12	R	CPD10	29	S	27	S	25	S	20	I
C30	27.5	S	18.5	S	15.5	I	23	S	C30	12	R	22.5	S	14.5	I	20	S	C30	13	I	25	S	11	R	15	I	C30	25.5	S	25	S	14.5	I	22	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	35	S	34	S	33.5	S	30	S	NOR10	27.5	S	30	S	23	S	24.5	S	NOR10	26	S	32	S	24	S	24	S	NOR10	34.5	S	35.5	S	29	S	24	S
ENR5	27	S	25.5	S	23	S	25	S	ENR5	25.5	S	25.5	S	22	I	25	S	ENR5	20	I	28	S	19	I	20	I	ENR5	27	S	24.5	S	22	I	**21.5**	I
OFX5	26.5	S	25	S	21	S	24	S	OFX5	24.5	S	24	S	23.5	S	23.5	S	OFX5	24	S	25	S	18	S	22	S	OFX5	24.5	S	24	S	22.5	S	21	S
CIP5	35.5	S	32.5	S	33.5	S	33	S	CIP5	31	S	29.5	S	29.5	S	33.5	S	CIP5	30	S	32	S	29	S	27	S	CIP5	34.5	S	36.5	S	30	S	26.5	S
NA30	31	S	21.5	S	23.5	S	25	S	NA30	30.5	S	28	S	28.5	S	30	S	NA30	24	S	28	S	21	S	24	S	NA30	25	S	28	S	23.5	S	18	I
TE30	9	R	0	R	0	R	9	R	TE30	10	R	0	R	0	R	0	R	TE30	0	S	10	R	0	R	0	R	TE30	12	R	**9**	R	**0**	R	0	R
CN10	31	S	26	S	26	S	24	S	CN10	25.5	S	26.5	S	27	S	25.5	S	CN10	25	S	25	S	25	S	23	S	CN10	25	S	27	S	**24**	S	**21.5**	S
S10	24	S	22.5	S	23.5	S	23	S	S10	25.5	S	23.5	S	21.5	S	21	S	S10	23	S	21	S	23	S	23	S	S10	26.5	S	21.5	S	**22.5**	S	23	S
AK30	29	S	24	S	25	S	23.5	S	AK30	30	S	25.5	S	27	S	25	S	AK30	22	S	22	S	24	S	24	S	AK30	25	S	26	S	**26**	S	23	S
AZM15	17	I	16.5	I	16	I	17.5	I	AZM15	25	S	17.5	I	19.5	S	16	I	AZM15	15	I	14	I	15	I	15	I	AZM15	16	I	18.5	R	**15.5**	I	20	S
E15	12	R	10	R	9	R	10.5	R	E15	13	R	13	R	9.5	R	10	R	E15	10	R	11	R	11	R	11	R	E15	12.5	R	**10**	R	9	R	**12**	R

M32									M39								M38								M58							
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	0	R	0	R	0	R	AMP10	9	R	12	R	11	R	10	R	AMP10	9.5	R	10.5	R	0	R	0	R
CPD10	30	S	24.5	S	**27.5**	S	24.5	S	CPD10	29.5	S	27	S	30	S	25.5	S	CPD10	28.5	S	30.5	S	26.5	S	29	S	CPD10	27.5	S	29	S	26	S	25.5	S
C30	19	S	18.5	S	13	I	**23.5**	S	C30	31	S	0	R	12	R	27	S	C30	21.5	S	27.5	S	12	R	29	S	C30	23.5	S	23.5	S	11.5	R	23.5	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	33	S	**28**	S	**29.5**	S	**29**	S	NOR10	27.5	S	29	S	27.5	S	25	S	NOR10	35	S	38	S	36	S	38	S	NOR10	34	S	33.5	S	32.5	S	31	S
ENR5	27.5	S	**24**	S	23	S	24.5	S	ENR5	27	S	26	S	22.5	I	22	I	ENR5	25.5	S	30.5	S	25	S	31	S	ENR5	25	S	23	S	23	S	24	S
OFX5	27.5	S	21.5	S	**20.5**	S	23	S	OFX5	27	S	25	S	21.5	S	23	S	OFX5	26	S	28.5	S	22.5	S	27	S	OFX5	24	S	21	S	21	S	23	S
CIP5	34.5	S	28.5	S	34	S	31.5	S	CIP5	34.5	S	31.5	S	33.5	S	31	S	CIP5	34	S	40	S	35.5	S	37.5	S	CIP5	34	S	33	S	34	S	32	S
NA30	30.5	S	26	S	**25.5**	S	25	S	NA30	27.5	S	27	S	23.5	S	23	S	NA30	30.5	S	31	S	26	S	28	S	NA30	23	S	28	S	24	S	24	S
TE30	9	R	0	R	**0**	R	0	R	TE30	0	R	0	R	0	R	19	S	TE30	9	R	15	I	0	R	0	R	TE30	0	R	0	R	0	R	9	R
CN10	29	S	24	S	**26**	S	25	S	CN10	29.5	S	12.5	I	25	S	21	S	CN10	28	S	28.5	S	27	S	24	S	CN10	26.5	S	25	S	25	S	25	S
S10	24	S	19.5	S	**21.5**	S	21	S	S10	21	S	0	R	21	S	25	S	S10	22.5	S	28	S	30	S	27.5	S	S10	25.5	S	27	S	25	S	25.5	S
AK30	29	S	24	S	26	S	24	S	AK30	28	S	23.5	S	24	S	24	S	AK30	24.5	S	27	S	30	S	25	S	AK30	25	S	25	S	25	S	24.5	S
AZM15	18.5	S	18	S	17	I	17.5	I	AZM15	18.5	S	12.5	R	13	R	20.5	S	AZM15	17.5	I	21.5	S	16	I	26	S	AZM15	20.5	S	18.5	S	16	I	13.5	R
E15	12.5	R	**12**	S	**10**	R	**14**	I	E15	0	R	9	R	0	R	15.5	I	E15	12	R	12.5	R	10	R	13	R	E15	13.5	R	11.5	R	10.5	R	12.5	S

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 4B: Resistance, Intermediate and susceptibility profile of *P. shigelloides* isolates

