

**CHARACTERIZATION OF FACTORS INVOLVED IN AND AFFECTING BIOFILM
FORMATION BY *Aeromonas* SPP. ISOLATES**

By

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of KwaZulu-Natal (Westville Campus)**

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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ABSTRACT

Aeromonas spp. isolates, which are fish and opportunistic human pathogens, form biofilms, however the factors involved in and affecting biofilm formation have not been fully elucidated. Biofilm formation is affected by motility, cell surface characteristics, and/or metabolism, thus it is important to identify factors potentially contributing to initial attachment and/or biofilm formation and their correlation with biofilm formation by *Aeromonas* spp. isolates. With knowledge of the stages of biofilm formation, mechanisms involved in biofilm formation and its physiology, various strategies may be applied to control aeromonad biofilms. Factors potentially involved in initial attachment and/or biofilm formation were investigated for 99 *Aeromonas* isolates obtained from seawater and cultured fish. Aeromonad biofilm formation was assessed using microtiter plate assays under varying physicochemical conditions. The disk diffusion method was used to determine the antimicrobial susceptibility profiles of isolates, for comparison to clinical and aquaculture isolates reported in other studies. The MICs and MBICs for antimicrobial agents (azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline) of planktonic cells and biofilm cells, respectively, were investigated using the broth microdilution and modified microtiter plate assays. The effect of sub-MIC ($0.5 \times \text{MIC}$) and supra-MIC ($2 \times \text{MIC}$) exposures on biofilm-forming cells was also determined using microtiter plate assays. The presence of efflux pump-mediated resistance in 45 *Aeromonas* spp. isolates was determined using the disk diffusion assay incorporating efflux pump inhibitors (EPIs) [carbonyl cyanide 3-chlorophenylhydrazone (CCCP), phenylalanine arginine β -naphthylamide (PA β N) and 1-(1-naphthylmethyl)-piperazine (NMP)]. Modified microtiter plate assays were used to determine the effect of EPIs [CCCP, PA β N, and NMP], matrix-degrading DNase I and quorum-sensing inhibitors (QSIs; vanillin, 2(5H)-furanone, S-adenosylhomocysteine and cinnamaldehyde) on initial attachment and mature biofilm. Majority of isolates were motile by swimming and swarming and displayed caseinase, gelatinase, and DNase activities, as well as an A-layer phenotype. Majority of isolates displayed high levels of autoaggregation and were hydrophilic. Isolates showed varying levels of adherence, but majority were strongly adherent in nutrient-rich media at 30 °C. Motility appeared to be a significant characteristic for biofilm formation. Majority of *Aeromonas* isolates spp. showed high levels of resistance to β -lactams, trimethoprim and sulphamethoxazole, and were susceptible to augmentin, piperacillin-tazobactam, aztreonam, 2nd and 3rd generation cephalosporins, carbapenems, macrolides, fluoroquinolones and aminoglycosides. High levels of resistance towards ceftazidime (MIC > 32 $\mu\text{g/ml}$) were observed for isolates, while levels of resistance towards remaining antimicrobial agents tested (tetracycline, azithromycin, ciprofloxacin, and gentamicin) were $\leq 32 \mu\text{g/ml}$. There was a ≥ 16 -fold increase in MBICs (4096 $\mu\text{g/ml}$) compared to the MICs for all the antimicrobial agents. The sub-MIC, MIC, and supra-MIC exposures of all antimicrobial agents had an inhibitory effect on both initial attachment and pre-formed biofilms by *Aeromonas* spp. isolates. Majority of isolates were more susceptible to tetracycline, norfloxacin, and azithromycin due to

CCCP and NMP inhibition of the efflux pumps eliminating these antimicrobial agents. Susceptibility to erythromycin was observed for 51% and 47% of isolates, respectively, due to NMP and PA β N inhibition of the efflux pump/s eliminating erythromycin. In the microtiter plate assays, CCCP, NMP and PABN exposures resulted in significant reduction of biofilm formation by majority of *Aeromonas* spp. isolates in both initial attachment and mature biofilm assays, with CCCP being more effective. DNase I was more effective in reducing mature biofilm, causing reduction for 60% of isolates, compared to its effect on initial attachment. QSIs were also more effective in reducing mature biofilm compared to inhibiting initial attachment. Although increased biofilm dispersal was observed with all QSIs, vanillin and 2(5H)-furanone were more effective compared to S-adenosylhomocysteine and cinnamaldehyde. Based on data obtained in this study, antimicrobial agents, EPIs and QSIs can be used as potential biofilm-inhibiting compounds in aquaculture to control aeromonad infections and may not only prevent disease outbreaks but they could also increase the effectiveness of existing therapeutic agents.

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CHAPTER ONE

Introduction and Literature Review

1.1. The genus *Aeromonas* and pathogenicity of *Aeromonas* spp.

Members of the genus *Aeromonas* belong to the family Aeromonadaceae (Abbott *et al.*, 2003; Ghenghesh *et al.*, 2008) and have been known to microbiologists since the early 1890s (Farmer *et al.*, 2006). Until 1984, only four phenospecies were identified, viz., *Aeromonas hydrophila*, *A. salmonicida*, *A. sobria* and *A. caviae* (Ghenghesh *et al.*, 2008). With the evolution of newer, more effective techniques over the years, molecular techniques such as DNA-DNA hybridization, have allowed the identification of 17 hybridization groups (genospecies) and 14 phenospecies, including *A. bestiarum*, *A. media*, *A. eucrenophila*, *A. veronii* biotype *veronii*, *A. veronii* biotype *sobria*, *A. salmonicida*, *A. hydrophila*, *A. schubertii*, *A. caviae*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *A. culicicola*, *A. simiae*, *A. jandaei* and *A. molluscorum* (Ghenghesh *et al.*, 2008; Soler *et al.*, 2004; Tacao *et al.*, 2005).

The genus *Aeromonas* comprises Gram-negative, non-spore-forming, rod-shaped, facultative anaerobes, which are oxidase- and catalase-positive. Species are motile by polar flagella (some species are non-polar) and they grow at a temperature of 22-28 °C (Belaluddin and Shahjahan, 2003; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Kirov *et al.*, 2002; Tacao *et al.*, 2005). Members of the genus *Aeromonas* are ubiquitous and autochthonous aquatic bacteria (Castro-Escarpulli *et al.*, 2003; Gordon *et al.*, 2008 and Kirov *et al.*, 2002; Tacao *et al.*, 2005) and occupy a wide variety of environmental niches including soil and water. They are also

pathogens of warm- and cold-blooded animals (Jacobs and Chenia, 2007). Because of this diversity in habitats, aeromonads have been observed to be associated with a wide variety of diseases.

Aeromonas spp. isolates are often associated with fish diseases, where *A. salmonicida* causes furunculosis, which affects salmon and trout at commercial farms (Austin, 1997; Farmer *et al.*, 2003; L’Abee-Lund and Sorum, 2001). *A. hydrophila* causes red sore disease of bass and also affects carp and cod causing ulcer disease (Farmer *et al.*, 2006). Channel fish, shad and centrarchid fish are also affected by *A. hydrophila* (Farmer *et al.*, 2006). *A. bestiarum* and *A. sobria* have been observed in frozen fish intended for human consumption in Mexico (Castro-Escarpulli *et al.*, 2003). These pathogenic aeromonads cause losses in farmed fish stocks and this results in economic losses in aquaculture. Humans may be exposed to pathogens of aquaculture origin via consumption of inadequately prepared fish products or by cross contamination of other foods by these fish bacteria (Abraham *et al.*, 2007; Austin, 1997; Castro-Escarpulli *et al.*, 2003; Davies *et al.*, 2001; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Hanninen *et al.*, 1997; Jacobs and Chenia, 2007).

Aeromonads have also been identified as an aetiological agent in human (food or water-borne) infections (Davies *et al.*, 2001; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Gordon *et al.*, 2008; Kirov *et al.*, 2002; Koksal *et al.*, 2007; Tacao *et al.*, 2005). In humans, they are the causative agents of gastroenteritis, wound infections (cellulitis, furunculosis and abscesses) and septicaemia (Ghenghesh *et al.*, 2008; Koksal *et al.*, 2007). They are also associated with respiratory, urinary tract and ocular infections, meningitis, endocarditis, peritonitis, hepatobiliary disease and endotoxic shock (Ghenghesh *et al.*, 2008; Koksal *et al.*, 2007). *A. hydrophila*, *A.*

veronii and *A. caviae* are normally the cause of extra-intestinal infections in immunocompromised patients (Abraham *et al.*, 2007; Castro-Escarpulli *et al.*, 2003; Ghengesh *et al.*, 2008; Gordon *et al.*, 2008; Koksai *et al.*, 2007). *A. sobria* and *A. schubertii* were isolated and were found to be associated with blood infection (Farmer *et al.*, 2006). *Aeromonas* spp. are also found in water (fresh, drinking, aquatic samples), in waste-water drainage, sewage and in swimming pools, with many cases of infection in humans and animals being traced to exposure to contaminated water (Abraham *et al.*, 2007; Farmer *et al.*, 2006; Gordon *et al.*, 2008; Hanninen *et al.*, 1997; Koksai *et al.*, 2007).

1.2. Bacterial biofilms and biofilm development

It has been observed that most bacterial infections involve biofilms (Behlau and Gilmore, 2008; Hoiby *et al.*, 2010). Aquatic fish pathogens such as *Vibrio*, *Yersinia*, and *Aeromonas* have been shown to form biofilm structures in aquaculture environments, and survival of these aquatic bacteria outside the fish host may be dependent on biofilm formation (Basson *et al.*, 2008). Biofilms are collections of microorganisms enclosed in a matrix known as extracellular polymeric substances (EPS) which consists of polysaccharides, proteins and DNA originating from the microbes; that provides structural stability, protection to the biofilm and also promotes adherence or initial attachment of microbes to smooth surfaces and to other cells (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and voan Holy, 2006; Hoiby *et al.*, 2010; Vu *et al.*, 2009). Bacterial biofilms form on biotic or abiotic surfaces, developing into large communities of complex architecture whereby there is cell-to-cell communication and co-ordinated behaviour, i.e., quorum-sensing (Behlau and Gilmore, 2008;

Hoiby *et al.*, 2010; Vu *et al.*, 2009). In these biofilms, microbes surround themselves with the slimy EPS, which protects them from their environment; conferring protection against phagocytosis, interference with the cellular immune response, and reduction of antimicrobial potency, making them hard to remove or kill with traditional chemical or mechanical methods. Due to this durability, biofilms are responsible for a wide range of industrial and health problems. Current technologies used to deal with biofilms are largely ineffective, costly and based on toxic biocides. Consequently, new strategies are being investigated with the main foci being the use of biosolutions (enzymes, phages, interspecies interactions and antimicrobial molecules from microbial origin) (Simoes *et al.*, 2010).

There are five basic stages (Fig. 1) involved in the development of biofilms, viz., adhesion (attachment), colonization, maturation, detachment and detachment (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). Adhesion or attachment, either reversible and irreversible, is the first stage in biofilm development. For reversible adhesion, it is the initial non-specific weak association of cells to the surface, and involves van der Waal's and hydrophobic interactions. At this stage bacteria are easily removable. Irreversible adhesion on the other hand involves firm adherence and involves hydrogen bonding, covalent and hydrophobic bonding. It results from the anchoring of appendages (pili, flagella) or production of extracellular polymers (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). This is followed by the formation of microcolonies given appropriate growth conditions, and results from simultaneous aggregation and growth of microorganisms and is accompanied by the production of the EPS (Chmielewski and Frank, 2003). This then leads to the maturation of these

microcolonies into organized three-dimensional structures. If conditions are suitable, which are enclosed and stabilized by the EPS. Lastly, the dispersal or detachment of the bacterial cells from the biofilm and there is subsequent transfer to other surfaces (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006).

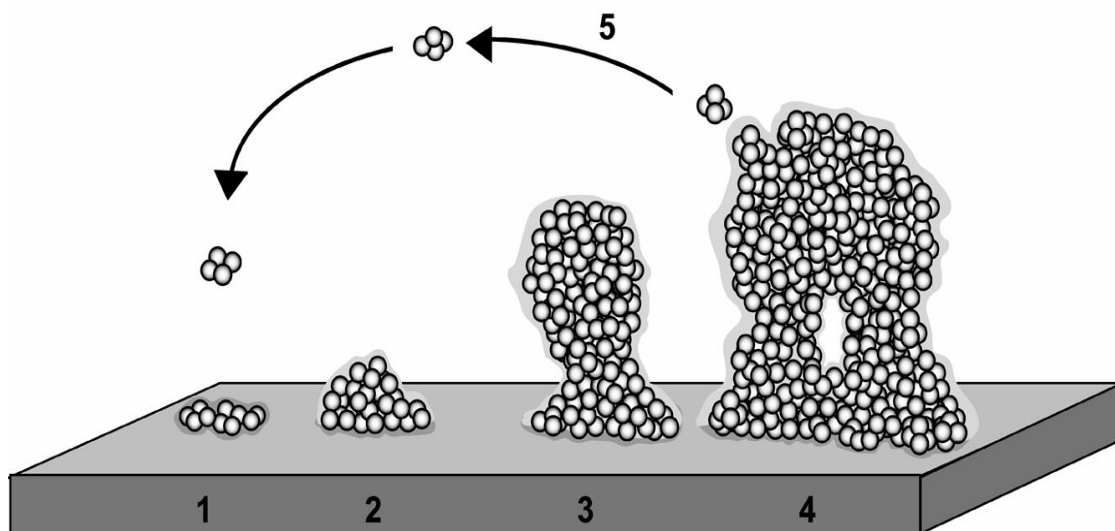


Figure 1.1: The development of a biofilm. Stage 1: initial attachment of cells to the surface; stage 2: microcolony formation and production of the extracellular polymeric substances (EPS); stage 3: early development of biofilm architecture; stage 4: maturation of biofilm architecture; and stage 5: dispersion of bacterial cells from the biofilm (Lasa, 2006).

1.2.1. Biofilm formation by *Aeromonas* spp.

Aeromonas spp. isolates have been identified from diverse biofilms. Bechet and Blondeau (2003) observed that *A. caviae* were capable of forming biofilms on the glass surface of cultured flasks in nutrient broth under agitation at 30 °C for 15 h. Aeromonads were also detected in water

distribution pipe biofilms in the United States where they were shown to be very persistent (Bomo *et al.*, 2004). *A. hydrophila* biofilms have been grown on chitin flakes suspended in tryptic soy broth (TSB) to improve antigen delivery for oral vaccination of catfish (Asha *et al.*, 2004; Azad *et al.*, 1999; Nayak *et al.*, 2004). Dogruoz *et al.* (2009) observed that *Pseudomonas* and *Aeromonas* spp. strains are pioneer colonizers and are predominant in biofilms on galvanized steel surfaces. *Aeromonas* spp. isolates, *A. hydrophila* in particular, have been described to form biofilms on microtiter plates in TSB when incubated for 48 h, without agitation at 30 °C (Gavin *et al.*, 2003; Merino *et al.*, 2001). They have also been shown to form biofilms in borosilicate glass tubes, when incubated for 30 h without agitation at 37 °C (Kirov *et al.*, 2004). Kozlova *et al.* (2008) revealed that *A. hydrophila* could form biofilms on glass cover slips and on plastic-cover slips after 24 h of incubation. *A. caviae* and *A. hydrophila* were able to grow biofilms on glass tube walls for 24 h at 30 °C in Luria Bertani (LB) medium (Li *et al.*, 2009). *A. hydrophila* was among the seven isolates described to be strong-biofilm formers (Li *et al.*, 2009).

1.3 Mechanism of biofilm formation and factors associated with initial attachment and/or biofilm formation

Initial attachment or adhesion has been shown to be a very important for most of microbial infections, being a crucial stage in biofilm formation (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). Initial attachment is affected by chemical and physical properties of the cell and surface, as well as the composition of the surrounding medium (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). According to Behlau and Gilmore (2008),

environmental signals including variation in temperature, nutrient availability, pH, osmolarity, iron and oxygen requirements enhances biofilm formation and are involved in the initiation of this process. For adhesion, cell surface components which include flagella, pili, fimbriae, adhesin proteins, proteases, capsules, EPS as well as surface charge (i.e., hydrophobicity), all play a huge role in initial attachment for biofilm formation and in pathogenicity (Behlau and Gilmore, 2008; Basson *et al.*, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006; Karatan and Watnick, 2009) and many of them are important components of bacterial motility (Basson *et al.*, 2008). Francolini and Donelli (2010) have suggested that possible anti-biofilm strategies should be based on the inhibition of microbial adhesion to the surface and of colonization.

1.3.1. Motility

Flagella are more than organelles of locomotion, they are important as they allow cells to move to a specific attachment site. They play an important role in motility and in attachment by overcoming repulsive forces associated with the surface and contribute to pathogenesis (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). In addition, motility is required to move along the surface, thereby, facilitating growth and spread of a developing biofilm; and also flagella themselves (as surface appendages) can directly mediate attachment to surfaces (Van Houdt and Michiels, 2010). According to Santos *et al.* (2010), swimming motility is important not only in the initial approach of bacterial cells to surfaces but also in the attachment of cells for the formation of biofilms.

Mesophilic *Aeromonas* express a single polar flagellum for swimming under all culture conditions and produce lateral flagella for swarming on solid media (Altarriba *et al.*, 2003; Gavin *et al.*, 2002; Kirov *et al.*, 2002; 2004; Kirov, 2003; Rabaan *et al.*, 2001). Altarriba *et al.* (2003) described the presence of a polar flagella operon (*flg*) of *Aeromonas* and its association with motility (swimming and swarming), adherence to Hep-2 cells as well their ability to form biofilms. Although *A. salmonicida* is classified as non-motile, several flagellar genes have also been identified (Altarriba *et al.*, 2003).

Swarming motility is known to facilitate rapid colonization of surfaces and is linked to virulence factor production (proteases), robust biofilm formation, antimicrobial resistance and pathogenicity (Connelly *et al.*, 2004). It permits fast and local colonization, where bacteria then proceed to form microcolonies, which is then followed by the formation of a mature biofilm (Santos *et al.*, 2010). Connelly *et al.* (2004) observed that extracellular proteolytic activity played a central role in swarming motility and was often associated with biofilm formation in *Bacillus subtilis*. Murray *et al.* (2010) observed that swarming motility was negatively associated with biofilm formation by clinical *P. aeruginosa* isolates; however, swarming motility was positively associated with the secretion of proteases and exoenzymes by these isolates. Kalmokoff *et al.* (2006) described the role of the motility complex in biofilm formation by *Campylobacter jejuni* 11168. Their findings demonstrated that the flagella complex FlaA, FlaB, FlaG, and FliD plays a crucial role in initial attachment of *C. jejuni* 11168 to solid surfaces during biofilm formation, as well as in the cell-cell interactions required for pellicle formation (Kalmokoff *et al.*, 2006).

The lateral flagella of *Aeromonas* species have been described as being essential for epithelial cell adherence and biofilm formation (Gavin *et al.*, 2002; 2003; Rabaan *et al.*, 2001). Kirov *et al.* (2004) has also described *Aeromonas* flagella (polar and lateral) as enterocyte adhesins that contribute to biofilm formation on surfaces. Furthermore, Imzilh *et al.* (1998) observed that in motile aeromonads, there was a significant correlation between most virulent factors, although some strains produced one virulence factor, which was independent of presence of the other. Santos *et al.* (2010) observed that *A. caviae* produce inducible lateral flagella (*laf*), involved in swarming motility which is associated with the area of colonization of surfaces, biomass production as well as biofilm formation. They provided evidence that both polar flagella and lateral flagella function in biofilm formation by *A. caviae* strains isolated from environment, food and human source.

1.3.2. Lipopolysaccharide (LPS) and other virulence properties

Lipopolysaccharide (LPS) and the Gram-negative outer membrane are also involved in adhesion and pathogenicity in many pathogenic bacteria. LPS is an amphiphilic antigen common to most Gram-negative bacteria such as *Aeromonas* spp; and is a potent immunogenic substance that plays a role in initial attachment of cells to host tissues, biofilm formation and pathogenicity in many Gram-negative bacteria (Asha *et al.*, 2004; Bandara *et al.*, 2010; Lee *et al.*, 2010). It consists of lipid A, a core oligosaccharide, and the O-antigen side chain polysaccharide, and all three parts are important (Lee *et al.*, 2010). The O-antigen has been shown to be involved in the colonization of the host and in resistance of bacteria to antimicrobial stress (Asha *et al.*, 2004; Lee *et al.*, 2010). Sawasdidoln *et al.* (2010) reported that apart from being immunogenic, LPS

also acts as a permeability barrier, at bacterial surfaces mostly to hydrophobic agents. It has been observed that the deficiency in the LPS negatively affects biofilm formation in most Gram-negative bacteria (Asha *et al.*, 2004; Lee *et al.*, 2010). Asha *et al.* (2004) compared the LPS of biofilms of *A. hydrophila* with that of planktonic cells, and observed that approximately 15 proteins were repressed while three new proteins were uniquely expressed in biofilm cells compared to the planktonic cells. The same changes in LPS moiety have also been observed during biofilm formation in *P. aeruginosa* (Hodgson *et al.*, 1995). The adhesive properties of the outer membrane and its role as an adhesion non-fimbrial protein involved in *A. veronii* adhesion have been described by Vacquez-Juarez *et al.* (2004).

Ljungh *et al.* (1985) observed that surface proteins (A-layer and proteolytic enzymes) contribute to high surface hydrophobicity of autoaggregating *Staphylococcus aureus* strains. It has been observed that the cell envelope of virulent *A. salmonicida* is composed of an A-layer (predominant cell surface protein) found beyond the outer membrane and the repeating O-antigen subunit of bacterial LPS (Fernandez *et al.*, 1995). The 50 kDA A-layer protein is also responsible for several cell surfaces properties such as autoaggregation and hydrophobicity, because it has a high proportion of hydrophobic amino acids which confer increased cell surface hydrophobicity (Bjornsdottir *et al.*, 1992; Fernandez *et al.*, 1995; Phipps *et al.*, 1983). Phipps *et al.* (1983) purified and characterized the A-layer from *A. salmonicida*, and observed that this protein was hydrophobic in composition and the N-terminal sequence was highly hydrophobic, as it lacks carbohydrate residues. The phenotype A-layer⁺ LPS⁺ is an essential virulence factor for *Aeromonas* spp., particularly *A. salmonicida* (Fernandez *et al.*, 1995). The presence of the A-layer, which is said to be absent in avirulent strains of *A. salmonicida* and the LPS, is essential

for agglutination, virulence and pathogenesis (Bernoth, 1990; Bjornsdottir *et al.*, 1992; Ellis *et al.*, 1997; Fernandez *et al.*, 1995). Van Alstine *et al.* (1986) observed that both the presence of the superficial protein A-layer and the O polysaccharide chains of lipopolysaccharide play an important role in the detachment behaviour of *A. salmonicida* cells and that the presence of the A-layer, which is crucial to the virulence of *A. salmonicida*, appeared to increase the surface hydrophobicity of this pathogen.

1.3.3. Cell surface hydrophobicity

Hydrophobicity of the cell surface is another important factor in adhesion and biofilm formation. This is because hydrophobic interactions of the cell surface tend to increase more with an increasing non-polar nature of one or both surfaces involved (Donlan, 2002). Although bacteria are negatively charged, they still contain hydrophobic surface components, which influence their surface attachment and play a role in pathogenicity and biofilm formation (Donlan, 2002; Dykes *et al.* 2003). Surface hydrophobicity is generally associated with bacterial adhesiveness; it varies from organism to organism, and from strain to strain and is influenced by the growth medium, bacterial age, and bacterial structure (Basson *et al.*, 2008). Fimbriae and pili contribute to cell surface hydrophobicity as these are also known to contain hydrophobic amino acid residues (Donlan, 2002). Xu *et al.* (2009) also reported that bacterial hydrophobic and hydrophilic properties are the result of proteins and polysaccharides on bacterial cell surface. According to Di Bonaventura *et al.* (2008), *Listeria monocytogenes* can adhere to and form biofilms on food-processing surfaces and this is significantly influenced by temperature and cell surface hydrophobicity. Pagedar *et al.* (2010) and Kouidhi *et al.* (2010) observed that substrate surface

hydrophobicity affected biofilm formation by *S. aureus* isolates as this was positively correlated to biofilm formation; hence hydrophobicity plays a critical role in the adherence of *S. aureus* to various surfaces, such as polystyrene surfaces. However, Auger *et al.* (2009) evaluated cell surface properties and biofilm formation by *B. cereus* and observed that cell surface hydrophobicity, the presence of an S layer, and adhesion on HeLa epithelial cells were not positively correlated to biofilm formation.

Bartkova and Ciznar (1994) evaluated the adherence pattern of non-piliated *A. hydrophila* and observed that hydrophobicity may be the major factor responsible for adherence to epithelial cells. Trust *et al.* (1983) observed differences in cell surface hydrophobicity of fish pathogenic *A. salmonicida* strains, which differed in their ability to produce the A-layer surface protein array. The presence of this superficial protein layer, as mentioned previously, is said to be crucial to the virulence of this organism and was found to coincide with a drastic increase in cell surface hydrophobicity. Bonet *et al.* (1993) also observed that cell surface properties, such as hydrophobicity, are strongly influenced by capsule production; and that increased capsular polysaccharide production by *A. salmonicida* is associated with enhanced cell hydrophilicity. Deree *et al.* (1997) also observed that the cell surface hydrophobicity of *A. salmonicida* was responsible for pathogenicity of fish pathogens as hydrophobic cells adhered to fish surfaces and macrophages. Jiwa (1983) investigated hydrophobicity in *Aeromonas* spp. and observed that *Aeromonas* spp., particularly *A. hydrophila* and *A. sobria*, can vary in their hydrophobicity and cells may express weak to strong hydrophobic cell surface properties. Scoaris *et al.* (2008) used the bacterial adherence to hydrocarbons test to evaluate the hydrophobicity of *Aeromonas* spp. isolates and its correlation to virulence and observed that most of the virulent strains were highly

hydrophilic. Elhariry *et al.* (2011) observed that *Aeromonas* strains were hydrophilic and no significance differences were observed between hydrophobicity of *Aeromonas* strains.

1.3.4. Autoaggregation and Coaggregation

Autoaggregation, the attachment of genetically identical cells to each other, and coaggregation, the adhesion of genetically distinct cells to each other, are common features of cells that are able to participate in biofilm formation (Basson *et al.*, 2008). Coaggregation appears to be a more widespread phenomenon and is an essential feature of many multi-species biofilms. It allows secondary colonizer cells in suspension to recognize and attach to cells on the surface of a pre-existing biofilm, and it also allows these secondary colonizers to coaggregate with each other whilst in suspension, followed by adhesion of these flocs to a biofilms. In both autoaggregation and coaggregation, co-adhesion of the suspended cells to the biofilms is necessary in order for the cells to become part of the biofilm (Rickard *et al.*, 2003). A larger portion of biofilm strains were able to coaggregate and autoaggregate compared to their planktonic counterparts (Basson *et al.*, 2008).

The processes that allow for autoaggregation and coaggregation are highly specific and are typically mediated by interactions between “adhesin” proteins found on one cell and complementary saccharide “receptors” borne on another. Typically the “adhesin” and the “receptor” components would be present on the same cell (Rickard *et al.*, 2003). Many bacterial adhesins are lipoproteins, and it is believed that specific receptor-ligand (protein-saccharide or protein-protein) interactions mediate the aggregation of bacterial cells allowing for adherence

and biofilm formation, since interruption of these interactions by the addition of sugars such as lactose and galactose has been observed. Del Re *et al.* (1998) observed that autoaggregation is an important trait contributing to the ability of *Bifidobacterium suis* to colonize the intestinal tract. Del Re *et al.* (2000) indicated that the ability to autoaggregate and cell surface hydrophobicity seem to be independent traits which were both necessary for *B. longum* adhesion. Rahman *et al.* (2008) observed that autoaggregation of *Bifidobacterium* was mediated by surface proteins. Tomich and Mohr (2003) observed that cable pilus expression played a role in mediating *Burkholderia cenocepacia* cell-cell interaction and thus autoaggregation and adherence. Kos *et al.* (2003) observed that there was a relationship between autoaggregation and adhesive ability of *Lactobacillus acidophilus* M92, which was mediated by the proteinaceous components of the cell surface. Felek *et al.* (2008) demonstrated that the autotransporter YapC (surface protein) of *Yersinia pestis*, can mediate adhesion to Hep-2 cells, which leads to autoaggregation and biofilm formation, all of which play a role in overcoming the host immune system. Iida *et al.* (2010) demonstrated that autoaggregation of enteropathogenic *E. coli* strains plays a role in adhesion of these to Hep-2 cells, and that a bundle-forming pilus is involved in bacteria-bacteria interaction and subsequent autoaggregation.

As previously mentioned, the A-layer is commonly responsible for several cell surfaces properties such as autoaggregation in *Aeromonas* spp. (Bjornsdottir *et al.*, 1992; Fernandez *et al.*, 1995; Phipps *et al.*, 1983). Ishiguro *et al.* (1981) observed that attenuated strains of *A. salmonicida* strains not possessing the A-layer did not autoaggregate. Fernandez *et al.* (1995) also reported that characteristics of autoaggregation are highly common among *Aeromonas* spp. and frequently associated with the A-layer⁺ LPS⁺ phenotype. Basson *et al.* (2008) observed that

Aeromonas spp. can coaggregate with *Flavobacterium johnsoniae*-like isolates which are also Gram-negative aquatic fish pathogens, although the rates of coaggregation were not high as the rate of coaggregation observed with Gram-positive bacteria. The same trend has also been observed with *Myroides odoratus* by Jacobs and Chenia (2009), where the rate of coaggregation of *Aeromonas* spp. with *M. odoratus* was not as high when compared to the rate of coaggregation observed with Gram-positive bacteria.