M45									M46								M9							
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN	
AMP10	0	R	13	R	0	R	7.5	R	AMP10	0	R	9	R	0	R	0	R	AMP10	0	R	0	R	0	R	0	R
CPD10	31	S	28.5	S	27.5	S	30	S	CPD10	0	R	11	R	10	R	0	R	CPD10	25	S	24	S	25	S	27	S
C30	27.5	S	28.5	S	22.5	S	28	S	C30	10	R	13	R	12	R	12.5	R	C30	17	I	17	I	12	R	18	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	39	S	40	S	36	S	39.5	S	NOR10	21	R	19	S	22	S	18.5	S	NOR10	27	S	35	S	28	S	33	S
ENR5	27	S	24.5	S	22.5	I	28.5	S	ENR5	19.5	I	19	I	20	I	21	I	ENR5	20	I	21	I	19	I	22	I
OFX5	26	S	26.5	S	20.5	S	25	S	OFX5	21	S	20	S	22	S	21	S	OFX5	25	S	23	S	22	S	24	S
CIP5	37	S	30.5	S	33.5	S	42.5	S	CIP5	24	S	27.5	S	28	S	26	S	CIP5	32	S	32	S	36	S	36	S
NA30	28	S	28	S	25	S	29	S	NA30	24	S	24.5	S	25.5	S	24.5	S	NA30	25	S	26	S	24	S	26	S
TE30	11	R	10	R	0	R	10	R	TE30	0	R	0	R	0	R	0	R	TE30	0	R	0	R	0	R	0	R
CN10	27	S	27	S	27.5	S	26	S	CN10	22.5	S	26	S	24	S	23.5	S	CN10	23	S	24	S	24	S	25	S
S10	27	S	25	S	27	S	26	S	S10	19.5	S	23	S	20	S	18.5	S	S10	18	S	21	S	20	S	20	S
AK30	26	S	26.5	S	25.5	S	26	S	AK30	24	S	25	S	22.5	S	23.5	S	AK30	22	S	24	S	23	S	24	S
AZM15	17.5	I	18	I	12.5	R	26.5	S	AZM15	28	S	18	S	16.5	I	16.5	I	AZM15	15	I	14	I	14	I	15	I
E15	12.5	R	12	R	10	R	15.5	I	E15	0	R	0	R	0	R	0	R	E15	0	R	0	R	0	R	0	R
M67									M66								M47							
	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN		CONTROL		CCCP		NMP		PABN	
AMP10	0	R	10	R	0	R	0	R	AMP10	0	R	0	R	0	R	0	R	AMP10	0	R	10	R	12	R	0	R
CPD10	27.5	S	28	S	27	S	25.5	S	CPD10	26	S	27	S	27	S	24.5	S	CPD10	25	S	27	S	27	S	25	S
C30	26.5	S	23.5	S	13	I	21	S	C30	26	S	23	S	15	I	20	S	C30	22	S	25	S	13	I	25	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	33.5	S	31.5	S	36.5	S	30.5	S	NOR10	32	S	33	S	35.5	S	32	S	NOR10	28	S	34	S	32	S	29	S
ENR5	25.5	S	24.5	S	22	I	24.5	S	ENR5	26	S	26	S	24	S	23	S	ENR5	25	S	26	S	22	S	24	S
OFX5	24.5	S	25	S	21.5	S	24	S	OFX5	23	S	24	S	21	S	24	S	OFX5	23	S	25	S	20	S	23	S
CIP5	32	S	36	S	31	S	36.5	S	CIP5	32	S	32.5	S	31.5	S	35	S	CIP5	28	S	32	S	32	S	29	S
NA30	25.5	S	25.5	S	25	S	25	S	NA30	26	S	25.5	S	25	S	24.5	S	NA30	25	S	28	S	22	S	24	S
TE30	0	R	0	R	0	R	0	R	TE30	0	R	0	R	0	R	0	R	TE30	0	R	9	R	0	R	0	R
CN10	24.5	S	25.5	S	26	S	26	S	CN10	25	S	25.5	S	26	S	27	S	CN10	25	S	26	S	24	S	23	S
S10	25	S	24	S	21.5	S	25.5	S	S10	25	S	24	S	20	S	26	S	S10	21	S	23	S	21	S	22	S
AK30	25	S	24	S	25	S	25	S	AK30	25	S	24	S	24	S	25	S	AK30	24	S	24	S	23	S	23	S
AZM15	20.5	S	24.5	S	16.5	I	20.5	S	AZM15	20	S	24.5	S	17	I	19.5	S	AZM15	18	S	17	I	14	I	14	I
E15	15	I	13.5	R	10.5	R	14	I	E15	20	I	20	I	9	R	15	I	E15	12	R	11	R	9	R	9	R

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 5B: Resistance, Intermediate and susceptibility profile of *Aeromonas* and *A. veronii*

Aeromonas spp									M26									M34								
M41	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	0	R	9	R	0	R	0	R	AMP10	0	R	0	R	11	R	23	S	AMP10	33	S	25	S	26	S	20	S
CPD10	33.5	S	26.5	S	36	S	33.5	S	CPD10	30	S	14	R	15.5	R	33.5	S	CPD10	0	R	0	R	9.5	R	0	R
C30	24	S	26.5	S	14.5	S	24	S	C30	22	S	13	I	28	S	30.5	S	C30	33	S	25.5	S	27	S	20.5	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	11.5	I	0	R	0	R
NOR10	32.5	S	33.5	S	31	S	32	S	NOR10	28	S	27.5	S	28	S	37	S	NOR10	24	S	29	S	29	S	24.5	S
ENR5	28	S	28.5	S	24.5	S	28.5	S	ENR5	30	S	27.5	S	22.5	I	33	S	ENR5	28	S	33	S	35	S	26.5	S
OFX5	26	S	29.5	S	24.5	S	27.5	S	OFX5	27.5	S	26	S	22	S	28.5	S	OFX5	24	S	33	S	36	S	24.5	S
CIP5	33	S	32.5	S	39	S	35.5	S	CIP5	35.5	S	31.5	S	39	S	40.5	S	CIP5	27.5	S	37	S	28	S	29	S
NA30	27.5	S	29	S	13.5	R	23	S	NA30	18.5	I	11	R	26	S	27	S	NA30	0	R	32	S	31	S	26	S
TE30	0	R	9	R	9	R	0	R	TE30	0	R	9	R	10	R	10	R	TE30	36.5	S	33	S	30	S	15.5	R
CN10	27	S	26	S	27	S	26	S	CN10	28.5	S	33	S	25.5	S	23.5	S	CN10	23	S	26	S	23	S	26	S
S10	20	S	23	S	23.5	S	20.5	S	S10	29	S	26	S	28	S	24	S	S10	14	I	9.5	R	21	S	28	S
AK30	26	S	25	S	28	S	24	S	AK30	28	S	28	S	26	S	27	S	AK30	14.5	R	27	S	32.5	S	25.5	S
AZM15	15.5	I	22.5	S	15	I	16.5	I	AZM15	24.5	S	27	S	27	S	24	S	AZM15	27	S	30.5	S	28	S	29.5	S
E15	0	R	11	R	9.5	R	9.5	R	E15	0	R	0	R	15.5	I	7	R	E15	27	S	39	S	27.5	S	26.5	S
A. veronii									M55									M63								
M57	CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN			CONTROL		CCCP		NMP		PABN	
AMP10	15.5	I	0	I	0	I	15.5	I	AMP10	0	R	0	R	0	R	9	R	AMP10	0	R	0	R	0	R	0	R
CPD10	30.5	S	29.5	S	17	R	12	R	CPD10	26	S	24	S	22.5	S	22.5	S	CPD10	14	R	13.5	R	13	R	13	R
C30	20	S	17.5	I	10	R	16	I	C30	21	S	25	S	9	R	22	S	C30	12	R	15	I	12	R	14	R
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	25	S	36.5	S	37	S	17	S	NOR10	35	S	29	S	31.5	S	31.5	S	NOR10	20.5	S	23.5	S	23	S	24.5	S
ENR5	25.5	S	24.5	S	23	S	21	I	ENR5	24	S	21.5	I	21.5	S	24	S	ENR5	19	I	19	I	19	I	18	I
OFX5	29	S	27	S	23.5	S	20	S	OFX5	24	S	25.5	S	20	S	22	S	OFX5	19.5	S	19	S	20	S	22	S
CIP5	34	S	38	S	35.5	S	30	S	CIP5	31	S	30.5	S	32	S	32.5	S	CIP5	31	S	30.5	S	30.5	S	31	S
NA30	32.5	S	31.5	S	27.5	S	28	S	NA30	27	S	22	S	22	S	25.5	S	NA30	12	R	12	R	11	R	12	R
TE30	0	R	0	R	0	R	0	R	TE30	0	R	0	R	0	R	0	R	TE30	8	R	8	R	0	R	9	R
CN10	29	S	27	S	27.5	S	26	S	CN10	23.5	S	21.5	S	24.5	S	24	S	CN10	16.5	S	23	S	15.5	S	15.5	S
S10	28	S	29	S	21.5	S	18	S	S10	24.5	S	20.5	S	23.5	S	22	S	S10	25	S	25	S	24.5	S	26.5	S
AK30	28	S	32	S	26	S	25	S	AK30	25	S	23	S	23	S	25	S	AK30	27	S	25	S	25.5	S	26	S
AZM15	17.5	R	20	S	13	R	15	I	AZM15	22	S	16.5	I	14.5	I	20	S	AZM15	24.5	S	26	S	23.5	S	27	S
E15	13	R	10	R	0	R	0	R	E15	12.5	R	11	R	9	R	13.5	R	E15	8	R	10	R	0	R	10	R