1.3.5. Extracellular DNA secretion

Bacterial EPS is generally composed of polysaccharides, proteins, phospholipids, humic substances and nucleic acids such extracellular DNA (eDNA) (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006; Hoiby *et al.*, 2010; Simões *et al.*, 2010; Vu *et al.*, 2009). It is generally assumed that nucleic acids are localized inside living cells and that their primary function is the storage of information. In contrast, eDNA is not considered to be a remnant of lysed cells but a major structural component of the EPS (Bockelmann *et al.*, 2006). Three hypotheses have been put forward for the origin of eDNA, i.e., either from the lysis of subpopulation, release through membrane vesicles from the cell and/or it could be secreted actively (Vilain *et al.*, 2009).

eDNA has been shown to play a major role in the structure of biofilms (Spoering and Gilmore, 2006). Biofilms provide a niche for horizontal gene transfer (HGT), as cells are close to one another, which allows them to transfer genes located in the eDNA, which may be required for antimicrobial resistance, and thus survival of biofilm (Allen-Holmes *et al.*, 2006; Donlan,

2002; Finkel and Kolter, 2001; Hannan *et al.*, 2010; Tetz *et al.*, 2009). Evidence that eDNA may function as a cell-to-surface adhesin and/or cell-to-cell adhesin in the initial phase of biofilm formation was observed in the Gram-negative bacterium, *P. aeruginosa* (Molin and Tolken-Nielsen, 2003; Steinberger and Holden, 2005). eDNA has previously been shown to be essential for saturated biofilm stability during early stages of biofilm growth (Witchurch *et al.*, 2002). Steinberg and Holden (2005) reported that eDNA was present in unsaturated *P. aeruginosa* biofilm and was maximally 50% more abundant than cellular DNA and that it played a role in initial attachment and early biofilm formation. Tetz *et al.* (2009) confirmed the role of eDNA in the EPS in the maintenance of biofilms in Gram-positive and Gram-negative bacteria. Vilain *et al.* (2009) also observed that biofilm formation by *Bacillus cereus* requires DNA as part of the EPS. According to Das *et al.* (2010), DNA released by autolysins acts as an adhesive and strengthens the biofilm, and that removal of eDNA from Gram-positive bacteria reduced initial adhesion and bacterial aggregation of cells to surfaces. eDNA has also been shown to be an important component of the extracellular matrix of *Neisseria meningitidis* biofilms, as it stabilized biofilm structures in the late stages of meningitidis biofilm formation (Lappann *et al.*, 2010). Kirkpatrick and Viollier (2010) reported the role of eDNA in *Caulobacter crescentus* biofilm dispersal. In *C. crescentus*, biofilm formation is facilitated through its asymmetric cell division, where one daughter cell becomes a motile flagellated swarmer cell able to colonize new surfaces, while the other remains a stalked cell attached to the substrate through an adhesive holdfast (Kirkpatrick and Viollier, 2010). When a threshold level of cell death is reached in the biofilm, in order to allow cells to escape from the biofilm, the concentration of eDNA from lysed cells becomes sufficient to bind newly synthesized holdfasts. This prevents further growth of the

biofilm without dispersing the stalked cells from the holdfast and eDNA, therefore, directly regulates the homeostasis of the biofilm (Kirkpatrick and Viollier, 2010).

1.3.6. Quorum sensing, different types of signals and QS detection

Quorum sensing (QS) is a mechanism of cell-to-cell communication via the production of compounds called autoinducers. This allows bacteria to sense their own population and as well as the population of other bacteria in the environment (Bi *et al.*, 2007). QS regulates functions like conjugation, secretion of virulence factors, antibiotic production and biofilm formation (Bi *et al.*, 2007). QS appears to regulate biofilm formation in most bacteria including *Aeromonas* spp. (Bi *et al.*, 2007; Garde *et al.*, 2010; Lynch *et al.*, 2002; Ponnusamy *et al.*, 2009).

N-acyl homoserine lactones (AHLs) are highly conserved, having the same homoserine lactone moiety but different acyl side chains and substitutions (carbonyl and hydroxyl) at the C3 carbon (Ponnusamy *et al.*, 2009). They are also chemically unstable and their production is affected by temperature and carbon source (Medina-Martinez *et al.*, 2006). Medina-Martinez *et al.* (2006) observed that carbon sources (glucose) and temperature have an effect on N-butanoyl-L-homoserine lactone (C4-HSL) produced by *A. hydrophila*.

Swift *et al.* (1997) observed that *A. hydrophila* and *A. salmonicida* produce diffusible AHLs, with N-butyryl homoserine lactone (BHL) being produced as the main signalling molecule by these species. *A. hydrophila* produces: N-octanoylhomoserine lactone (OHL), N-dodecanoylhomoserine lactone (d-DHL) and t-DHL N-tetradecanoylhomoserine lactone while *A. salmonicida* produces OHL, d-DHL, t-DHL and N-decanoylhomoserine lactone (DHL) (Cataldi

et al., 2007). *Aeromonas* spp. isolates isolated from patients with malaria were shown to produce C4-HSL and C6-HSL as their two major types of AHLs. *A. sobria* and *A. hydrophila* isolated from patients in Malaysia produced C4-HSL and C6-HSL, respectively (Chan *et al.*, 2010). *A. hydrophila* isolated from faeces, fish and meat was shown to produce C4-HSL, as the major AHL, and *A. caviae* isolated from vegetables was shown to produce 3-oxo-C6-HSL (Chan *et al.*, 2010; Medina-Martínez *et al.*, 2006). *Aeromonas* spp. isolates isolated from municipal activated sludge produced C4-HSL and C6-HSL (Morgan-Sagastume *et al.*, 2005).

In *A. hydrophila* and *A. salmonicida*, the genes responsible for QS are *ahyRI* and *asaRI* (Swift *et al.*, 1997). *A. hydrophila* possesses homologues of the *V. fischeriluxI* and *luxR* QS genes termed *ahyI* and *ahyR*, respectively (Bi *et al.*, 2007; Garde *et al.*, 2010; Ponnusamy *et al.*, 2009). When the bacterial population has reached a minimum population size, the appropriate target gene is activated via transcriptional activator AhyR, which acts as both negative and positive regulator of *ahyI* (Bi *et al.*, 2007; Garde *et al.*, 2010).

The autoinducer that is responsible for cell-to-cell communication between both Gram-negative and Gram-positive bacteria is AI-2 (Kozlova *et al.*, 2008). The gene responsible for production of AI-2 in *A. hydrophila* is *luxS*, while in *Vibrio* spp. the receptors of LuxS protein are recognized as LuxP, however in *A. hydrophila* the receptors have not been identified (Kozlova *et al.*, 2011). In *A. hydrophila*, AI-2 together with AI-1 is responsible for production of virulence factors (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).

Different strategies have been used to identify AHL production, including the use of biosensors, thin layer chromatography and/or high performance liquid chromatography (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 (C6, C6-3-oxo, C8, C8-3-oxo, C4) and the latter detects a broad range of AHLs (all 3-oxo, C6, C8, C10, C12, C14, C6-3-hydroxy, C8-3-hydroxy and C10-3-hydroxy) (Steindler and Venturi, 2007). Production of AHLs by *C. violaceum* CV026 is indicated by the production of a purple violacein pigment, while in *A. tumefaciens* A136, it is indicated by the presence of blue colour which appears after this bacterium utilizes 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal). The *C. violaceum* CV026 reporter strain was constructed by inserting a transposon in the *cvil* 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 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*, and as a result the reporter can utilize X-gal, and produce a detectable blue color (Steindler and Venturi, 2007).

1.3.6.1. QS, expression of virulence factors and biofilm formation in bacteria

For most pathogens, secretion of virulence factors and initiation of biofilm formation is important to cause infections and is regulated by QS. QS is shown to be linked to protease production, virulence and biofilm formation in many bacteria including *Aeromonas* spp. Vivas *et*

al. (2004) reported that the production of proteases, particularly in *A. hydrophila*, is under the control of QS mechanisms. Extracellular enzymes, such as proteases, play a huge role in invasion, virulence, and in the establishment of infections by overcoming initial host defences by providing nutrients for cell proliferation (Sakai, 1985; Zacaria *et al.*, 2010). Vivas *et al.* (2004) reported that the role of proteases is to provide nutrients by breaking host proteins into small molecules, capable of entering the bacterial cell. Moreover, these extracellular proteases contribute to the metabolic versatility that allows *Aeromonas* to persist in the different habitats and that facilitate ecological interactions with other organisms (Zacaria *et al.*, 2010). Proteases, gelatinases, haemolysins, and elastases have been identified to be essential for virulence and pathogenicity in *A. hydrophila* (Cascon *et al.*, 2000; Poobalane *et al.*, 2008; Vivas *et al.*, 2004). Sechi *et al.* (2002) analysed the production of virulence factors in *Aeromonas* spp. from Sardinian waters and from patients with diarrhoea and found that the cytopathic effect induced on Hep-2 cells was due to toxins, haemolysins, proteases and gelatinase produced by *Aeromonas* spp. The expression of the gelatinase enzyme (GeE) and extracellular proteases has also been described to play a role in initial adhesion, colonization of surfaces, as well as in biofilm formation by *Enterococcus faecalis* (Thomas *et al.*, 2009; Van Merode *et al.*, 2006). This enzyme has been shown to hydrolyse small, biologically active peptides such as gelatine and collagen (Van Merode *et al.*, 2006). Snowden *et al.* (2006) tested the most prevalent and persistent strains of *Aeromonas* in waterways of the Sunshine Coast region, and found that isolates possessed different virulence properties (toxins and proteases), which appeared to be associated with the ability to adhere to and translocate to the human gut epithelial cell model. Two proteases that have been identified in *A. hydrophila* include metalloproteases and serine proteases (Cascon *et al.*, 2000; Harf-Monteil *et al.*, 2004; Imzih *et al.*, 1998) which are said to

be produced under the control of QS mechanism (Vivas *et al.*, 2004). Nitta *et al.* (2007) also purified a 65kDA-serine protein (ASP) produced by *A. sobria*, which is more virulent than other *Aeromonas* species, and is involved in septic shock and other diseases conditions. Yu *et al.* (2007) characterized extracellular proteases produced by *A. hydrophila* AH-1 using proteomics, and determined that the serine protease was involved in the processing of a toxin, and secreted enzymes such as haemolysin and metalloprotease. Khajanchi *et al.* (2009) also reported the role of N-acyl homoserine lactones in quorum sensing, biofilm formation, protease production and virulence of clinical isolate *A. hydrophila*.

1.4. Antimicrobial resistance in biofilms

In biofilms, bacteria are usually embedded within the EPS produced by them and this matrix affects the penetration of an externally applied antimicrobial stress to cells buried in the depth. Additionally, most antimicrobial agents target actively growing cells which are found in the periphery of the biofilm and the inner inactive cells are not affected by the application of the antimicrobial stress (Del Pozo and Patel, 2007). Lewis (2008) also described that cells within biofilms prevent an antimicrobial agent from binding to the target, which leads to the increase in the minimum inhibitory concentration (MIC). This causes a problem for antimicrobial therapy and results in the re-occurrence of disease outbreaks by most pathogens.

1.4.1. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of bacteria in biofilms

Clinical biofilm infections are marked by symptoms that typically recur even after repeated treatment with the antimicrobial agents. Standard antimicrobial therapy is only able to eliminate the planktonic cells, leaving the sessile (surface-attached) forms to spread within the biofilm and to continue to disseminate when therapy is terminated (Davey and O'Toole, 2000). According to Dal Sasso *et al.* (2003) when faced with bacterial infections, antimicrobial therapy is usually used in order to reach the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MICs are defined as the lowest concentration of an antimicrobial agent that will inhibit visible growth of a microorganism after overnight incubation while MBCs are defined as the lowest concentrations that will prevent growth of an organism after subculture to an antimicrobial-free media (Andrews, 2001). The MICs and MBCs of antimicrobial agents to biofilm-growing bacteria may be up to 100-1000-fold higher compared with planktonic bacteria (Hoiby *et al.*, 2010).

Based on antimicrobial susceptibility studies, planktonic *Aeromonas* spp. isolates are often resistant to β -lactams, tetracycline, quinolones, and second- and third-generation cephalosporins (Castro-Escarpulli *et al.*, 2003; Farmer *et al.*, 2006; Jacobs and Chenia, 2007). Many strains of *Aeromonas* spp. display resistance to piperacillin and mezlocillin, while being susceptible to gentamicin, ciprofloxacin, chloramphenicol, amikacin, trimethoprim sulfamethoxazole, nitrofurantoin, and tobramycin (Castro-Escarpulli *et al.*, 2003; Farmer *et al.*, 2006; Jacobs and Chenia, 2007).

1.4.2. Mechanisms of antimicrobial resistance in biofilms

In biofilms, bacteria are usually embedded within the EPS produced by them and this matrix affects the penetration of an externally applied antimicrobial stress to cells buried in the depth. Additionally, most antimicrobial agents target actively growing cells which are found in the periphery of the biofilm (Fig. 2) and the inner inactive cells are not affected by the application of the antimicrobial stress (Del Pozo and Patel, 2007). Lewis (2008) also described that cells within biofilms prevent an antimicrobial agent from binding to the target, which leads to the increase in the MIC. Another problem is that the increased bacterial density within biofilm microcolonies results in waste accumulation (Fig. 2) and a distorted microenvironment, which may have an effect on antimicrobial agent action deep within the biofilm (Del Pozo and Patel, 2007). One mechanism for multidrug tolerance of biofilms involves the production of persister cells (Fig. 2). These are produced in small numbers and exhibit multidrug tolerance (Lewis, 2008). The number of persisters in a growing population of bacteria rises at mid-log phase and reaches a maximum of approximately 1% at stationary state (Lewis, 2008). Persisters are not mutants but rather phenotypic variants of the wild-type that upon re-inoculation produce a culture with similar levels of tolerant (Shah *et al.*, 2006). Other mechanisms of resistance (Fig. 2) that are considered likely when bacteria assume the biofilm lifestyle may also include phenotypic changes in bacteria that result in resistance while bacteria are within the biofilm environment, including inactivation of antimicrobial agents by extracellular polymers or modifying enzymes (Davey and O'Toole, 2000; Hoiby, 2010) and regulated efflux pumps (Hoiby, *et al.*, 2010).

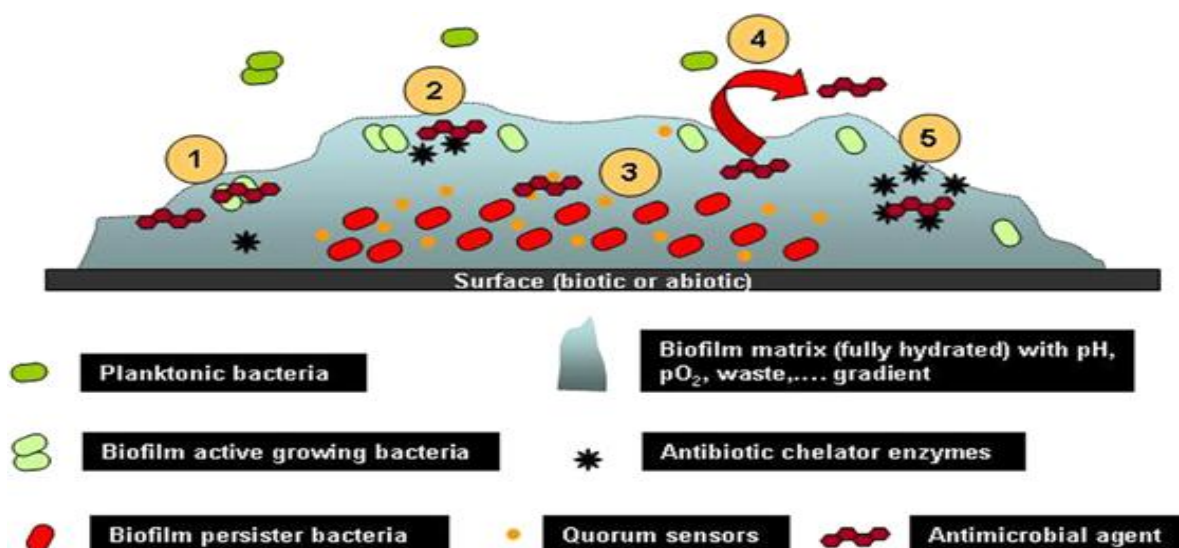


Figure 1.2: Some proposed-biofilm associated resistance mechanisms: (1) Antimicrobial agents may fail to penetrate beyond the surface layers of the biofilm. Outer layers of biofilm cells absorb damage. Antimicrobial agent's action may be impaired in areas of waste accumulation or altered environment (pH, pCO₂, pO₂, etc). (2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix. (3) Altered growth rate inside the biofilm. Antimicrobial agents may not be active against non-growing microorganisms (persister cells). (4) Expression of biofilm-specific resistance genes (*e.g.*, efflux pumps). (5) Stress response to hostile environmental conditions leading to an over-expression of antimicrobial agent-destroying enzymes (Del Pozo and Patel, 2007).