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 6B: Resistance, Intermediate and susceptibility profile of *A. jandae, A. sobria* and *A. caviae*

A. Jandaei

41	CONTROL		CCCP		NMP		PABN	
AMP10	9	R	10	R	9	R	0	R
CPD10	28.5	S	23.5	S	24	S	20	I
C30	19	S	24.5	S	11.5	R	19	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	30.5	S	31	S	31	S	28	S
ENR5	29	S	22	I	25	S	25.5	S
OFX5	27	S	21.5	S	25	S	22.5	S
CIP5	32	S	33	S	31	S	25	S
NA30	29	S	23	S	25	S	24.5	S
TE30	0	R	0	R	0	R	0	R
CN10	26	S	23	S	24	S	27	S
S10	26	S	20.5	S	22	S	20.5	S
AK30	29	S	23	S	28.5	S	21	S
AZM15	19	S	16.5	I	20	S	15	I
E15	12	R	10	R	11.5	R	10.5	R

A. sobria

M49	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	10	R	10	R	0	R
CPD10	0	R	27	S	18	I	0	R
C30	19	S	26	S	16	I	18	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	22	S	26	S	25	S	21	S
ENR5	19	I	23	S	13	R	17	I
OFX5	20	S	23	S	14	I	19	S
CIP5	23	S	25.5	S	25	S	22	S
NA30	14	I	18	I	14	I	13	R
TE30	9	R	10.5	R	0	R	0	R
CN10	21	S	24	S	19	S	19	S
S10	19	S	24	S	20	S	19	S
AK30	21	S	23	S	21	S	19	S
AZM15	15	I	16	I	12	R	14	I
E15	12	R	11	R	10	R	10	R

A.caviae

M18	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	9	R	0	R	0	R
CPD10	24	S	26	S	26	S	26	S
C30	15	I	24	S	10	R	22	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	27	S	30	S	23	S	30	S
ENR5	22	S	35	S	25	S	29	S
OFX5	27	S	27	S	24	S	26	S
CIP5	27	S	30	S	25	S	36	S
NA30	14	I	23	S	0	R	29	S
TE30	0	R	12	R	0	R	9	R
CN10	25	S	24	S	26	S	26	S
S10	22	S	19	S	23	S	23	S
AK30	24	S	21	S	25	S	24	S
AZM15	13	R	14	I	13	R	23	S
E15	0	R	9	R	0	R	0	R

M68	CONTROL		CCCP		NMP		PABN	
AMP10	13	R	13	R	10	R	0	R
CPD10	29	S	33.5	S	26.5	S	28.5	S
C30	21.5	S	21.5	S	17	I	24	S
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	38	S	35.5	S	33.5	S	36	S
ENR5	25.5	S	25	S	23	S	25	S
OFX5	24.5	S	25	S	23	S	25	S
CIP5	34	S	33	S	35	S	30	S
NA30	28.5	S	14	I	26	S	24	S
TE30	11	R	9	R	0	R	0	R
CN10	18	S	19	S	17.5	S	22.5	S
S10	26.5	S	26.5	S	25	S	27.5	S
AK30	25.5	S	27	S	26	S	26	S
AZM15	16	I	19	S	19.5	S	25	S
E15	12	R	13	R	10	R	13	R

M59	CONTROL		CCCP		NMP		PABN	
AMP10	0	R	0	R	0	R	0	R
CPD10	25.5	S	18	I	19.5	I	0	R
C30	24	S	18.5	S	11	R	16	I
W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R
NOR10	26.5	S	29.5	S	20.5	S	22.5	S
ENR5	24	S	21	I	20	I	21.5	I
OFX5	24	S	21	S	19.5	S	23	S
CIP5	27.5	S	31	S	26	S	25.5	S
NA30	26.5	S	27.5	S	21	S	20	S
TE30	0	R	0	R	0	R	0	R
CN10	25.5	S	22	S	22	S	21	S
S10	22.5	S	21	S	22	S	21	S
AK30	24	S	19.5	S	23	S	20	S
AZM15	20	S	20	S	16	I	16.5	I
E15	10.5	R	10.5	R	0	R	0	R

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 7B: Resistance, Intermediate and susceptibility profile of *A. allosacharophila* and *A. salmonicida*

A. salmonicida									M76								
M77	**CONTROL**		**CCCP**		**NMP**		**PABN**			**CONTROL**		**CCCP**		**NMP**		**PABN**	
AMP10	8	R	9	R	0	R	0	R	AMP10	10	R	9.5	R	0	R	11	R
CPD10	20	I	22	S	18	I	24	S	CPD10	28	S	25	S	23.5	S	26	S
C30	21	S	24	S	18	S	20	S	C30	26.5	S	26.5	S	11.5	R	23.5	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	0	R	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	27	S	26	S	29	S	29	S	NOR10	34.5	S	33.5	S	30	S	36	S
ENR5	23	S	23	S	18	I	22	I	ENR5	25	S	22	I	20.5	I	23	S
OFX5	23	S	23	S	17	S	24	S	OFX5	23.5	S	22.5	S	20	S	22	S
CIP5	28	S	30	S	29	S	30	S	CIP5	36	S	34.5	S	30.5	S	36.5	S
NA30	24	S	25	S	19	S	24	S	NA30	27	S	23	S	22.5	S	25.5	S
TE30	0	R	10	R	0	R	0	R	TE30	0	R	0	R	0	R	0	R
CN10	23	S	24	S	23	S	23	S	CN10	26	S	23	S	22	S	23.5	S
S10	19	S	22	S	22	S	20	S	S10	24.5	S	26.5	S	23.5	S	24.5	S
AK30	23	S	23	S	23	S	23	S	AK30	26	S	27	S	23.5	S	25	S
AZM15	16	I	17	I	13	R	20	S	AZM15	19.5	S	22	S	13.5	R	20	S
E15	10	R	12	R	0	R	12	R	E15	11	R	12	R	12	R	14	I
A.allosaccharophila									*A.allosacharophila*								
M92	**CONTROL**		**CCCP**		**NMP**		**PABN**		**M8**	**CONTROL**		**CCCP**		**NMP**		**PABN**	
AMP10	16	I	14	I	0	R	0	R	AMP10	0	R	0	R	0	R	0	R
CPD10	27	S	28	S	27	S	28	S	CPD10	10	R	21	S	14.5	R	15	R
C30	14	I	18	S	11	R	21	S	C30	12	R	29.5	S	15	I	26	S
W1.25	0	R	0	R	0	R	0	R	W1.25	0	R	25.5	S	0	R	0	R
RL25	0	R	0	R	0	R	0	R	RL25	0	R	0	R	0	R	0	R
NOR10	35	S	36	S	33	S	40	S	NOR10	21	S	18.5	S	17	S	15	I
ENR5	25	S	24	S	22	I	26	S	ENR5	26	S	13	R	10.5	R	16	I
OFX5	27	S	27	S	23	S	27	S	OFX5	24	S	14	I	15	I	15.5	I
CIP5	36	S	38	S	35	S	36	S	CIP5	26.5	S	19	I	22	S	14	R
NA30	30	S	33	S	27	S	28	S	NA30	0	R	0	R	0	R	34	S
TE30	0	R	0	R	0	R	0	R	TE30	18.5	I	0	R	0	R	20.5	S
CN10	25	S	23	S	25	S	24	S	CN10	28.5	S	31	S	28.5	S	25.5	S
S10	21	S	21	S	22	S	23	S	S10	24.5	S	27	S	22.5	S	26	S
AK30	24	S	23	S	27	S	26	S	AK30	28.5	S	32.5	S	29	S	29.5	S
AZM15	13	R	10	R	13	R	14	I	AZM15	21	S	22	S	25	S	29.5	S
E15	0	R	0	R	0	R	0	R	E15	10	R	14.5	I	9	R	20.5	S

*R = Reistant, *I = intermediately susceptible, *S = susceptible

Table 8B: EPIs treatments absorbance readings after 24 h

	Average	stDev	Average	StDev	Average	StDev	Average	StDev	Average		Average	Stdv	Average	Stdv		Stdv
	UNTREATED		CCCP		UNTREATED		PAβN		UNTREATI	Stdv	NMP		UNTREATED		DNase I	
TSB 30 SH	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001	0.145	0.001
M2	1.176	0.126	0.148	0.048	1.176	0.126	0.185	0.062	1.176	0.126	0.153	0.075	1.176	0.126	0.419	0.093
M5	0.866	0.090	0.124	0.050	0.866	0.090	0.262	0.109	0.866	0.090	0.165	0.078	0.866	0.090	0.297	0.087
M6	1.063	0.085	0.129	0.035	1.063	0.085	1.031	0.102	1.063	0.085	0.124	0.014	1.063	0.085	0.454	0.055
M13	1.078	0.067	0.179	0.153	1.078	0.067	0.850	0.115	1.078	0.067	0.184	0.078	1.078	0.067	1.009	0.096
M14	1.015	0.103	0.550	0.058	1.015	0.103	0.766	0.108	1.015	0.103	0.382	0.078	1.015	0.103	0.951	0.095
M17	0.896	0.115	0.158	0.049	0.896	0.115	0.762	0.270	0.896	0.115	0.345	0.093	0.896	0.115	1.378	0.223
M50	0.840	0.111	0.133	0.046	0.840	0.111	1.306	0.114	0.840	0.111	0.172	0.073	0.840	0.111	1.137	0.067
M51	0.676	0.083	0.179	0.145	0.676	0.083	0.521	0.070	0.676	0.083	0.219	0.056	0.676	0.083	0.597	0.043
M52	1.420	0.105	0.236	0.058	1.420	0.105	0.678	0.127	1.420	0.105	0.428	0.091	1.420	0.105	0.757	0.079
M53	0.928	0.089	0.336	0.220	0.928	0.089	0.157	0.044	0.928	0.089	0.164	0.071	0.928	0.089	0.806	0.080
M60	0.517	0.086	0.177	0.096	0.517	0.086	0.731	0.104	0.517	0.086	0.272	0.054	0.517	0.086	1.205	0.057
M62	0.980	0.107	0.183	0.005	0.980	0.107	1.039	0.093	0.980	0.107	0.437	0.198	0.980	0.107	0.958	0.088
M64	0.843	0.092	0.143	0.036	0.843	0.092	0.948	0.082	0.843	0.092	0.184	0.066	0.843	0.092	0.876	0.087
M65	1.363	0.169	0.132	0.043	1.363	0.169	0.693	0.107	1.363	0.169	0.150	0.030	1.363	0.169	1.419	0.185
M86	0.828	0.172	0.144	0.045	0.828	0.172	0.989	0.053	0.828	0.172	0.177	0.065	0.828	0.172	0.105	0.005
M94	0.257	0.059	0.124	0.009	0.257	0.059	0.769	0.073	0.257	0.059	0.322	0.109	0.257	0.059	0.632	0.080
M95	0.827	0.105	0.182	0.041	0.827	0.105	0.764	0.081	0.827	0.105	0.221	0.082	0.827	0.105	0.591	0.096
ATCC 7966T	0.072	0.072	0.089	0.008	0.072	0.072	0.373	0.029	0.072	0.072	0.118	0.036	0.072	0.072	0.389	0.063
M70	0.822	0.079	0.453	0.054	0.822	0.079	0.304	0.008	0.822	0.079	0.261	0.094	0.822	0.079	0.442	0.050
M72	0.973	0.078	0.258	0.089	0.973	0.078	0.307	0.096	0.973	0.078	0.142	0.063	0.973	0.078	0.651	0.086
M80	1.171	0.062	0.208	0.012	1.171	0.062	0.942	0.053	1.171	0.062	0.154	0.027	1.171	0.062	1.118	0.123
M81	1.071	0.099	0.104	0.017	1.071	0.099	0.543	0.069	1.071	0.099	0.102	0.014	1.071	0.099	0.269	0.069
M88	0.624	0.033	0.637	0.191	0.624	0.033	0.350	0.076	0.624	0.033	0.465	0.083	0.624	0.033	0.337	0.094
M90	0.509	0.022	0.180	0.040	0.509	0.022	0.815	0.097	0.509	0.022	0.234	0.051	0.509	0.022	1.120	0.037
M96	1.010	0.060	0.169	0.068	1.010	0.060	0.961	0.105	1.010	0.060	0.159	0.057	1.010	0.060	0.848	0.079
M99	0.839	0.120	0.202	0.017	0.839	0.120	0.377	0.047	0.839	0.120	0.241	0.051	0.839	0.120	1.009	0.086
M22	0.902	0.151	0.181	0.080	0.902	0.151	1.223	0.219	0.902	0.151	0.144	0.028	0.902	0.151	0.786	0.096
M23	0.992	0.167	1.075	0.041	0.992	0.167	0.918	0.133	0.992	0.167	0.239	0.030	0.992	0.167	0.882	0.069
M25	0.719	0.078	0.235	0.063	0.719	0.078	0.180	0.025	0.719	0.078	0.169	0.092	0.719	0.078	0.995	0.104
M31	0.798	0.059	0.594	0.033	0.798	0.059	0.193	0.013	0.798	0.059	0.201	0.053	0.798	0.059	0.382	0.022
M32	0.898	0.100	0.317	0.089	0.898	0.100	0.672	0.017	0.898	0.100	0.189	0.047	0.898	0.100	0.735	0.052
M38	1.210	0.020	1.015	0.110	1.210	0.020	0.997	0.132	1.210	0.020	0.203	0.047	1.210	0.020	0.615	0.127
M39	0.817	0.078	0.321	0.103	0.817	0.078	0.781	0.046	0.817	0.078	0.313	0.088	0.817	0.078	0.975	0.087
M58	0.625	0.059	0.128	0.012	0.625	0.059	1.043	0.066	0.625	0.059	0.390	0.039	0.625	0.059	0.956	0.088
M26	0.921	0.072	0.259	0.106	0.921	0.072	0.986	0.079	0.921	0.072	0.685	0.090	0.921	0.072	1.086	0.093
M34	1.322	0.147	0.128	0.018	1.322	0.147	0.841	0.150	1.322	0.147	0.163	0.038	1.322	0.147	0.864	0.059
M41	0.619	0.082	1.109	0.111	0.619	0.082	0.768	0.087	0.619	0.082	0.440	0.038	0.619	0.082	0.834	0.110
M18	0.519	0.066	0.193	0.092	0.519	0.066	0.424	0.073	0.519	0.066	0.224	0.013	0.519	0.066	0.514	0.052
M59	0.909	0.249	0.377	0.089	0.909	0.249	1.254	0.131	0.909	0.249	0.222	0.052	0.909	0.249	1.062	0.115
M68	0.878	0.098	0.220	0.088	0.878	0.098	0.984	0.090	0.878	0.098	0.679	0.023	0.878	0.098	0.919	0.043
ATCC15468T	0.044	0.044	0.084	0.004	0.044	0.044	0.171	0.072	0.044	0.044	0.126	0.035	0.044	0.044	0.238	0.048
M55	0.891	0.061	0.323	0.025	0.891	0.061	0.133	0.013	0.891	0.061	0.274	0.084	0.891	0.061	0.454	0.071
M57	0.541	0.045	0.515	0.064	0.541	0.045	0.669	0.079	0.541	0.045	0.254	0.094	0.541	0.045	0.503	0.121
M63	0.545	0.018	0.199	0.051	0.545	0.018	0.825	0.092	0.545	0.018	0.320	0.082	0.545	0.018	1.000	0.074
M8	0.519	0.047	0.124	0.028	0.519	0.047	0.764	0.149	0.519	0.047	0.144	0.019	0.519	0.047	0.835	0.062
M92	0.921	0.208	0.160	0.022	0.921	0.208	1.175	0.044	0.921	0.208	0.267	0.107	0.921	0.208	0.126	0.009
M76	0.928	0.049	0.179	0.037	0.928	0.049	0.149	0.032	0.928	0.049	0.370	0.109	0.928	0.049	0.765	0.029
M77	0.804	0.150	0.375	0.066	0.804	0.150	0.733	0.097	0.804	0.150	0.269	0.041	0.804	0.150	0.683	0.081
M28	0.797	0.082	0.460	0.112	0.797	0.082	0.116	0.002	0.797	0.082	0.198	0.068	0.797	0.082	0.427	0.031
M49	0.989	0.143	0.504	0.082	0.989	0.143	0.206	0.091	0.989	0.143	0.185	0.036	0.989	0.143	0.441	0.130
M9	0.498	0.085	0.367	0.139	0.498	0.085	0.399	0.041	0.498	0.085	0.229	0.024	0.498	0.085	0.465	0.050
M45	0.919	0.032	0.184	0.027	0.919	0.032	0.758	0.031	0.919	0.032	0.238	0.021	0.919	0.032	1.186	0.065
M46	1.184	0.241	0.328	0.091	1.184	0.241	0.713	0.005	1.184	0.241	0.277	0.085	1.184	0.241	1.118	0.073
M47	0.982	0.056	0.309	0.106	0.982	0.056	0.892	0.070	0.982	0.056	0.221	0.092	0.982	0.056	0.852	0.096
M66	0.752	0.087	0.760	0.029	0.752	0.087	0.872	0.071	0.752	0.087	0.321	0.017	0.752	0.087	1.043	0.022
M67	1.035	0.166	0.140	0.046	1.035	0.166	0.427	0.101	1.035	0.166	0.136	0.033	1.035	0.166	0.777	0.063