1.4.2.1. Efflux pumps systems and biofilm formation

Multidrug efflux pumps have emerged as relevant elements in the intrinsic and acquired antimicrobial resistance of bacterial pathogens (Martinez *et al.*, 2009) and pose a huge threat for

antimicrobial therapy and human health. Multidrug efflux pumps are transport proteins that extrude chemically unrelated antimicrobial agents from the cell into the environment (Nelson, 2002; Mah and O'Toole, 2001). They act like bilge pumps that decrease the intracellular concentration of the drug to sub-toxic levels (Borges-Walmsley *et al.*, 2003). The genes that code for these multidrug efflux pumps are present in the chromosomes of all living organisms but can also be found on plasmids (Pidcock, 2006a). The genes are highly conserved (members of the same species contain the same efflux pumps) and their expression is tightly regulated (Martinez *et al.*, 2009). Nelson (2002) reported that efflux proteins can be specific, facilitating the efflux of only one compound or a class of compounds or they can be non-specific exhibiting broad specificity for chemical compounds that are structurally unrelated. Martinez *et al.* (2009) reported that in clinical environments, multidrug efflux pumps are involved in resistance to antimicrobial agents present on mucosal surfaces and this resistance allows bacteria to grow, form biofilms and colonize these surfaces (Fig. 3). Furthermore, they could efflux virulence factors, and are also involved in the QS-regulated expression of virulence factors (Martinez *et al.* 2009). All of these are important traits required for the survival of pathogenic and virulent bacteria in clinical environments. In non-clinical environments (Fig. 3), they may allow for heavy-metal resistance, resistance to organic solvents and in resistance to antimicrobial agents produced by plants, and this may also have relevant outcomes for the environment (Fernandes *et al.*, 2003; Martinez *et al.* 2009).

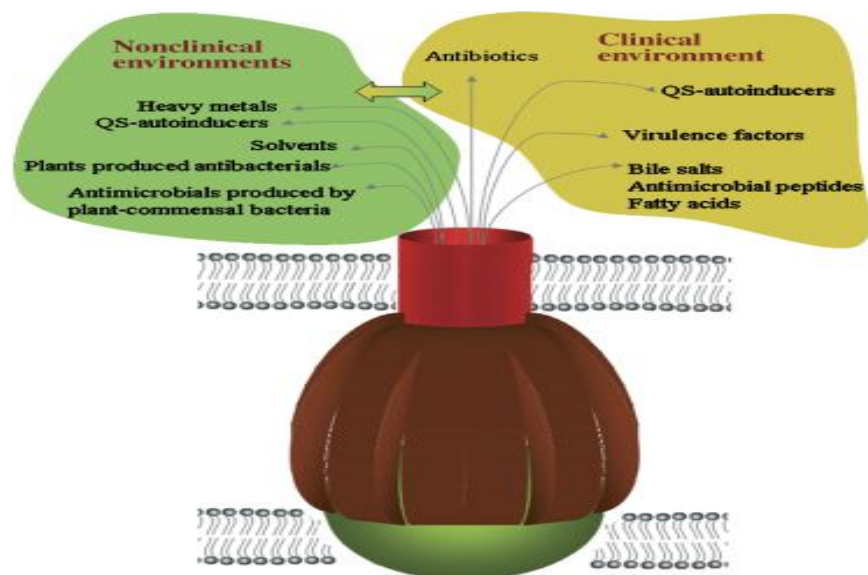


Figure 1.3: Functional role of multidrug efflux pumps in clinical and nonclinical environments (Martinez *et al.*, 2009).

1.4.2.2. Classes and organization of efflux pump systems

Multidrug efflux pumps are subdivided into different families (Fig. 4), based upon their molecular architecture, mechanisms of action, energization requirements and biochemical constitution (Nelson, 2002). In most bacteria, the extrusion of drugs and other cytotoxic compounds is conferred by pumps in which the drug efflux process is coupled to the influx of a proton [H^+] (Borges-Walmsley *et al.*, 2003; Martinez *et al.*, 2009). These are called proton-driven antiporters, and are divided into a number of families namely: the MF (major facilitator), SMR (small multidrug resistance) family, and the RND (resistance-nodulation-division) families.

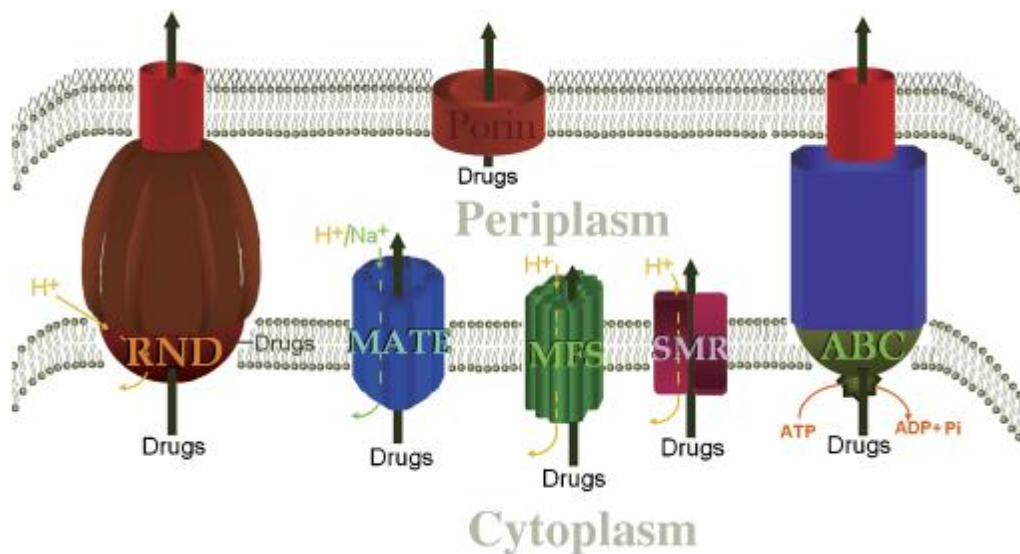


Figure 1.4: Diagrammatic representation of the five families of efflux pumps (Martinez *et al.*, 2009).

In Gram-negative bacteria the expression of efflux pumps is often induced through sub-lethal exposure of bacteria to lethal agents (Gilbert *et al.*, 2002). It is reported that active efflux usually confers a moderate level of resistance, causing a 2-fold increase in the MIC upon the expression of these pumps (Piddock, 2006b; Van Bambeke *et al.*, 2006). A key characteristic of the efflux pumps is the variety of molecules they can transport, which in turn relates to their poor substrate specificity (Van Bambeke *et al.*, 2000). Piddock (2006a) also reported that a single organism can possess multiple multidrug efflux pumps. Moreover, the pumps may also transport different classes of antimicrobial agents (Fig. 5), not within the same class (Van Bambeke *et al.*, 2000). Multidrug efflux pumps can extrude compounds from antimicrobial agents, to disinfectants, dyes, and detergents, and the substrates for each pump are said to be different

depending on the pump and on the bacterial species (Piddock, 2006a). The drug substrate profile of bacteria that overexpress an efflux pump generally includes structurally diverse antimicrobial agents such as chloramphenicol, quinolones (nalidixic acids, ciprofloxacin, norfloxacin), tetracyclines, aminoglycosides, β -lactams, dyes such as ethidium bromide, detergents (sodium dodecyl sulphate) and biocides such triclosan (Piddock, 2006a). Although the specific substrates that are transported by different efflux pump systems are difficult to predict, it has been shown that most transporters recognize molecules with a polar, slightly charged head associated with a hydrophobic domain (Van Bambeke *et al.*, 2000). Van Bambeke *et al.* (2000) reported that a given antimicrobial agent can be a substrate for different types of pumps, so that it may be expelled by different bacteria, such that no common pump been identified so far. The modulation of the activity of a given transporter may be compensated for by a modulation in the opposite direction of another transporter, with, therefore, no or little change in the expulsion of the drug and thus giving false results that the drug is not transported (Piddock, 2006a; Van Bambeke *et al.*, 2000).

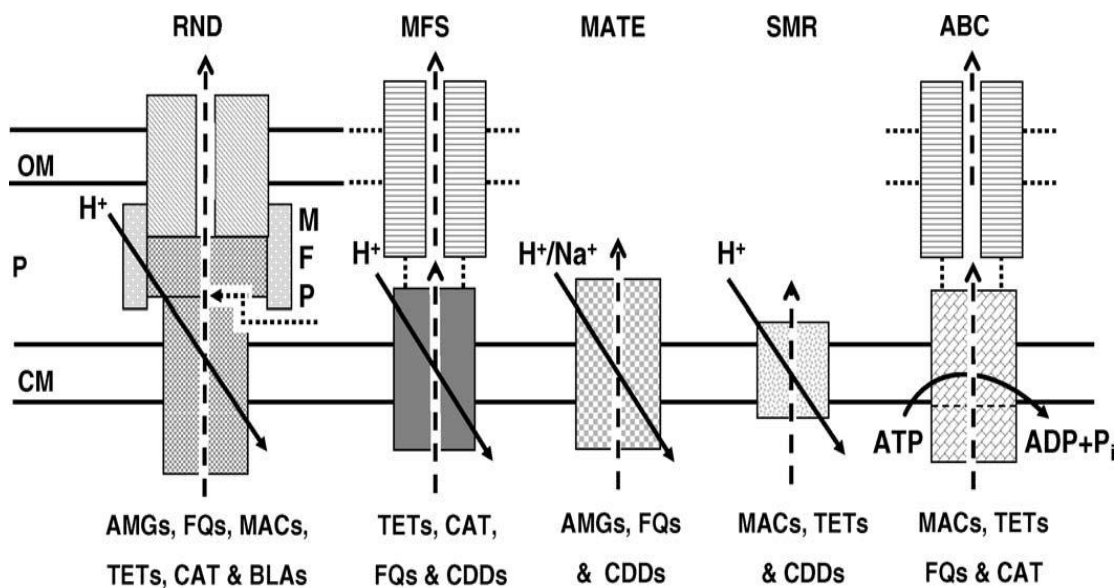


Figure 1.5: Different substrates for multidrug efflux pumps (Lynch, 2006). Abbreviations employed: OM: outer membrane; P: periplasm; CM: cytoplasmic membrane; MFP: membrane fusion protein; TETs: tetracyclines; CAT: chloramphenicol; FQs: fluoroquinolones; CDDs: cationic dyes and detergents; AMGs: aminoglycosides; MACs: macrolides; BLAs: β -lactams.

1.4.2.3. Prevalence of RND efflux pumps in Gram-negative bacteria

A wide variety of efflux pump systems have been identified in many bacteria, including *Aeromonas* spp. isolates, causing multidrug resistance. Bornet *et al.* (2003) reported on the expression of a multidrug efflux pump (AcrA) responsible for resistance to β -lactams, quinolones, tetracycline and chloramphenicol in *Enterobacter aerogenes* due to the repeated use of imipenem to treat patients infected by *E. aerogenes*. Efflux pump genes of the RND family were also shown to be present in the genome of *Burkholderia cenocepacia* and *Burkholderia pseudomallei* isolates (Gugliera *et al.*, 2006; Kumar *et al.*, 2008). Sanchez-Céspedes and Vila

(2007) reported on the presence of the *acrA* and *acrB* genes of *Citrobacter freundii* which were shown to be similar to those described in *E. coli*, and their overexpression was found play an important role in the resistance of *C. freundii* to quinolones. Huang *et al.* (2008) also reported on the increased expression of the AdeABC efflux pump, which caused decreased susceptibility to carbapenems among *Acinetobacter baumannii* isolates in a Chinese hospital.

A multi-drug efflux pump, AheABC, belonging to the RND family was observed in *Aeromonas* spp. isolates specifically, *A. hydrophila* and *A. salmonicida* (Giraud *et al.*, 2004; Hernould *et al.*, 2008). This pump is involved in resistance to the antimicrobial agents erythromycin, trimethoprim, fusidic acid, rifampicin as well as to quinolones (nalidixic acid and oxolinic acid) and fluoroquinolones (ofloxacin and ciprofloxacin).

1.5. Biofilm Control Strategies

Diverse strategies are being applied to control bacterial biofilms due to their increased resistance to antimicrobial agents, including the use of quorum-quenching enzymes, phytochemicals, bacteriophages, nanoparticles, varying concentrations of antimicrobial agents, efflux pump inhibition, matrix-degrading enzymes, quorum-sensing inhibitors (Francolini and Donelli, 2010; Xiong and Liu, 2010).

1.5.1. Targeting biofilms with antimicrobial agents

Antimicrobial agents are designed to target different components of the cell structure including cell wall, cell membrane and intracellular organelles (Cloete, 2003), efflux pump systems (Van

Bambeke *et al.*, 2000) as well as QS signals (Rasmussen and Givskov, 2006). Many studies have focused on biofilm formation due to their increased resistance to antimicrobials which causes problems in treatment of biofilm-associated diseases (Hoiby *et al.*, 2009). Treating diseases of cells living within biofilm does not lead to the complete elimination of the biofilms but only cells on the surface may be eliminated leaving cells in the middle to gain resistance against these antimicrobial agents. These cells may either need a longer exposure time to the antimicrobial agents or higher concentrations of antimicrobial agents to be effective in removing them (Cogan, 2006). Targeting the attachment of cells to the surface where the cells are still accessible to elimination by antimicrobial agents is a good strategy because once cells have already been attached to the surface; it is not easy to eliminate them. The attached cells display reduced susceptibility to antimicrobial agents due to increased production of EPS, reduction of the growth rate as a result of accumulation of waste products and other attachment-specific phenotypes that are observed when cells reside within biofilms (Takahashi *et al.*, 2007). In the case of pathogens, once the cells have already adhered to the host tissue, it not easy to eliminate them because the infection process has already been triggered (Takahashi *et al.*, 2007).

Another possibility is to use a combination of two or more drugs that have different modes of action to target different sites, but this has been proven to have a slower mechanism of action compared to the single drug therapies (Quave *et al.*, 2008). Because cells within biofilm demonstrate different resistance mechanisms, the additive effect of two or more drugs has been proposed to be effective against pathogens (Tabak *et al.*, 2009). Tabak *et al.* (2009) observed that biofilms of *Salmonella enterica* serovar Typhimurium were more susceptible to double treatment with ciprofloxacin and triclosan because the double attack by these antimicrobials is beyond the capability of their defence system. In addition, triclosan weakens the cell membrane and allows

entry of ciprofloxacin into the cell and act on its target site. The weakening of the cell membrane by triclosan is caused by reduction of Van der Waal's forces/interactions between the adjacent phospholipid molecules of the cell membrane. Exposure of cells to triclosan also increases mutations of genes required for the formation of membrane structure of cells. This demonstrates that synergistic action of two or more antimicrobials is more effective compared to a single drug therapy (Tabak *et al.*, 2009).

Lewis (2007) observed that fluoroquinolone agents are effective against biofilm formation but once the biofilm community is matured, the antimicrobial agent is restricted in its ability to diffuse through the biofilm and reach its target site. Quinolones are a large group of excellent, synthetic antimicrobial agents that are known to have a broad spectrum of activity against a wide variety of infections (Chen and Lo, 2003). They are also used as drugs of choice for the treatment of *Aeromonas* infections in humans and in the treatment of bacterial fish diseases (Alcaide *et al.*, 2010). These drugs can persist in the environment for a long time, consequently leading to the emergence of resistant strains (Alcaide *et al.*, 2010). Their mechanism of action involves bacterial DNA damage which leads to defects in negative supercoiling and cell death, and this is linked to the inhibition of DNA gyrase activity, which plays a role in DNA packaging, replication and transcription as well as topoisomerase IV activity which mediates the relaxation of DNA duplex and unlinking of daughter chromosomes following replication (Martinez *et al.*, 2006).

In addition, some cells within biofilms possess the enzyme β -lactamase that allows them to degrade antimicrobial agents containing the β -lactam ring reducing their effectiveness (Lewis, 2007). Some antimicrobial agents are effective in preventing the attachment of biofilms on the

surface while others can cause detachment from biofilm that have already been attached to the surface. Tetracycline and monocycline were able to inhibit the early phase of biofilm growth as they inhibit attachment of *Actinobacillus actinomycetemcomitans* to the surface preventing formation of biofilms (Takahashi *et al.*, 2007).