Table 9 B: EPIs treatments absorbance readings after 48 h

	Average UNTREATED	stDev	Average CCCP	StDev	Average UNTREATED	StDev	Average PAβN	StDev	Average UNTREATI	Stdv	Average NMP	Stdv	Average UNTREATED	Stdv	DNase I	Stdv
TSB 30 SH	0.170	0.040	0.170	0.040	0.170	0.040	0.170	0.040	0.170	0.040	0.170	0.040	0.170	0.040	0.170	0.040
M2	0.667	0.077	0.213	0.027	0.667	0.077	0.154	0.038	0.667	0.077	0.171	0.032	0.667	0.077	0.530	0.050
M5	0.388	0.145	0.239	0.088	0.388	0.145	0.203	0.039	0.388	0.145	0.183	0.033	0.388	0.145	0.456	0.079
M6	1.673	0.198	0.507	0.043	1.673	0.198	0.417	0.064	1.673	0.198	0.165	0.023	1.673	0.198	1.083	0.098
M13	0.779	0.103	0.168	0.018	0.779	0.103	0.753	0.157	0.779	0.103	0.246	0.084	0.779	0.103	1.074	0.087
M14	1.677	0.039	1.408	0.200	1.677	0.039	0.426	0.038	1.677	0.039	0.560	0.109	1.677	0.039	0.929	0.078
M17	1.006	0.065	0.154	0.019	1.006	0.065	1.377	0.190	1.006	0.065	0.350	0.084	1.006	0.065	1.252	0.051
M50	0.449	0.082	0.273	0.047	0.449	0.082	0.199	0.060	0.449	0.082	0.175	0.062	0.449	0.082	0.397	0.082
M51	0.618	0.137	0.199	0.057	0.618	0.137	0.570	0.099	0.618	0.137	0.193	0.084	0.618	0.137	0.663	0.093
M52	1.253	0.099	0.507	0.085	1.253	0.099	0.437	0.030	1.253	0.099	0.358	0.099	1.253	0.099	0.323	0.076
M53	0.827	0.145	0.198	0.007	0.827	0.145	0.199	0.027	0.827	0.145	0.174	0.047	0.827	0.145	0.324	0.100
M60	0.948	0.077	0.316	0.048	0.948	0.077	0.574	0.326	0.948	0.077	0.318	0.097	0.948	0.077	0.412	0.027
M62	1.387	0.256	0.538	0.033	1.387	0.256	0.050	0.001	1.387	0.256	0.326	0.034	1.387	0.256	0.735	0.046
M64	0.848	0.201	0.597	0.088	0.848	0.201	0.607	0.104	0.848	0.201	0.197	0.048	0.848	0.201	0.729	0.125
M65	0.337	0.099	0.315	0.064	0.337	0.099	0.190	0.085	0.337	0.099	0.182	0.042	0.337	0.099	0.366	0.058
M86	0.557	0.093	0.430	0.088	0.557	0.093	0.318	0.097	0.557	0.093	0.271	0.027	0.557	0.093	0.454	0.099
M94	0.658	0.078	0.241	0.067	0.658	0.078	0.638	0.023	0.658	0.078	0.295	0.104	0.658	0.078	0.852	0.045
M95	0.604	0.183	0.543	0.089	0.604	0.183	0.603	0.049	0.604	0.183	0.191	0.077	0.604	0.183	0.585	0.094
ATCC 7966T	2.243	0.198	0.800	0.167	2.243	0.198	0.088	0.009	2.243	0.198	0.297	0.030	2.243	0.198	0.727	0.053
M70	1.153	0.185	0.218	0.073	1.153	0.185	0.346	0.184	1.153	0.185	0.339	0.057	1.153	0.185	0.432	0.057
M72	0.573	0.033	0.409	0.095	0.573	0.033	0.314	0.044	0.573	0.033	0.273	0.074	0.573	0.033	0.357	0.175
M80	0.532	0.065	0.346	0.073	0.532	0.065	0.217	0.068	0.532	0.065	0.154	0.027	0.532	0.065	0.360	0.076
M81	1.713	0.140	0.672	0.121	1.713	0.140	0.351	0.092	1.713	0.140	0.345	0.076	1.713	0.140	0.340	0.063
M88	0.899	0.050	0.393	0.014	0.899	0.050	0.356	0.087	0.899	0.050	0.342	0.018	0.899	0.050	1.135	0.060
M90	0.469	0.105	0.461	0.081	0.469	0.105	0.163	0.004	0.469	0.105	0.201	0.024	0.469	0.105	0.546	0.094
M96	0.400	0.061	0.343	0.038	0.400	0.061	0.214	0.066	0.400	0.061	0.166	0.068	0.400	0.061	0.532	0.142
M99	0.469	0.088	0.533	0.107	0.469	0.088	0.327	0.097	0.469	0.088	0.209	0.030	0.469	0.088	0.488	0.074
M22	0.416	0.122	0.425	0.084	0.416	0.122	0.377	0.099	0.416	0.122	0.231	0.089	0.416	0.122	0.474	0.079
M23	1.486	0.130	0.157	0.018	1.486	0.130	0.624	0.063	1.486	0.130	0.201	0.048	1.486	0.130	0.697	0.058
M25	0.639	0.055	0.367	0.067	0.639	0.055	0.318	0.046	0.639	0.055	0.250	0.080	0.639	0.055	0.643	0.092
M31	1.189	0.169	0.838	0.043	1.189	0.169	0.449	0.059	1.189	0.169	0.336	0.036	1.189	0.169	0.625	0.105
M32	0.861	0.051	0.669	0.074	0.861	0.051	0.323	0.026	0.861	0.051	0.376	0.061	0.861	0.051	0.863	0.015
M38	1.119	0.100	0.221	0.072	1.119	0.100	0.913	0.089	1.119	0.100	0.251	0.106	1.119	0.100	0.605	0.117
M39	0.955	0.062	0.807	0.077	0.955	0.062	0.462	0.102	0.955	0.062	0.579	0.098	0.955	0.062	0.870	0.004
M58	1.185	0.041	0.566	0.149	1.185	0.041	0.848	0.053	1.185	0.041	0.192	0.092	1.185	0.041	0.549	0.211
M26	0.703	0.076	0.690	0.043	0.703	0.060	0.048	0.000	0.703	0.060	0.320	0.045	0.703	0.060	1.035	0.144
M34	0.635	0.064	0.366	0.027	0.635	0.064	0.158	0.028	0.635	0.064	0.139	0.027	0.635	0.064	0.946	0.114
M41	1.567	0.060	0.987	0.011	1.567	0.076	0.473	0.108	1.567	0.076	0.495	0.083	1.567	0.076	0.713	0.027
M18	1.848	0.053	0.764	0.031	1.848	0.053	0.291	0.021	1.848	0.053	0.434	0.090	1.848	0.053	1.564	0.056
M59	0.845	0.084	0.762	0.097	0.845	0.084	0.240	0.064	0.845	0.084	0.336	0.036	0.845	0.084	0.667	0.049
M68	1.537	0.083	0.819	0.071	1.537	0.083	0.397	0.040	1.537	0.083	0.470	0.104	1.537	0.083	0.942	0.060
ATCC15468T	1.537	0.001	0.845	0.036	1.537	0.001	0.103	0.008	1.537	0.001	0.355	0.030	1.537	0.001	0.681	0.156
M55	1.240	0.116	0.909	0.006	1.240	0.116	0.215	0.110	1.240	0.116	0.326	0.036	1.240	0.116	0.583	0.136
M57	1.595	0.422	0.636	0.098	1.595	0.422	0.270	0.005	1.595	0.422	0.380	0.090	1.595	0.422	1.056	0.115
M63	0.542	0.083	0.741	0.072	0.542	0.083	0.546	0.068	0.542	0.083	0.359	0.089	0.542	0.083	0.434	0.064
M8	0.834	0.194	0.416	0.096	0.834	0.194	0.250	0.052	0.834	0.194	0.223	0.077	0.834	0.194	0.728	0.048
M92	0.888	0.114	0.644	0.142	0.888	0.114	0.229	0.022	0.888	0.114	0.339	0.057	0.888	0.114	1.165	0.083
M76	1.625	0.099	0.878	0.079	1.625	0.099	1.200	0.030	1.625	0.099	0.342	0.018	1.625	0.099	0.514	0.206
M77	0.595	0.135	0.721	0.052	0.595	0.135	0.330	0.064	0.595	0.135	0.237	0.034	0.595	0.135	0.512	0.080
M28	1.436	0.271	0.448	0.142	1.436	0.271	0.155	0.015	1.436	0.271	0.434	0.090	1.436	0.271	0.611	0.258
M49	1.148	0.110	0.942	0.029	1.148	0.110	0.175	0.046	1.148	0.110	0.380	0.090	1.148	0.110	1.173	0.258
M9	1.495	0.255	0.883	0.051	1.495	0.255	0.275	0.036	1.495	0.255	0.326	0.036	1.495	0.255	1.034	0.004
M45	1.259	0.182	0.690	0.093	1.259	0.182	0.548	0.011	1.259	0.182	0.232	0.027	1.259	0.182	0.290	0.008
M46	0.832	0.043	0.215	0.021	0.832	0.043	0.384	0.082	0.832	0.043	0.249	0.080	0.832	0.043	0.853	0.072
M47	0.992	0.096	0.671	0.098	0.992	0.096	0.432	0.037	0.992	0.096	0.498	0.077	0.992	0.096	0.863	0.016
M66	1.343	0.068	0.306	0.096	1.343	0.068	0.638	0.120	1.343	0.068	0.243	0.087	1.343	0.068	0.518	0.130
M67	2.061	0.119	0.319	0.076	2.061	0.119	0.172	0.014	2.061	0.119	0.200	0.053	2.061	0.119	0.796	0.063