By understanding bacterial cell physiology, antimicrobial agents have been designed to target these appendages, which indirectly interfere with biofilm formation stages as well as the maintenance of the mature biofilm. Braga *et al.* (2000) observed a reduction in adhesiveness of pathogenic *E. coli* strains to human epithelial cells. This reduction was correlated with the filamentation of *E. coli* cells when exposed to cefodizime because it binds to penicillin binding protein 3 (PBP3) that plays a very important role in adhesion to the epithelial cells. The binding of cefodizime to PBP3 exposed on the surface prevented surrounding *E. coli* cells from recognising PBP3, which is crucial for biofilm formation thus interfering with a pathogenesis process. An initial stage of biofilm formation by these cells is also prevented due to inhibition of adhesion to epithelial cells by cefodizime. Cefodizime reduced hydrophobicity, which is required for biofilm formation and fimbriation which prevents fimbriated cells to bind into non-fimbriated cells and form biofilms in a process called autoggregation. Exposure of cells to cefodizime reduced motility which is important for spreading of the infection from one point into another and if this process is prevented biofilm formation can be controlled (Braga *et al.*, 2000).

Vidya *et al.* (2005) observed that exposure of *E. coli* to gentamicin, ceftazidime, ampicillin, ciprofloxacin and co-trimoxazole resulted in varying levels of adhesion. Co-trimoxazole caused the greatest suppression of adhesion compared to other tested antimicrobial agents followed by ceftazidime while ciprofloxacin, gentamicin and ampicillin cause

filamentation of *E. coli* cells. Adherence inhibition of these tested antimicrobial agents were dose-dependent indicating the importance of concentration of antimicrobial agents in treatment of diseases caused by bacterial pathogens. Inhibition of adherence during infection is important because adhesion to the surface is the first step that leads to the colonization and initiation of the infection process (Vidya *et al.*, 2005). Ciprofloxacin and gentamicin reduced biofilm formation through the reduction of exopolysaccharide production in *S. enterica* serovar Typhimurium which is required for the maintenance of the 3-D structure of biofilm by serving as the protective layer of cells within biofilms (Majtan *et al.*, 2007). Thus antimicrobial agents can have different modes of action depending on the bacterial species or genera they act on as differences are observed with *E. coli* and *S. enterica* serovar Typhimurium where there was reduction of adherence for *E. coli* and thinning of EPS for *S. enterica* serovar Typhimurium following ciprofloxacin and gentamicin exposures (Vidya *et al.*, 2005; Majtan *et al.*, 2007).

1.5.1.1. Effect of varying concentrations of diverse antimicrobial agents on planktonic and biofilm cells

The effective therapeutic result of antimicrobial agents is best when the concentration is above the MIC. However, after a certain period of time following dosing, antimicrobial agent concentrations within many tissues become lower than the MIC (Pompillo *et al.*, 2010) and these are called sub-inhibitory concentrations (sub-MIC). So depending on the type of the antimicrobial agent used or their modes of action, sub-MIC exposure can either eliminate or reduce the ability of pathogens to form biofilms by interfering with different stages of biofilm formation resulting in the elimination of these pathogens. When cells are exposed to

antimicrobial agents especially at sub-MIC levels, regardless of whether they are planktonic or biofilm cells, there are morphological changes that occur in response to the exposure to the antimicrobials (Dynes *et al.*, 2009). In planktonic cells, morphological changes include changes in cell shape, width and surface composition. They become longer; reduce their width and change their surface composition by changing the chemical composition on their surface. These changes allow the cells to adapt and survive in the presence of antimicrobial agents. Changes in surface composition that mostly occur in the planktonic cells is the increase in lipid deposition on the cell membrane which is thought to be important for the maintenance of the cell membrane integrity during exposure to antimicrobial agents as well as preventing leakage of the intracellular components. Dynes *et al.* (2009) observed that where there was a greater lipid content in *Pseudomonas fluorescens* cells in response to exposure to chlorhexidine, triclosan and benzalkonium chloride (Dynes *et al.*, 2009). Microorganisms often grow in the presence of sub-MICs, which although not able to inactivate the microorganism, are potentially capable of altering the chemical and physical cell-surface characteristics and consequently the functionality and expression of some virulence properties such as adhesion, biofilm formation, hydrophobicity and motility (Pompillo *et al.*, 2010). Pompillo *et al.* (2010) observed that sub-concentrations of moxifloxacin decreased adhesion and biofilm formation of *Stenotrophomonas maltophilia*. Braga and Piatti (1993) observed that sub-MIC rifloxacin concentrations played a role in decreasing pathogen-host interactions. Matjan *et al.* (2007) also observed that sub-MICs of antimicrobial agents affected biofilm formation of clinical strains of *Salmonella enterica* serovar Typhimurium. For some microorganisms being exposed to sub-MIC of antimicrobial agents can stimulate biofilm formation because they do not kill cells within biofilms but only inhibit their growth, this has been observed for which cells exposed to cefalexin (Haddadin *et al.*, 2009).

1.5.2. Efflux Pump Inhibition

Efflux pump inhibitors (EPIs) have been used to inhibit different efflux pump systems used by diverse bacterial species thus preventing elimination of antimicrobial agents within the cells. It is thought that by inhibiting these efflux pump systems, the antimicrobial agent will remain within the cells to act on their targets sites thus eliminating or inhibiting cell growth causing reduction of these biofilms (Hirakata *et al.*, 2009). Efflux pump inhibition represents one of the most promising strategies to control bacterial biofilms because cells in biofilm over-express these efflux pump systems compared to planktonic cells. This has been observed in *E. coli* and *Klebsiella* spp. strains during biofilm phase of growth, where *aaeX* and *yqgA* which encodes the membrane protein component of the AaeAB efflux pump and a putative transport protein required for the efflux pump mechanism, respectively, were up-regulated in biofilms (Kvist *et al.*, 2008).

Since there are different efflux pump systems, different inhibitors inhibit specific efflux pumps depending on their spectrum of activity (Kvist *et al.*, 2008). There are six different mechanisms of efflux pump inhibition that have been described (Poole and Lomovskaya, 2006); the first mechanism is through targeting the substrate binding sites of the efflux pump system. EPIs are designed to competitively inhibit the binding of the drug into their site causing the drug to remain within the cell. The second mechanism is inhibition of the pump modulation sites where the inhibitors act through allosteric inhibition by binding to sites other than the binding site and alter the activity of the efflux pump system. The third mechanism is uncoupling of drug efflux from proton influx. This occurs in secondary transporters that require energy to pump the drug out and by uncoupling proton influx and drug efflux, thus the efflux pump can be inhibited.

Protein-protein interaction is required for the assembly of any multi-components proteins in the cell including the efflux pump systems, thus by inhibiting the interaction between different components of the efflux pump, drug efflux can be prevented because the protein will be missing some of its vital parts. Inhibition of the exit pore is another mechanism of efflux pump inhibition and represents one of the components of efflux pump system in Gram-negative bacteria. This component adheres to the outer membrane of the cell wall while other components are required to serve as the passage, which connects the inner membrane and the outer membrane. The last component adheres to the inner membrane and stabilizes it (Poole and Lomovskaya, 2006). The TolC component is the exit pore that opens in the periplasm for elimination of the antimicrobial agent. Opening of TolC components can be inhibited by antimicrobial agents, e.g., divalent and trivalent cations, thus inhibiting the efflux pump systems in microorganisms. The last mechanism is through the inhibition of the efflux pump gene expression because efflux pump systems are encoded chromosomally and their expression is controlled by regulatory proteins that regulate if they are expressed or not and to what extent (Poole and Lomovskaya, 2006).

There are different compounds that are potential EPIs. The first class are the tetracycline analogues. These analogues are able to bind into efflux systems that are responsible for elimination of tetracycline antibiotics. The most effective inhibition by this class is obtained through 6-(alkylthio)-methyl-doxycycline compounds. These compounds are most potent inhibitors of both class A and B efflux pumps that are abundant in *E. coli* and at the same time they are less effective on class K and L efflux systems which are found in Gram-positive bacteria (Van Bambeke *et al.*, 2006). Aminoglycoside analogues provide another class of EPIs but they have been proven to be poor inhibitors due to their ability to being highly hydrophilic in nature

(Van Bambeke *et al.*, 2006). However, aminoglycosides have been used as inhibitors as they are able to reduce MICs of antimicrobial agents from one to four-fold decrease when tested in *Haemophilus influenzae*. They are also able to increase susceptibility of the wild type strain to different antimicrobial agents (Van Bambeke *et al.*, 2006). Fluoroquinolone analogues are also commonly used EPIs that are effective in inhibiting efflux pumps in both Gram-positive and Gram-negative bacteria (Van Bambeke *et al.*, 2006). The use of fluoroquinolones analogues was able to restore macrolide activity in streptococci that were over-expressing the Mef pumps. Piperazinyl-linked fluoroquinolone displayed potent antibacterial activity against *Staphylococcus aureus*, some of which were resistant to antimicrobial agents due to the presence of the NorA pumps (Van Bambeke *et al.*, 2006). Indioles, ureas and aromatic amides all belong to another class of EPIs. These inhibitors have a broad structural diversity and are able to inhibit multiple efflux pump systems demonstrating a low structural specificity for the pumps. Such inhibitors can be very useful for multiple efflux pump systems because they have a broad spectrum of activity. Other efflux pump inhibitors include piperadine-carboxylic acid derivatives, alkylaminoquinole, thioalkoxyquinolone, alkoxyquinolone and peptidometrics. Efflux pump inhibition using the above mentioned inhibitors and other additional inhibitors such as phenylalanine arginine β -naphthylamide (PABN), 1-(1-naphthylmethyl)-piperazine (NMP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) are promising candidates to be used in managing bacterial associated diseases (Van Bambeke *et al.*, 2006).

When an EPI is added together with other antimicrobial compounds, this causes a reduction in the MIC values of different antimicrobial agents because cells become more susceptible to these antimicrobials. Bina *et al.* (2009) observed that the MIC of different

antimicrobials compounds was reduced in the presence of two EPIs (PA β N and NMP). Both PA β N and NMP inhibit RND pumps that are abundant in Gram-negative bacteria including *Vibrio cholerae*, in which the multiple RND pumps, not only pump out antimicrobial agents but also modulate the expression of virulence genes (Bina *et al.*, 2009). Because RND pumps are found in cell membrane and are not regulatory proteins, they modulate gene expression through the efflux of QS molecules that reach the recipient cells and induce expression of these virulence genes (Bina *et al.*, 2009). PA β N and NMP inhibit the RND pumps by inhibiting RND-dependent gene expression resulting in reduction of MIC of the antimicrobial agents. This is important since the adhesion factor and virulence factors required for biofilm formation and virulence in *V. cholerae* are dependent on these genes being functional. The inhibition of the efflux pumps results in the reduction of virulence of *V. cholerae* strains due to the inhibition of adhesion to the host epithelial cells thus interfering with biofilm formation. This is why EPIs have the potential to be used in clinical drug therapy together with the antimicrobial agents (Bina *et al.*, 2009).

Hirakata *et al.* (2009) observed that *P. aeruginosa* invasiveness was reduced upon exposure to PA β N, which inhibits the MexAB-OprM efflux pump system in *P. aeruginosa* strains and also damages the outer membrane of Gram-negative organisms. This suggests that the inhibition of these efflux pump systems prevents the elimination of an antimicrobial agent allowing the antimicrobial agent to act on its target site (Hirakata *et al.*, 2009). Efflux pump systems that are dependent on proton motive force (PMF), MexAB-OprM, and others largely contribute to the efflux of antimicrobial agents and other compounds. Inhibition of these efflux pump systems interferes with biofilm formation by causing the accumulation of autoinducers within the cell (Ikonomidis *et al.*, 2008). Ikonomidis *et al.* (2008) observed that the addition of

the PMF inhibitor CCCP at the beginning of biofilm growth resulted in reduced growth compared to the untreated cells. This is because CCCP interferes with the PMF-dependent transporters that are abundant in bacterial cells. It was also observed that a lower concentration of CCCP did not have much effect on biofilm formation of *P. aeruginosa*. This organism might require higher concentrations of efflux inhibitors or might be using other types of efflux pump systems that are not entirely inhibited by CCCP (Ikonomidis *et al.*, 2008). The addition of different EPIs, PA β N and NMP, resulted in the inhibition of biofilm formation by *P. putida* and *Staphylococcus aureus* (Kvist *et al.*, 2008). Biofilm formation of uropathogenic *Klebsiella* spp. isolates and *E. coli* was also reduced by the addition of the EPI thioridazine, which inhibits the MFS pump. Multiple EPIs may be used to inhibit different efflux pumps because the organism might possess different kinds of efflux pumps that may contribute to resistance against different types of antimicrobial agents. That is why a combination of two or more types of EPIs have been used to synergistically act together in inhibiting different kinds of efflux pumps found in different bacterial species. The combination of thioridazine and PA β N resulted in an even greater effect on *Klebsiella* spp. and *E. coli* biofilm inhibition compared to the individual EPIs because these individual bacterial strains possess more than one type of efflux pump system (Kvist *et al.*, 2008). Based on the proteomic and transcriptomic studies of *F. johnsoniae*-like isolates, an upregulation of RND-, MATE-, MFS-efflux pump proteins was observed during biofilm phase of growth (Flemming, 2010) indicating the ability of these strains to be resistant to multiple antimicrobial agents in the biofilm stage of growth compared to planktonic cells (Flemming, 2010).

The efflux pump resistance mechanism is one of multiple resistance mechanisms observed in bacterial species and inhibition of these efflux pump systems does not necessarily mean that the bacteria will become completely susceptible to a range of antimicrobial agents. This was demonstrated in a multidrug-resistant *P. aeruginosa* isolate that produces β -lactamase. In this organism, inhibition of the MexAB-OprM efflux pump did not reduce or hinder resistance of *P. aeruginosa* to β -lactams since they are able to degrade and inactivate β -lactams (Poole and Lomovskaya, 2006). This means that efflux pump inhibition alone is not sufficient to reduce resistance to antimicrobial agents but can be used in combination with other antimicrobial agents. Since these efflux pumps are responsible for waste management in cells, it is possible that the cell can have multiple efflux pump systems to eliminate different waste and antimicrobial compounds, thus different inhibitors can be used at the same time or inhibitors with a broad spectrum of activity can be used to target these different pump systems. This is very useful when dealing with multidrug resistance organisms such as *P. aeruginosa*. Because EPIs can be used together with antimicrobial agents, these agents must not be cell wall-acting antimicrobials because they will tend to compete with the EPIs resulting in the reduction of their effectiveness (Poole and Lomovskaya, 2006).

1.5.3. Quorum sensing inhibition as strategy to control biofilm formation

The interference of bacterial QS is acknowledged to attenuate virulence and is considered to be a potential new therapy to treat bacterial infections caused by pathogenic bacteria (Rasch *et al.*, 2005; Truchado *et al.*, 2009). If cells cannot efficiently communicate, then they cannot form biofilms, which allows drugs to easily attack cells because they will be existing as planktonic

cells. Thus, QS inhibition has been proposed to be a promising way of managing bacterial infections (Ni *et al.*, 2009). There are a variety of mechanisms associated with the inhibition of bacterial QS (Fig. 6), viz., (1) inhibition of autoinducer synthesis, (2) autoinducer receptor antagonism, (3) inhibition of targets downstream of receptor binding, (4) sequestration of autoinducers, (5) degradation of autoinducers, (6) inhibition of autoinducer transport, and (7) antibodies that “cover” and therefore, block autoinducer receptors (Ni *et al.*, 2009).