APPENDIX C: DATA FOR CHAPTER 4

Table 1 C: QSIs treatments absorbance readings after 24 h

	UNTREATED		CINNAMALDEHYDE		UNTREATED		FURANONE		UNTREATED		SAHC		UNTREATED		VANILLIN	
	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev
TSB 30 SH	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038
M2	0.754	0.083	0.774	0.084	0.754	0.084	0.768	0.039	0.754	0.084	0.681	0.070	0.754	0.084	0.703	0.050
M5	0.382	0.027	0.386	0.060	0.382	0.060	0.499	0.056	0.382	0.060	0.404	0.071	0.382	0.060	0.362	0.067
M6	0.868	0.078	0.882	0.090	0.868	0.090	0.726	0.146	0.868	0.090	0.460	0.062	0.868	0.090	0.381	0.041
M13	0.407	0.024	0.264	0.086	0.407	0.086	0.406	0.069	0.407	0.086	0.512	0.027	0.407	0.086	0.377	0.077
M14	0.624	0.134	0.557	0.015	0.624	0.015	0.757	0.060	0.624	0.015	0.359	0.015	0.624	0.015	0.466	0.061
M17	0.336	0.043	0.330	0.089	0.336	0.089	0.543	0.103	0.336	0.089	0.274	0.065	0.336	0.089	0.324	0.069
M50	1.282	0.136	1.277	0.118	1.282	0.118	0.896	0.051	1.282	0.118	0.828	0.043	1.282	0.118	1.355	0.111
M51	0.386	0.046	0.341	0.079	0.386	0.079	0.324	0.081	0.386	0.079	0.217	0.077	0.386	0.079	0.405	0.064
M52	1.238	0.016	0.862	0.205	1.238	0.205	0.932	0.095	1.238	0.205	1.211	0.109	1.238	0.205	0.900	0.062
M53	0.492	0.051	0.463	0.093	0.492	0.093	0.369	0.076	0.492	0.093	0.353	0.058	0.492	0.093	0.698	0.067
M60	1.516	0.493	1.242	0.164	1.516	0.164	0.585	0.099	1.516	0.164	0.708	0.093	1.516	0.164	0.439	0.067
M62	1.267	0.196	1.308	0.200	1.267	0.200	0.920	0.091	1.267	0.200	1.213	0.159	1.267	0.200	1.872	0.023
M64	0.421	0.076	0.318	0.065	0.421	0.065	0.269	0.080	0.421	0.065	0.380	0.094	0.421	0.065	0.338	0.095
M65	0.381	0.039	0.778	0.073	0.381	0.073	0.338	0.046	0.381	0.073	0.382	0.090	0.381	0.073	0.364	0.030
M86	0.871	0.048	0.350	0.005	0.871	0.005	0.243	0.050	0.871	0.005	0.267	0.048	0.871	0.005	0.311	0.029
M94	0.248	0.085	0.211	0.054	0.248	0.054	0.287	0.091	0.248	0.054	0.448	0.097	0.248	0.054	0.434	0.067
M95	0.406	0.081	0.538	0.082	0.406	0.082	0.564	0.072	0.406	0.082	0.511	0.090	0.406	0.082	0.386	0.090
ATCC 7966^T	0.637	0.094	0.622	0.064	0.637	0.064	1.058	0.155	0.637	0.064	0.772	0.078	0.637	0.064	0.854	0.093
M70	1.359	0.054	0.788	0.468	1.359	0.468	0.582	0.142	1.359	0.468	0.812	0.082	1.359	0.468	0.536	0.084
M72	1.238	0.101	1.114	0.207	1.238	0.207	1.115	0.073	1.238	0.207	0.826	0.071	1.238	0.207	1.369	0.220
M80	0.625	0.095	0.645	0.061	0.625	0.061	0.701	0.105	0.625	0.061	0.409	0.019	0.625	0.061	0.634	0.091
M81	0.987	0.037	0.867	0.050	0.987	0.050	0.378	0.063	0.987	0.050	0.520	0.166	0.987	0.050	0.929	0.083
M88	0.472	0.087	0.304	0.069	0.472	0.069	0.453	0.051	0.472	0.069	0.395	0.076	0.472	0.069	0.517	0.087
M90	0.907	0.039	0.689	0.087	0.907	0.087	0.588	0.069	0.907	0.087	0.751	0.074	0.907	0.087	0.888	0.097
M96	0.744	0.054	0.749	0.045	0.744	0.045	1.015	0.115	0.744	0.045	0.636	0.027	0.744	0.045	0.639	0.027
M99	1.181	0.047	0.708	0.113	1.181	0.113	0.895	0.048	1.181	0.113	0.960	0.029	1.181	0.113	1.223	0.182
M22	0.762	0.006	0.289	0.024	0.762	0.024	0.366	0.026	0.762	0.024	0.566	0.048	0.762	0.024	0.521	0.056
M23	0.414	0.065	0.402	0.085	0.414	0.085	0.283	0.055	0.414	0.085	0.316	0.068	0.414	0.085	0.217	0.014
M25	0.360	0.074	0.281	0.068	0.360	0.068	0.437	0.093	0.360	0.068	0.599	0.091	0.360	0.068	0.535	0.084
M31	0.350	0.121	0.384	0.105	0.350	0.105	0.386	0.143	0.350	0.105	0.281	0.034	0.350	0.105	0.283	0.022
M32	0.638	0.050	0.770	0.087	0.638	0.087	0.574	0.040	0.638	0.087	0.732	0.095	0.638	0.087	0.629	0.056
M38	0.329	0.098	0.443	0.095	0.329	0.095	0.414	0.103	0.329	0.095	0.349	0.088	0.329	0.095	0.298	0.017
M39	0.686	0.043	0.714	0.078	0.686	0.078	0.445	0.026	0.686	0.078	0.287	0.042	0.686	0.078	0.477	0.035
M58	0.469	0.100	0.429	0.077	0.469	0.077	0.357	0.051	0.469	0.077	0.284	0.083	0.469	0.077	0.323	0.063
M26	0.718	0.074	0.639	0.059	0.718	0.059	0.578	0.041	0.718	0.059	0.623	0.063	0.718	0.059	0.428	0.011
M34	0.968	0.068	0.801	0.024	0.968	0.024	0.413	0.075	0.968	0.024	0.558	0.078	0.968	0.024	0.736	0.039
M41	0.698	0.083	0.838	0.045	0.698	0.045	0.732	0.024	0.698	0.045	1.158	0.237	0.698	0.045	0.734	0.048
M18	0.932	0.051	0.917	0.063	0.932	0.063	0.576	0.026	0.932	0.063	0.745	0.070	0.932	0.063	0.865	0.030
M59	0.769	0.023	0.328	0.018	0.769	0.018	0.656	0.103	0.769	0.018	0.377	0.020	0.769	0.018	0.538	0.094
M68	0.854	0.071	0.702	0.057	0.854	0.057	0.738	0.082	0.854	0.057	0.755	0.106	0.854	0.057	0.542	0.041
ATCC15468^T	0.838	0.075	0.725	0.033	0.838	0.033	0.725	0.085	0.838	0.033	0.827	0.095	0.838	0.033	0.768	0.029
M55	0.536	0.010	0.282	0.087	0.536	0.087	0.321	0.003	0.536	0.087	0.248	0.040	0.536	0.087	0.395	0.199
M57	0.653	0.083	0.572	0.084	0.653	0.084	0.658	0.062	0.653	0.084	0.349	0.045	0.653	0.084	0.394	0.057
M63	0.695	0.044	0.583	0.075	0.695	0.075	0.476	0.078	0.695	0.075	0.518	0.090	0.695	0.075	0.198	0.025
M8	0.480	0.030	0.348	0.066	0.480	0.066	0.551	0.030	0.480	0.066	0.795	0.113	0.480	0.066	0.588	0.048
M92	0.793	0.092	0.880	0.053	0.793	0.053	0.531	0.090	0.793	0.053	0.626	0.054	0.793	0.053	0.378	0.098
M76	0.244	0.093	0.300	0.108	0.244	0.108	0.316	0.094	0.244	0.108	0.255	0.029	0.244	0.108	0.478	0.068
M77	0.450	0.083	0.740	0.033	0.450	0.033	0.675	0.091	0.450	0.033	0.891	0.079	0.450	0.033	1.088	0.110
M28	0.637	0.054	0.246	0.024	0.637	0.024	0.292	0.107	0.637	0.024	0.243	0.016	0.637	0.024	0.327	0.077
M49	0.963	0.146	1.104	0.142	0.963	0.142	0.942	0.006	0.963	0.142	0.735	0.032	0.963	0.142	0.841	0.090
M9	1.173	0.069	0.672	0.148	1.173	0.148	0.402	0.012	1.173	0.148	0.319	0.066	1.173	0.148	0.569	0.066
M45	1.266	0.100	0.778	0.273	1.266	0.273	0.797	0.109	1.266	0.273	0.654	0.034	1.266	0.273	0.903	0.080
M46	0.392	0.029	0.365	0.043	0.392	0.043	0.346	0.063	0.392	0.043	0.290	0.049	0.392	0.043	0.374	0.117
M47	0.443	0.061	0.374	0.051	0.443	0.051	0.506	0.055	0.443	0.051	0.600	0.070	0.443	0.051	0.448	0.095
M66	0.276	0.032	0.331	0.097	0.276	0.097	0.259	0.030	0.276	0.097	0.457	0.074	0.276	0.097	0.371	0.040
M67	0.668	0.078	0.465	0.323	0.668	0.323	0.608	0.271	0.668	0.323	0.418	0.074	0.668	0.323	0.307	0.017