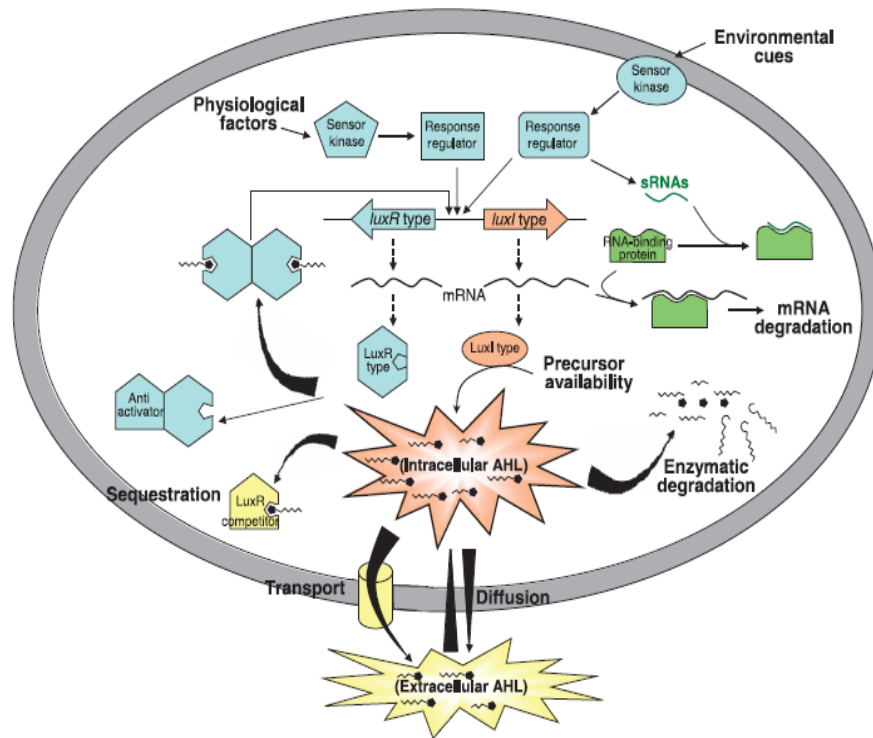


Figure 1.6: Different mechanisms of quorum sensing inhibition in microorganisms (Boyer and Wisniewski-Dye, 2009).

Enzyme molecules produced by different bacterial species have also been found to be effective as quorum sensing inhibitors (QSIs). AHL-lactonases are a group of enzymes produced by different bacterial species and have a quorum-quenching activity. This is because they hydrolyse the homoserine lactone ring of AHL molecules making them unavailable to act on recipient cells. Long chain AHL signals can also be degraded with paraoxonase enzymes that are found in mammalian cells as part of the defence system of the mammalian tissue. They silence bacterial communication due to the hydrolysis of homoserine lactone rings (Dong *et al.*, 2007). AHL-acylase is another type of enzyme produced by species like *P. aeruginosa* and has the ability to silence communication between bacterial species due to its ability to hydrolyse the amide bond producing corresponding fatty acids and homoserine lactone (Dong *et al.*, 2007).

QSIs include the molecules that structurally mimic QS signals such as halogenated furanones, which have a similar structure to AHLs. Furanone, first isolated from *Delisea pulcra*, was observed to be effective against Gram-negative bacteria, with susceptibility of the isolates to antimicrobial agents being increased in the presence of furanones. Furanones inhibit QS by interfering with AI-2 pathways but the mechanism of action of these furanone molecules is still unclear (Vestby *et al.*, 2010). There are different types of furanones that have been used for QS inhibition but the brominated furanones have been predominately used due to their ability to strongly inhibit communication because bacterial species thus inhibiting biofilm formation. These molecules competitively prevent binding of the QS molecules to the receptor or decrease the receptor concentration thus QS molecules cannot initiate the transduction pathway that leads to the response (Dong *et al.*, 2007). Beside inhibition of QS through competitive competition, furanones are known to be able to cause protein degradation of the LuxR protein, which is

required for the expression of genes required for QS. The degradation of LuxR causes reduced expression of the QS molecules resulting in reduced communication between the cells and inhibits the multi-cellular behaviour of microorganisms (Manefield *et al.*, 2002).

The second group are the enzymes inhibitors that interfere with the enzyme activities that are required for QS. These enzymes inhibitors include triclosan that inhibits enoyl-ACP reductase activity whose product is an essential intermediate for AHL biosynthesis and closantel, which is a potent inhibitor of histidine kinase sensor of the two-component system that is crucial for QS in different bacterial species (Dong *et al.*, 2007).

In the study performed by Kai *et al.* (2009), exposure of cells to sub-MIC of azithromycin resulted in a small down-regulation of AHL synthesis enzymes that resulted in a larger effect on AHL production thus interfering with bacterial communication. This was achieved by inhibition of intracellular S-adenosylmethionine, which decreases AHL production by transcriptional regulators (LasR and RHIR) during exposure of *P. aeruginosa* to azithromycin (Kai *et al.*, 2009). Beside azithromycin, different QSIs have been used to inhibit bacterial QS thus controlling biofilm formation. These QSIs have different mechanisms of action causing variations in the level of QS inhibition. Cinnamaldehyde acts by competitively binding to the QS receptor sites and prevents the binding of the QS molecule only when an organism is using short chain QS molecules but fails to cause inhibition of long chain QS molecules (Brackman *et al.*, 2009). Due to the low effectiveness of cinnamaldehyde, it can be used in combination with other antimicrobial agents to increase its effectiveness. This was observed in biofilms of *Salmonella enterica* serovar Typhimurium where the combination of cinnamaldehyde and thymol greatly reduced biofilm formation. Similar results were observed with biofilms of *Staphylococcus*,

Micrococcus, *Bacillus* and *Enterobacter* spp., where the combination of cinnamaldehyde and eugenol was effective against biofilms of these isolates as well as preventing biofilm formation by these isolates. However, cinnamaldehyde was observed to be less effective against already formed biofilms compared to planktonic cells (Zhou *et al.*, 2006). Ponnusamy *et al.* (2009) discovered the potential use of vanillin as a quorum sensing inhibitor for *A. hydrophila* biofilm formation, as it has been observed to inhibit short-chain (C4-) and long-chain (3-oxo-C8-) AHL molecules.

Microorganisms do not only use the AHLs as their QS molecules but also use autoinducer-2 (AI-2). Molecules such as *S*-adenosylhomocysteine, sinefungin and butyryl-SAM have been used for QS inhibition because they are *S*-adenosylmethionine (SAM) analogues that interfere with QS signalling by inhibiting the synthesis of SAM, a QS intermediate (Schauder *et al.*, 2001).

1.5.4. Use of DNase matrix-dispersing enzymes

The EPS is the outermost layer of the biofilm and consists of a wide variety of polysaccharides, proteins, glycoproteins, glycolipids and often, large amounts of extracellular DNA (eDNA) (Kaplan, 2009). eDNA has been identified as an important stabilizing factor in biofilms, although it does play very different structural roles in biofilms formed by different organisms.

Tetz and Tetz (2010) observed the effects of eDNA destruction by DNase I on the characteristics of bacterial biofilms and found that DNase I (5.0 µg/ml) was effective on bacterial biofilms causing bacterial biofilms to be less tolerant to antimicrobial agents. However, the role

of eDNA in early stages of biofilm formation is poorly understood (Tetz and Tetz, 2010). DNase I was effective against both Gram-negative (*P. aeruginosa*, *H. influenzae*, etc.) and Gram-positive (*Staphylococcus epidermidis*, *Streptococcus intermedius*, etc.) bacteria indicating the broad-spectrum of activity and can potentially be used for therapeutic purposes to eliminate bacterial biofilms. When DNase I was added at the time of inoculation, it resulted in up to 99% reduction of biofilm formation, as observed after crystal violet staining. The action of DNase I on biofilms depends on its concentration and the type of species that are exposed to the enzymes. Inhibition of biofilms by DNase I is not through inhibition of cell growth but one possible explanation is the degradation of cell surface nucleic acid that function as adhesins, since inhibiting adhesion to the surface results in prevention of biofilm formation. Supporting this hypothesis of using eDNA as an adhesin is the fact that when exogenous DNA was added to the culture medium of *P. aeruginosa*, it resulted in increased adhesion to the surface. Another role of eDNA within biofilm matrix may be through cell-to-cell interactions that causes the cells to form aggregates resulting in biofilm formation. Less coaggregated cells of *P. aeruginosa* and *S. aureus* were observed when these cells were exposed to DNase I, which is correlated with reduction in biofilm formation (Kaplan, 2009).

DNase I can also be effective on pre-formed biofilms (12-60 hour old), reducing biofilms of different bacterial strains. However, less effectiveness was observed for established (80 hour old) biofilms (Kaplan, 2009). The effectiveness of DNase I in different microorganisms is affected by other factors such as the type of isolate exposed to the enzyme, the media used to grow the bacterial isolates, the origin of the enzymes and other cell characteristics (Kaplan, 2009). Due to increased resistance of the cells within biofilms, effectiveness of the DNase I on

pre-formed biofilms is thus important. This was observed with *S. epidermidis* where detachment was observed for 6 hour old biofilm but a 24 hour old biofilm was completely resistant to DNase I treatment (Kaplan, 2009). This may be due to the fact that cells in young biofilms may be completely relying on DNA as an adhesin but as they grow older they produce other adhesins that are used for biofilm maintenance. Another possible explanation is that cells in mature biofilm might contain stabilized DNA that is very resistant to the DNase I attack or the DNA may be sheltered by other components in the biofilm matrix reducing effectiveness of the DNase I in matured biofilms (Kaplan, 2009).

DNase I can also increase the bactericidal activity of different antimicrobial agents by sensitizing the bacterial cells to antimicrobial agents, thus making them more susceptible to antimicrobial agents (Kaplan, 2009). This was observed with *P. aeruginosa* and *S. aureus* where the effectiveness of levofloxacin, rifampin, benzalkonium chloride, cetylpyridinium chloride, bleach, and chlorhexidine gluconate was increased when these bacterial cells were pre-exposed to the DNase I prior to the treatment with these bactericidal agents. The mechanism by which the DNase I sensitizes the cells is not known, however, it is hypothesized that DNA acts as a barrier for the diffusion of bactericidal agents so by degradation this DNA allows for the entry of these antimicrobial agents to act on bacterial cells and eliminates them (Kaplan, 2009). Another possibility is that DNA that is surface-attached restricts the entry of the antimicrobial agents, so by eliminating this surface-exposed DNA, the antimicrobial agent gains entry to the cell and act on their targets site (Kaplan, 2009).

1.6. Rationale of the study

Biofilm formation is not only an important stage in the pathogenicity of organisms, but biofilm establishment on host tissue (human or fish) or on inanimate surfaces also inhibits the effectiveness of antimicrobial therapy, protects against host defence mechanisms and also facilitates bacterial communication (QS) leading to the expression of virulence determinants. Thus, apart from characterizing *Aeromonas* spp. isolates, identifying species, which have the ability to form biofilms and the manner in which they form biofilms is critical. Additionally, although *Aeromonas* spp. biofilms have been studied, the focus has been on specific species, i.e., *A. hydrophila* and *A. caviae*. Thus, studying biofilm formation by a variety of members of this genus could provide more relevant information on the behaviour of diverse species and identify phenotypic adherence and/or biofilm formation characteristics of *Aeromonas* spp. This, together with understanding the factors involved in and affecting biofilm formation by *Aeromonas* spp. isolates is also important. Evaluating the antimicrobial concentrations required to eradicate biofilms by *Aeromonas* spp. isolates could facilitate effective treatment of these biofilms using antimicrobial agents, as cells in a biofilm appear to be more resistant to antimicrobial agents and/or biofilm inhibition molecules. Understanding and evaluating the effect of different biofilm inhibitors on biofilm formation by *Aeromonas* spp. isolates could facilitate effective removal of these biofilms and provide strategies to solve some of the infections caused by these biofilms.

It is hypothesized that *Aeromonas* spp. isolates from diverse cultured fish will display biofilm-forming abilities, which are species-specific and are associated with their pathogenicity. It is further hypothesized that characteristics such as motility, hydrophobicity, autoaggregation, proteolytic ability, extracellular DNA secretion may be correlated with the initiation of biofilm

formation and/or biofilm maturation by *Aeromonas* spp. isolates. Additionally, it is hypothesized that exposure to antimicrobial agents, efflux pump inhibitors and quorum-sensing inhibitors will control *Aeromonas* spp. biofilm formation.

1.7. Objectives

The following objectives have been established:

- a. To evaluate the ability of *Aeromonas* spp. isolates from diverse fish species to form biofilms;
- b. To investigate factors potentially involved in initiating biofilm formation, i.e., determining the correlation between motility, cell surface characteristics, and metabolism with biofilm formation by *Aeromonas* spp. isolates;
- c. To determine the antimicrobial resistance profiles of *Aeromonas* spp. isolates;
- d. To determine efflux pump-mediated resistance in *Aeromonas* spp. isolates and its potential contribution to antimicrobial resistance in biofilms;
- e. To identify the ability of *Aeromonas* spp. isolates to communicate by producing signaling molecules (AHL production);
- f. To determine the effect of varying concentrations of diverse antimicrobial agents on planktonic and biofilm cells; and
- g. To determine the effect of efflux pump inhibitors, lytic enzymes and quorum-sensing inhibitors on *Aeromonas* spp. biofilm-forming ability.

1.8. Aims of present study

The following aims were pursued:

- a. To screen *Aeromonas* spp. isolates obtained from diverse fish species for biofilm formation using microtiter plate assays, under different physicochemical parameters including temperature, agitation, and nutrient availability;
- b. To identify factors potentially contributing to initial attachment and/or biofilm formation, i.e., capsule production, motility (swimming/swarming), hydrophobicity, autoaggregation, protease activity, and extracellular DNA production;
- c. To determine the antimicrobial susceptibility of *Aeromonas* spp. isolates using the disk diffusion method;
- d. To determine the prevalence and diversity of efflux pumps in *Aeromonas* spp. using the disk diffusion assay on efflux inhibitor-containing Mueller Hinton (MH) medium;
- e. To identify the expression of QS signaling molecules by *Aeromonas* spp. isolates using biosensors: *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136;
- f. To determine the effect of antimicrobial agents (azithromycin, ceftazidime, ciprofloxacin, gentamicin, tetracycline), i.e., the minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of planktonic and biofilm-forming isolates, respectively, using the broth microdilution and modified microtiter plate assay;

- g. To determine the effect of sub-minimum inhibitory concentration (sub-MIC), MIC and supra-minimum inhibitory concentration (supra-MIC) of planktonic and biofilm-forming cells, respectively, using microtiter plate assays; and
- h. To determine the effect of efflux pump inhibitors [carbonyl cyanide 3-chlorophenylhydrazone (CCCP), phenylalanine arginine β -naphthylamide (PA β N) and 1-(1-naphthylmethyl)-piperazine (NMP)], lytic enzymes (DNase I) and quorum-sensing inhibitors [cinnamaldehyde, (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, S-adenosylhomocysteine, vanillin] on planktonic and biofilm-forming isolates (initial attachment and/or mature biofilm) using microtiter plate assays.

CHAPTER TWO

Phenotypic investigation of factors potentially involved in initial attachment and/or biofilm formation by *Aeromonas* spp. isolates

2.1. Introduction

Members of the genus *Aeromonas* are ubiquitous and autochthonous aquatic bacteria (Castro-Escarpulli *et al.*, 2003; Gordon *et al.*, 2008; Kirov *et al.*, 2002; Tacao *et al.*, 2005) that occupy a wide variety of environmental niches including soil and water. They are also pathogens of warm- and cold-blooded animals, often associated with fish and human (food or water-borne) infections. They are implicated as possible threats to human health, and also result in economic losses in aquaculture and fish-farming industries (Davies *et al.*, 2001; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Gordon *et al.*, 2008; Kirov *et al.*, 2002; Koksai *et al.*, 2007; Tacao *et al.*, 2005).