Table 2 C: QSIs treatments absorbance readings after 48 h

	UNTREATED		CINNAMALDEHYDE		UNTREATED		FURANONE		UNTREATED		SAHC		UNTREATED		VANILLIN	
	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev	Average	StDev
TSB 30 SH	0.163	0.038	0.163	0.038	0.163	0.142	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038	0.163	0.038
M2	0.754	0.051	0.774	0.083	0.754	0.082	0.768	0.039	0.754	0.051	0.681	0.070	0.754	0.084	0.703	0.077
M5	0.382	0.105	0.386	0.027	0.382	0.087	0.499	0.056	0.382	0.105	0.404	0.071	0.382	0.060	0.362	0.069
M6	0.868	0.045	0.882	0.078	0.868	0.038	0.726	0.146	0.868	0.045	0.460	0.062	0.868	0.090	0.381	0.064
M13	0.407	0.086	0.264	0.024	0.407	0.068	0.406	0.069	0.407	0.086	0.512	0.027	0.407	0.086	0.377	0.095
M14	0.624	0.087	0.557	0.134	0.624	0.086	0.757	0.060	0.624	0.087	0.359	0.015	0.624	0.015	0.466	0.067
M17	0.336	0.089	0.330	0.043	0.336	0.024	0.543	0.103	0.336	0.089	0.274	0.065	0.336	0.089	0.324	0.090
M50	1.282	0.142	1.277	0.136	1.282	0.089	0.896	0.051	1.282	0.142	0.828	0.043	1.282	0.118	1.355	0.014
M51	0.386	0.079	0.341	0.046	0.386	0.084	0.324	0.081	0.386	0.079	0.217	0.077	0.386	0.079	0.405	0.017
M52	1.238	0.108	0.862	0.016	1.238	0.105	0.932	0.095	1.238	0.108	1.211	0.109	1.238	0.205	0.900	0.063
M53	0.492	0.068	0.463	0.051	0.492	0.018	0.369	0.076	0.492	0.068	0.353	0.058	0.492	0.093	0.698	0.068
M60	1.516	0.084	1.242	0.493	1.516	0.108	0.585	0.099	1.516	0.084	0.708	0.093	1.516	0.164	0.439	0.040
M62	1.267	0.024	1.308	0.196	1.267	0.079	0.920	0.091	1.267	0.024	1.213	0.159	1.267	0.200	1.872	0.117
M64	0.421	0.065	0.318	0.076	0.421	0.063	0.269	0.080	0.421	0.065	0.380	0.094	0.421	0.065	0.338	0.025
M65	0.381	0.018	0.778	0.039	0.381	0.045	0.338	0.046	0.381	0.018	0.382	0.090	0.381	0.073	0.364	0.057
M86	0.871	0.063	0.350	0.048	0.871	0.065	0.243	0.050	0.871	0.063	0.267	0.048	0.871	0.005	0.311	0.199
M94	0.248	0.054	0.211	0.085	0.248	0.054	0.287	0.091	0.248	0.054	0.448	0.097	0.248	0.054	0.434	0.077
M95	0.406	0.082	0.538	0.081	0.406	0.051	0.564	0.072	0.406	0.082	0.511	0.090	0.406	0.082	0.386	0.090
ATCC 7966^T	0.637	0.273	0.622	0.094	0.637	0.273	1.058	0.155	0.637	0.273	0.772	0.078	0.637	0.064	0.854	0.022
M70	1.359	0.468	0.788	0.054	1.359	0.087	0.582	0.142	1.359	0.468	0.812	0.082	1.359	0.468	0.536	0.084
M72	1.238	0.066	1.114	0.101	1.238	0.033	1.115	0.073	1.238	0.066	0.826	0.071	1.238	0.207	1.369	0.087
M80	0.625	0.087	0.645	0.095	0.625	0.057	0.701	0.105	0.625	0.087	0.409	0.019	0.625	0.061	0.634	0.066
M81	0.987	0.033	0.867	0.037	0.987	0.468	0.378	0.063	0.987	0.033	0.520	0.166	0.987	0.050	0.929	0.030
M88	0.472	0.069	0.304	0.087	0.472	0.207	0.453	0.051	0.472	0.069	0.395	0.076	0.472	0.069	0.517	0.094
M90	0.907	0.095	0.689	0.039	0.907	0.095	0.588	0.069	0.907	0.095	0.751	0.074	0.907	0.087	0.888	0.098
M96	0.744	0.057	0.749	0.054	0.744	0.069	1.015	0.115	0.744	0.057	0.636	0.027	0.744	0.045	0.639	0.110
M99	1.181	0.207	0.708	0.047	1.181	0.024	0.895	0.048	1.181	0.207	0.960	0.029	1.181	0.113	1.223	0.182
M22	0.762	0.075	0.289	0.006	0.762	0.066	0.366	0.026	0.762	0.075	0.566	0.048	0.762	0.024	0.521	0.048
M23	0.414	0.060	0.402	0.065	0.414	0.078	0.283	0.055	0.414	0.060	0.316	0.068	0.414	0.085	0.217	0.097
M25	0.360	0.024	0.281	0.074	0.360	0.015	0.437	0.093	0.360	0.024	0.599	0.091	0.360	0.068	0.535	0.095
M31	0.350	0.064	0.384	0.121	0.350	0.075	0.386	0.143	0.350	0.064	0.281	0.034	0.350	0.105	0.283	0.067
M32	0.638	0.043	0.770	0.050	0.638	0.060	0.574	0.040	0.638	0.043	0.732	0.095	0.638	0.087	0.629	0.035
M38	0.329	0.093	0.443	0.098	0.329	0.064	0.414	0.103	0.329	0.093	0.349	0.088	0.329	0.095	0.298	0.067
M39	0.686	0.078	0.714	0.043	0.686	0.093	0.445	0.026	0.686	0.078	0.287	0.042	0.686	0.078	0.477	0.084
M58	0.469	0.015	0.429	0.100	0.469	0.053	0.357	0.051	0.469	0.015	0.284	0.083	0.469	0.077	0.323	0.061
M26	0.718	0.148	0.639	0.074	0.718	0.085	0.578	0.041	0.718	0.148	0.623	0.063	0.718	0.059	0.428	0.041
M34	0.968	0.053	0.801	0.068	0.968	0.043	0.413	0.075	0.968	0.053	0.558	0.078	0.968	0.024	0.736	0.048
M41	0.698	0.085	0.838	0.083	0.698	0.148	0.732	0.024	0.698	0.085	1.158	0.237	0.698	0.045	0.734	0.017
M18	0.932	0.087	0.917	0.051	0.932	0.087	0.576	0.026	0.932	0.087	0.745	0.070	0.932	0.063	0.865	0.029
M59	0.769	0.045	0.328	0.023	0.769	0.045	0.656	0.103	0.769	0.045	0.377	0.020	0.769	0.018	0.538	0.030
M68	0.854	0.097	0.702	0.071	0.854	0.097	0.738	0.082	0.854	0.097	0.755	0.106	0.854	0.057	0.542	0.027
ATCC15468^T	0.838	0.323	0.725	0.075	0.838	0.205	0.725	0.085	0.838	0.323	0.827	0.095	0.838	0.033	0.768	0.083
M55	0.536	0.200	0.282	0.010	0.536	0.164	0.321	0.003	0.536	0.200	0.248	0.040	0.536	0.087	0.395	0.056
M57	0.653	0.205	0.572	0.083	0.653	0.323	0.658	0.062	0.653	0.205	0.349	0.045	0.653	0.084	0.394	0.067
M63	0.695	0.164	0.583	0.044	0.695	0.050	0.476	0.078	0.695	0.164	0.518	0.090	0.695	0.075	0.198	0.091
M8	0.480	0.024	0.348	0.030	0.480	0.200	0.551	0.030	0.480	0.024	0.795	0.113	0.480	0.066	0.588	0.039
M92	0.793	0.050	0.880	0.092	0.793	0.024	0.531	0.090	0.793	0.050	0.626	0.054	0.793	0.053	0.378	0.220
M76	0.244	0.090	0.300	0.093	0.244	0.090	0.316	0.094	0.244	0.090	0.255	0.029	0.244	0.108	0.478	0.062
M77	0.450	0.061	0.740	0.083	0.450	0.061	0.675	0.091	0.450	0.061	0.891	0.079	0.450	0.033	1.088	0.080
M28	0.637	0.118	0.246	0.054	0.637	0.118	0.292	0.107	0.637	0.118	0.243	0.016	0.637	0.024	0.327	0.020
M49	0.963	0.084	1.104	0.146	0.963	0.073	0.942	0.006	0.963	0.084	0.735	0.032	0.963	0.142	0.841	0.111
M9	1.173	0.113	0.672	0.069	1.173	0.059	0.402	0.012	1.173	0.113	0.319	0.066	1.173	0.148	0.569	0.041
M45	1.266	0.033	0.778	0.100	1.266	0.005	0.797	0.109	1.266	0.033	0.654	0.034	1.266	0.273	0.903	0.056
M46	0.392	0.073	0.365	0.029	0.392	0.113	0.346	0.063	0.392	0.073	0.290	0.049	0.392	0.043	0.374	0.011
M47	0.443	0.077	0.374	0.061	0.443	0.084	0.506	0.055	0.443	0.077	0.600	0.070	0.443	0.051	0.448	0.050
M66	0.276	0.005	0.331	0.032	0.276	0.077	0.259	0.030	0.276	0.005	0.457	0.074	0.276	0.097	0.371	0.029
M67	0.668	0.059	0.465	0.078	0.668	0.033	0.608	0.271	0.668	0.059	0.418	0.074	0.668	0.323	0.307	0.093