Most bacterial infections involve biofilms (Behlau and Gilmore, 2008; Hoiby *et al.*, 2010). Aquatic fish pathogens such as *Aeromonas* spp. have been shown to form biofilm structures in aquaculture environments and on abiotic surfaces, and their survival outside the fish host may be dependent on biofilm formation (Basson *et al.*, 2008). Bacterial biofilms form on biotic or abiotic surfaces, developing into large communities of complex architecture where there is cell-to-cell communication and co-ordinated behaviour, i.e., quorum sensing (Behlau and Gilmore, 2008; Hoiby *et al.*, 2010; Vu *et al.*, 2009). *Aeromonas* spp. isolates form biofilms in diverse environments, i.e., glass surfaces, galvanized steel surfaces, and on microtiter plate wells

(Asha *et al.*, 2004; Azad *et al.*, 1999; Dogruoz *et al.*, 2009; Gavin *et al.*, 2004; Li *et al.*, 2009; Merino *et al.*, 2001; Nayak *et al.*, 2004). In these biofilms, bacteria surround themselves with a slimy EPS, which protects them from their environment; conferring protection against phagocytosis in the host, interference with the cellular immune response, and reduction of antimicrobial potency. Due to this durability, biofilms are responsible for a wide range of industrial and health problems (Asha *et al.*, 2004; Azad *et al.*, 1999; Dogruoz *et al.*, 2009; Gavin *et al.*, 2004; Li *et al.*, 2009; Merino *et al.*, 2001; Nayak *et al.*, 2004).

Initial attachment or adhesion is very important for most microbial infections and a crucial stage in biofilm formation (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). It is affected by chemical and physical properties of the cell and surface, as well composition of the surrounding medium (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006). Environmental signals also promote biofilm formation and are involved in the initiation of this biofilm process including variation in temperature, nutrient availability, pH, osmolarity, iron and oxygen requirements (Behlau and Gilmore, 2008). For adhesion, cell surface components (flagella, pili, fimbriae, adhesin proteins), proteases, capsules, LPS, EPS, surface charge (i.e., hydrophobicity), autoaggregation and coaggregation, eDNA, QS, all play a huge role in initial attachment and/or biofilm formation and in pathogenicity of most pathogens (Asha *et al.*, 2004; Bandara *et al.*, 2010; Basson *et al.*, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006; Karatan and Watnick, 2009). Identifying *Aeromonas* species, which have the ability to form biofilms is critical since the manner in which they actually form biofilms has not been fully elucidated. Additionally,

although *Aeromonas* spp. biofilms have been studied, the focus has been on specific species, i.e., *A. hydrophila* and *A. caviae* (Asha *et al.*, 2004; Azad *et al.*, 1999; Dogruoz *et al.*, 2009; Gavin *et al.*, 2004; Li *et al.*, 2009; Merino *et al.*, 2001; Nayak *et al.*, 2004). Thus studying biofilm formation by a variety of members of this genus could provide more relevant information on the behaviour of diverse species and identify phenotypic adherence and/or biofilm formation characteristics displayed by *Aeromonas* spp. isolates. Therefore, the aim of this study was to screen *Aeromonas* spp. isolates isolated from diverse fish species for biofilm formation, using different physicochemical parameters including temperature, agitation, and nutrient availability, in order to determine the optimal conditions for biofilm formation. Since biofilm formation is affected by motility, cell surface characteristics, and/or metabolism, identifying factors potentially contributing to initial attachment and/or biofilm formation and their correlation with biofilm formation by *Aeromonas* spp. isolates was also assessed.

2.2. Materials and Methods

2.2.1. Bacterial isolates and growth conditions

Three-hundred and seventy-one cultures, obtained from South African catfish, koi carp, tilapia, trout, abalone and seawater, were screened to identify presumptive *Aeromonas* spp. Bacterial colonies were grown on tryptic soy broth (TSB) agar plates and transferred to the selective medium Rimmler-Shotts (RS) agar (Farmer *et al.*, 2006) to identify presumptive *Aeromonas* spp. isolates. These isolates were then subjected to Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-ToF) mass spectrometry analysis for species identification. All

Aeromonas spp. isolates, as well as two type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) were maintained on tryptic soy broth (TSB) agar plates at 4 °C and for long-term storage in TSB containing 20% glycerol at -70 °C (Jacobs and Chenia, 2007).

2.2.2. Biochemical and physiological characterization of *Aeromonas* isolates

Preliminary identification of isolates included screening by oxidase test, gelatin hydrolysis, A-layer phenotypic detection test, motility, and casein tests to further identify presumptive *Aeromonas* spp. isolates and to phenotypically detect the presence of factors associated with initial attachment and/or biofilm formation.

A-layer presence test was determined by streaking 24 hour cultures on TSB agar plates, supplemented with 0.1 mg/ml Coomassie brilliant blue R250 (Merck Chemicals, Gauteng, South Africa). Plates were incubated at 30 °C for 4 days, and observed for blue and/or white colonies, indicative of the presence and/or absence of the A-layer protein, respectively (Evernberg *et al.*, 1985; Bernoth, 1990).

Motility [swimming (0.25% agar) and swarming (0.6% agar)] was determined by measuring the zone or the migration of cell suspension (inoculated in the centre) through the agar from the centre to towards the periphery of the plate (Altarriba *et al.*, 2003; Wilhelms *et al.*, 2009). Plates were inoculated with five microlitres of cell suspension, standardized equivalent to a 0.5 McFarland standard, and incubated for 24 hours at 30 °C. Colony diameters were measured and averaged. All assays were carried out in triplicate.

Caseinolytic (proteolytic) activity was determined by the casein hydrolysis test. The substrate used for the test was bacteriological agar supplemented with 1% skim milk (Zacaria *et al.*, 2010). Plates were inoculated as described above for the motility tests. Development or appearance of either a clear halo or zones of clearance around a colony were taken as a positive result. Colony diameters as well as zone diameters were measured and the relative caseinolytic activity (RCA) was determined using the formula: $RCA = \frac{\text{halo diameter (zone)} - \text{bacterial growth diameter}}{\text{bacterial growth diameter}}$ (Zacaria *et al.*, 2010).

2.2.3. Determination of the effect of physicochemical conditions for optimal biofilm formation by *Aeromonas* spp. isolates

The effect of different temperatures (37 °C, 30 °C, or room temperature, \approx 21 °C), nutrient conditions (nutrient-rich or -poor), agitation (dynamic or static) for optimal biofilm formation was determined using the microtiter plate assay as described by Basson *et al.* (2008) with a few modifications. All isolates were cultured overnight in enriched TSB broth (TSB; Merck Chemicals, Gauteng, South Africa), cells were washed three times and resuspended in sterilized distilled water to a turbidity equivalent to a 0.5 MacFarland standard (Basson *et al.*, 2008). Wells of sterile 96-well bottomed microtiter plates were each filled with 90 μ l TSB (nutrient-rich) or Enriched Anacker and Ordal's broth (EAOB; nutrient-poor) and 10 μ l of each cell suspension, in triplicate. Negative control wells contained TSB or EAOB only. Plates were placed on a microtiter shaker (45 rpm, Labnet) and/or benchtop to stimulate dynamic and/or static conditions, respectively. These were incubated aerobically at 37 °C, 30 °C, and room temperature (\approx 21 °C) for 24 hours.

Contents of each well were aspirated, washed three times with 250 μ l of sterile distilled water and the remaining cells were fixed with 200 μ l of methanol for 15 min. After air-drying, wells were stained with 150 μ l 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 μ l of 33% (v/v) glacial acetic acid, and the optical density (OD) of each well was obtained at 595 nm using the Fluoroskan reader (Ascent F1, Thermolabsystems). Tests were done in triplicate, on two separate occasions and the results were averaged. The cut-off OD (OD_c) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control (Basson *et al.*, 2008). Isolates were classified as follows: $OD \leq OD_c$ = non-adherent, $OD_c < OD \leq (2 \times OD_c)$ = weakly adherent, $(2 \times OD_c) < OD \leq (4 \times OD_c)$ = moderately adherent, and $(4 \times OD_c) < OD$ = strongly adherent (Basson *et al.*, 2008; Stepanović *et al.*, 2000).

2.2.4. Cell surface hydrophobicity assay

Surface hydrophobicity was determined using the bacterial adherence to hydrocarbons (BATH) test as described by Basson *et al.* (2008). Bacteria grown in TSB, were harvested during the exponential growth phase (18 hour old cultures), washed three times and resuspended in sterile 0.1 M phosphate buffer (pH 7) to an OD of 0.8 at a wavelength of 550 nm (OD_0). Samples (3 ml) of bacterial suspension were placed in glass tubes with 400 μ l of the hydrocarbon, xylene, equilibrated in a water bath at 25 °C for 10 min and vortexed. After a 15 min phase separation, the lower aqueous phase was removed and its OD_{550} (OD_1) was determined. Values were expressed as the percentage of bacteria adhering to hydrocarbon (A) compared with the control

suspension as follows: $A = [(OD_0 - OD_1 / OD_0) \times 100\%$. Each value represented the mean experiments done in triplicate, on two separate occasions. Isolates were considered strongly hydrophobic when values were $> 50\%$, moderately hydrophobic if values were in the range of 20-50%, and hydrophilic if values were $< 20\%$. Phosphate buffered saline (PBS) was used as a negative control.

2.2.5. Autoaggregation assay

Autoaggregation was determined according to the protocol described by Basson *et al.* (2008). Bacteria were grown in 20 ml TSB, harvested after 36 hours, washed three times, and resuspended in sterile distilled water to an OD of 0.3 at a wavelength of 660 nm. The percentage of autoaggregation for study isolates was measured by transferring the bacterial suspension to a sterile cuvette and measuring the OD after 60 min at a wavelength of 660 nm (Basson *et al.*, 2008). The degree of autoaggregation was determined as the percent decrease of optical density after 60 min using the equation: $\% \text{ Autoaggregation} = [(OD_0 - OD_{60} / OD_0) \times 100\%$, where OD_0 referred to the initial OD of the organism measured, while OD_{60} was obtained after 60 min at room temperature. Experiments were done in triplicate, on two separate occasions (Basson *et al.*, 2008). Isolates were considered to have strong autoaggregation ability when values were $> 50\%$, moderate autoaggregation ability when values were in the range of 20-49%, and weak autoaggregation ability when values were $< 20\%$.

2.2.6. Statistical analyses

Statistical significance of differences ($p < 0.05$) caused by altered variables (temperature, nutrient medium, agitation) in the microtiter adherence assays were determined using one-way repeated measures analysis of variance (OWR-ANOVA). Correlation analyses for the different parameters affecting biofilm formation were performed using the Pearson's Correlation Coefficient, where a p value < 0.05 was considered significant.

2.3. Results

2.3.1. Bacterial isolates and growth conditions

Ninety-nine presumptive *Aeromonas* spp. isolates (Table 2.1.) from catfish ($n = 14$), koi carp ($n = 32$), tilapia ($n = 33$) and seawater ($n = 20$), respectively, were selected following the production of yellow colonies without black centre on RS agar as well as two type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T). These isolates, M1–M99, were classified into 10 species by MALDI-ToF analyses as described in Table 2.1.

Table 2.1. Selection of presumptive *Aeromonas* spp. isolates, using selective media and MALDI –TOF analysis

Fish host/origin	<i>n</i> = 99	Species identification
Catfish	14	<i>A. culicicola</i> , <i>A. bestiarum</i> , <i>A. allosaccharophila</i> , <i>A. jandaei</i> , <i>A. hydrophila</i>
Koi carp	32	<i>A. caviae</i> , <i>A. bestiarum</i> , <i>A. allosaccharophila</i> , <i>A. salmonicida</i> , <i>A. culicicola</i> , <i>A. hydrophila</i>
Tilapia	33	<i>A. caviae</i> , <i>A. ichthiosmia</i> , <i>A. allosaccharophila</i> , <i>A. sobria</i> , <i>A. culicicola</i> , <i>A. hydrophila</i> , <i>A. jandaei</i> , <i>A. veronii</i>
Seawater	20	<i>A. culicicola</i> , <i>A. jandaei</i>

2.3.2. Biochemical and physiological characterization of *Aeromonas* isolates

All 99 study isolates were oxidase-positive, while varied results were obtained for gelatin hydrolysis, A-layer phenotypic, motility (swimming and swarming), and casein hydrolysis tests, for all isolates from the five different fish hosts or origin (Table 2.2.). Majority of the isolates (86.9%; 86/99) were capable of degrading gelatin while 80.8% (80/99) of the isolates were capable of producing the A-layer protein (A-layer protein positive). All isolates (100%) were motile by swimming, with zone diameters ranging from 14 – 61 mm and 98% (97/99) were motile by swarming, with zone diameters ranging from 5.7 – 67.3 mm (Table A1). Partial degradation of casein was observed for 90.9% (90/99) of isolates, with zone diameters ranging from 9.0 – 22 mm and the relative caseinolytic activity (RCA) ranged from 0 – 1.1 (Table A2). The two type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) demonstrated

positive results for all biochemical and physiological test, i.e., gelatin hydrolysis, A-layer phenotypic, motility (swimming and swarming), and casein hydrolysis tests.

A negative correlation was observed between casein hydrolysis and swarming motility ($r = -0.205$, $p = 0.0414$). Swarming and swimming motility were positively correlated ($r = 0.203$, $p = 0.0436$). There was also a positive correlation between the fish host/origin of the isolates and swimming motility ($r = 0.221$, $p = 0.0279$).

Table 2.2. Biochemical and physiological characterization of 99 *Aeromonas* spp. isolates obtained from 5 different fish hosts or origin

Phenotypic tests	Number (%)				
	Number positive Total ($n=99$)	Catfish ($n=14$)	Koi carp ($n=32$)	Tilapia ($n=33$)	Seawater ($n=20$)
Swimming	99 (100)	14 (100)	32 (100)	33 (100)	20 (100)
Swarming	97 (98.0)	13 (92.9)	32 (100)	32 (97.0)	20 (100)
Casein hydrolysis	90 (90.9)	13 (92.9)	27 (84.4)	30 (90.9)	20 (100)
Gelatin hydrolysis	86 (86.9)	11 (78.6)	23 (71.9)	32 (97.0)	20 (100)
A-layer presence	80 (80.8)	10 (71.4)	25 (78.1)	29 (87.9)	16 (80.0)

2.3.3. Determination of the effect of physicochemical conditions for optimal biofilm formation by *Aeromonas* spp. isolates

Isolates showed varying levels of adherence, but 92.9% (92/99) were strongly adherent in nutrient-rich medium (TSB) at 30 °C, under static conditions (Table 2.3, Table A3). Biofilm formation was influenced more by altered nutrient levels rather than temperature. Majority of the isolates adhered strongly at 30 °C, 37 °C and room temperature ($\approx 21^\circ\text{C}$) under both static and shaking conditions (Table 2.3), and showed a preference for nutrient-rich (TSB) medium (Table A5). The type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) also demonstrated strong-biofilm abilities.

At room temperature ($\approx 21^\circ\text{C}$), biofilm formation ranged from 0.273–1.301 in EAOB under shaking conditions, with 6.1% (6/99) of isolates being non-adherent; while under static conditions, biofilm formation ranged from 0.286–1.221, with 3% (3/99) of isolates appearing non-adherent (Table 2.3, Table A3). Using TSB, biofilm formation ranged from 0.289–1.311, with agitation; while under static conditions it ranged from 0.816–1.460 under static conditions (Table A4). At 30 °C, biofilm formation ranged from 0.221–1.302 under shaking conditions in EAOB, with 8.1% (8/99) of isolates considered non-adherent; while under static conditions, biofilm formation ranged from 0.473–1.206, with 18.2% (18/99) of isolates considered non-adherent (Table A3). Similarly, for TSB under shaking conditions, biofilm formation ranged from 0.371–1.457; while under static conditions, it ranged from 0.560–1.482 (Table A4). At 37°C, biofilm formation ranged from 0.393 to 0.777 in EAOB under shaking conditions, with 8.1% (8/99) of isolates considered non-adherent; while under static conditions, biofilm formation ranged from 0.260–0.779, with 2% (2/99) of isolates considered non-adherent (Table A3). Using

TSB, biofilm formation ranged from 0.275–1.005 with agitation; while it ranged from 0.411–1.411 under static conditions (Table A4).

Stronger biofilm formation in this study was documented at all temperature tested in nutrient-rich TSB compared to nutrient-poor EAOB as shown in Table 2.3. In nutrient-poor EAOB, stronger adherence ability of the isolates was observed under static conditions compared to shaking conditions.

Correlation analyses for the different parameters affecting biofilm formation were performed using the Pearson's Correlation Coefficient, where a p value < 0.05 was considered significant. A significant negative correlation was observed between fish host/origin and biofilm formation (37 °C, TSB, shaking; $r = -0.0233$, $p = 0.0203$), while a positive correlation was observed between fish host/origin and biofilm formation was observed (RT, EAOB, static; $r = 0.217$, $p = 0.0311$). Positive correlations were observed between swimming motility and biofilm formation (30 °C, and RT, TSB, static; 30 °C, EAOB, shaking; $r = 0.199$, $p = 0.0486$; $r = 0.254$, $p = 0.0113$; $r = 0.217$, $p = 0.0306$). Swarming motility was also positively correlated with biofilm formation (37 °C, TSB, static; 37 °C, EAOB, shaking; $r = 0.229$, $p = 0.0229$; $r = 0.258$, $p = 0.0100$). A negative weak correlation was observed between casein hydrolysis and biofilm formation (37 °C, EAOB, static; $r = -0.330$, $p = 0.0008$) while a negative correlation was also observed between autoaggregation and biofilm formation (37 °C, TSB, shaking; $r = -0.213$, $p = 0.0344$). Furthermore, significant negative weak correlations were observed between gelatinase activity and biofilm formation (30 °C, TSB, shaking and static; 30 °C, 37 °C, EAOB, shaking and static; $r = -0.360$, $p = 0.0002$; $r = -0.271$, $p = 0.0066$; $r = -0.228$, $p = 0.0232$, $r = -0.231$, $p = 0.0212$).

Table 2.3. Biofilm formation by *Aeromonas* spp. isolates (n=99) following incubation at room temperature (RT, ≈21 °C), 30 °C, or 37 °C, under shaking or static conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively

Parameters	Number of isolates (%)							
	Non-adherent		Biofilm formation*				Strong	
	No. (%)	Average optical density OD ± SD	No. (%)	Average optical density OD ± SD	No. (%)	Average optical density OD ± SD	No. (%)	Average optical density OD ± SD
RT EAOB Shaking	6 (6.1)	0.146±0.009	21 (21.2)	0.273±0.051	25 (25.3)	0.528±0.049	47 (47.5)	1.301±0.041
RT EAOB Static	3 (3.0)	0.136±0.012	29 (29.3)	0.286±0.050	20 (20.2)	0.481±0.050	47 (47.5)	1.221±0.040
RT TSB Shaking	-	-	2 (2.0)	0.289±0.032	21 (21.2)	0.714±0.063	76 (76.8)	1.311±0.078
RT TSB Static	-	-	-	-	19 (19.2)	0.816±0.043	82 (82.8)	1.460±0.036
30°C EAOB Shaking	8 (8.1)	0.158±0.017	29 (29.3)	0.221±0.051	30 (30.3)	0.512±0.035	32 (32.3)	1.032±0.042
30 °C EAOB Static	18 (18.2)	0.249±0.032	34 (34.3)	0.473±0.046	18 (18.2)	0.714±0.039	29 (29.3)	1.206±0.048
30 °C TSB Shaking	-	-	4 (4.0)	0.371±0.047	15 (15.2)	0.766±0.044	80 (80.8)	1.457±0.047
30 °C TSB Static	-	-	-	-	7 (7.1)	0.560±0.030	92 (92.9)	1.482±0.048
37 °C EAOB Shaking	8 (8.1)	0.181±0.024	42 (42.4)	0.393±0.027	38 (38.4)	0.443±0.069	11 (11.1)	0.777±0.041
37 °C EAOB Static	2 (2.0)	0.189±0.013	37 (37.4)	0.260±0.038	47 (47.5)	0.453±0.062	13 (13.1)	0.779±0.045
37 °C TSB Shaking	-	-	4 (4.1)	0.275±0.014	12 (12.1)	0.604±0.018	83 (83.8)	1.005±0.040
37 °C TSB Static	-	-	4 (4.1)	0.411±0.013	24 (24.2)	0.731±0.029	71 (71.7)	1.411±0.030

*Biofilm formation assay data are the mean of three independent experiments carried out in triplicate ± standard deviation (Stepanovic *et al.* 2000).

2.3.4. Correlation of cell surface hydrophobicity of *Aeromonas* spp. with biofilm formation

Overall, the BATH values for isolates ranged from 0.27–69.1% (Table 2.4). Majority of isolates (74.8%; 74/99) were classified as hydrophilic (0.27 – 19.27), 21.2% (21/99) as moderately hydrophobic (20.31 – 45.80), and 4% (4/99) as hydrophobic (52.64-69.09), respectively (Table 2.4). The type strains, *A. caviae* ATCC 15468^T (19%) and *A. hydrophila* ATCC 7966^T (17.3%) were also classified as hydrophilic. Majority of hydrophilic isolates belonged to four species, i.e., *A. hydrophila*, *A. jandaei*, *A. bestiarum*, and *A. culicicola*. No specific association was observed between BATH hydrophobicity and specific fish host/origin. It was not possible to correlate *Aeromonas* spp. isolates' hydrophilic nature with biofilm formation or with any of the phenotypes associated with biofilm formation.

Table 2.4. BATH hydrophobicity results obtained for 99 *Aeromonas* spp. isolates, according to their fish hosts/origin

Isolates (fish host/origin)	No. (%) HPL*	No. (%)MHPB*	No. (%) HPB*
Koi carp (n = 32)	25 (78.1)	5 (15.6)	2 (6.3)
Catfish (n = 14)	9 (64.3)	4 (28.6)	1 (7.1)
Tilapia (n = 33)	25 (75.8)	7 (21.2)	1 (3.0)
Seawater (n = 20)	15 (75.0)	5 (25.0)	-
All isolates	74 (74.8)	21 (21.2)	4 (4.0)

*HPL=hydrophilic, MHPB=moderately hydrophobic, and HPB=hydrophobic (Basson *et al.*, 2008).

2.3.5. Correlation of autoaggregation of *Aeromonas* spp. with biofilm formation

Autoaggregation indices ranged from 26 – 84% (Table 2.5). Majority of isolates displayed high levels of autoaggregation, with 84.8% (84/99) of the isolates displaying strong autoaggregation abilities (50 – 84%) and 15.2% (15/99) of the isolates displaying moderate autoaggregation ability (26 – 48%). No specific association was observed between autoaggregation ability and specific *Aeromonas* species. The type strains, *A. caviae* ATCC 15468^T (69%) and *A. hydrophila* ATCC 7966^T (82%) also displayed high levels of autoaggregation. No significant correlation was observed with biofilm formation.

Table 2.5. Autoaggregation results obtained for 99 *Aeromonas* spp. isolates, according to their fish hosts/origin

Isolates (fish host/origin)	No. (%) SA*	No. (%) MA*	No. (%) WA*
Koi carp (n = 32)	29 (90.6)	3 (9.4)	-
Catfish (n = 14)	11 (78.6)	3 (21.4)	-
Tilapia (n = 33)	29 (87.9)	4 (12.1)	-
Seawater (n = 20)	15 (85.0)	5 (25.0)	-
All isolates	84 (84.8)	15 (15.2)	-

*SA=strong autoaggregation ability, MA=moderate autoaggregation ability, WA=weak autoaggregation ability (Basson *et al.*, 2008).

2.4. Discussion

Members of the genus *Aeromonas* are often associated with fish diseases and are also human opportunistic pathogens causing food or water-borne infections in humans (Davies *et al.*, 2001; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Gordon *et al.*, 2008; Kirov *et al.*, 2002; Koksai *et al.*, 2007; Tacao *et al.*, 2005). The ability of most bacterial pathogens, such as *Aeromonas* spp., to cause disease is usually linked to their ability to form biofilms (Behlau and Gilmore, 2008; Hoiby *et al.*, 2010).

Aeromonas spp. isolates displayed strong adherence profiles with a preference for adherence at moderate temperatures (30 °C), and nutrient-rich environments with low hydrodynamic forces (static conditions). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed strong adherence profiles under these conditions. These conditions may be correlated with isolation of majority of study isolates from aquaculture tanks with high nutrient availability and steady, slow water flow (Jacobs and Chenia, 2009; Giaouris *et al.*, 2005; Williams *et al.*, 2009). All isolates in this study displayed strong adherence characteristics at room temperature (\approx 21 °C) and 30 °C in nutrient-rich environment under static and shaking conditions, as a range of 22-28 °C is their optimal temperature, according to the literature (Belaluddin and Shahjahan, 2003; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008; Kirov *et al.*, 2002; Tacao *et al.*, 2005). *Aeromonas* spp. isolates such *A. hydrophila* have been described to form biofilms on microtiter plates in TSB (nutrient-rich) when incubated for 48 hours, without agitation at 30 °C (Gavin *et al.*, 2003; Elhariry, 2011; Merino *et al.*, 2001). Based on this study, strong adherence profiles were also observed at 37°C in nutrient-rich environments under static and dynamic (shaking) conditions correlating with conditions often associated with clinical isolation sites (Belaluddin and Shahjahan, 2003; Farmer *et al.*, 2006; Ghenghesh *et al.*, 2008;

Kirov *et al.*, 2002; Tacao *et al.*, 2005). *Aeromonas* spp. isolates have been shown to form biofilms at this temperature (37 °C) in borosilicate glass tubes, when they were incubated for 30 hours without agitation (Kirov *et al.*, 2004). Biofilm formation appeared to be influenced more by nutrient-rich conditions suggesting that nutrient limitation is not a cue in the switch to a sessile lifestyle for *Aeromonas* spp. isolates. The ability to form good biofilms in nutrient-rich medium has been previously observed for other species such as *Myroides odoratus*, *Salmonella* spp. and non-tuberculous *Mycobacterium* spp., and is consistent with their ability to cause disease (Jacobs and Chenia, 2009; Giaouris *et al.*, 2005; Williams *et al.*, 2009).

Majority of the isolates in the present study displayed motility, both swimming and swarming. Motility is usually mediated by flagella amongst other components in *Aeromonas* spp. isolates and is required to move along the surface thereby, facilitating growth and spread of a developing biofilm. These flagella themselves (as surface appendages) can also directly mediate attachment to surfaces (Van Houdt and Michiels, 2010). Mesophilic *Aeromonas* express a single polar flagellum for swimming in all culture conditions and produce lateral flagella for swarming on solid media (Altarriba *et al.*, 2003; Gavin *et al.*, 2002; Kirov *et al.*, 2002, 2004; Kirov, 2003; Rabaan *et al.*, 2001). A significant positive correlation was observed between swimming motility and biofilm formation while swarming motility was also positively correlated with biofilm formation. A significant positive significant correlation between motility and biofilm formation was also observed for aquatic *M. odoratus* (Jacobs and Chenia, 2009).

Majority of the *Aeromonas* spp. isolates in the present study were capable of producing the A-layer protein, as has been observed previously by Fernandez *et al.* (1995). The cell envelope of virulent *A. salmonicida* is composed of A-layer (predominant cell surface protein) beyond the outer membrane and the repeating O-antigen subunit of bacterial LPS (Fernandez *et*

al., 1995). The presence of this protein, which is said to be absent in avirulent strains of *A. salmonicida*, together with the LPS was suggested to be essential for agglutination, virulence and pathogenesis (Bernoth, 1990; Bjornsdottir *et al.*, 1992; Ellis *et al.*, 1997; Fernandez *et al.*, 1995). The A-layer is responsible for several cell surfaces properties such as cell-surface hydrophobicity and autoaggregation in *Aeromonas* spp. (Bjornsdottir *et al.*, 1992; Fernandez *et al.*, 1995; Phipps *et al.*, 1983), but no correlations with biofilm formation were observed in the present study.

Study isolates displayed considerable variability in their autoaggregating abilities suggesting differences between *Aeromonas* spp. and between isolates. The type strains, *A. caviae* ATCC 15468^T (69%) and *A. hydrophila* ATCC 7966^T (82%) also displayed high levels of autoaggregation. A significant negative correlation was observed between autoaggregation and biofilm formation for *Aeromonas* spp. isolates in the present study.

Majority of the study isolates were hydrophilic, with 74.8% (74/99) being classified as hydrophilic (0.27 – 19.27). The type strains, *A. caviae* ATCC 15468^T (19%) and *A. hydrophila* ATCC 7966^T (17.3%) were also classified as hydrophilic. Scoaris *et al.* (2008) used the bacterial adherence to hydrocarbons test to evaluate the hydrophobicity of *Aeromonas* spp. isolates and its correlation to virulence and observed that most of the virulent strains were hydrophilic. Elhariry *et al.* (2011) also studied hydrophobicity patterns amongst *Aeromonas* strains, and observed that most *Aeromonas* strains were hydrophilic and no significance differences in hydrophobicity were observed among the *Aeromonas* strains. Although the general rule has been that adhesiveness increases with increasing or decreases with decreasing hydrophobicity, a number of studies have shown contradictory results, where no relationship was found between the bacterial strain's surface hydrophobicity and the extent of initial binding to either a hydrophilic or hydrophobic

substrate (Basson *et al.*, 2008). It was not possible to correlate *Aeromonas* spp. biofilm formation and the strongly hydrophilic nature demonstrated by these isolates in present study.

The presence of *Aeromonas* spp. isolates in mixed species biofilms has been reported previously from the clinical and industrial settings (Asha *et al.*, 2004; Azad *et al.*, 1999; Bomo *et al.*, 2004; Dogruoz *et al.*, 2009; Nayak *et al.*, 2004). *Aeromonas* spp. isolates from seawater and diverse cultured fish displayed different levels of biofilm formation on polystyrene, which was affected by nutrient alteration, but not by temperature. Since biofilms have been studied with members of this genus, although the focus has been on specific species (*A. hydrophila* and *A. caviae*), studying biofilm formation with a variety of members of this genus provided more relevant information on the behaviour of diverse species from diverse sources. Additionally, the ability of *Aeromonas* spp. to autoaggregate, rather than its hydrophilic nature, appears to be a significant characteristic in biofilm formation. The role of motility and other surface-associated appendages (A-layer) and extra-cellular enzymes (protease and gelatinase) in adherence and biofilm formation requires further investigation. These all play a role in the adherence and/or biofilm formation by *Aeromonas* spp. isolates, and have implications in antimicrobial resistance by these species, which requires investigation.

CHAPTER THREE

Antimicrobial resistance of planktonic and biofilm-associated *Aeromonas* spp. isolates

3.1. Introduction

Most aquatic bacterial pathogens, such as *Aeromonas* spp., in aquatic environments are often associated with surfaces (biofilm state) rather than in a planktonic state (Basson *et al.*, 2008). Antimicrobial resistance studies have shown that planktonic *Aeromonas* spp. isolates are often resistant to β -lactams, tetracycline, quinolones, and second- and third-generation cephalosporins (Castro-Escarpulli *et al.*, 2003; Farmer *et al.*, 2006; Jacobs and Chenia, 2007). Many strains of *Aeromonas* spp. display resistance to piperacillin and mezlocillin, while being susceptible to gentamicin, chloramphenicol, amikacin, trimethoprim sulfamethoxazole, nitrofurantoin, and tobramycin (Castro-Escarpulli *et al.*, 2003; Farmer *et al.*, 2006; Jacobs and Chenia, 2007). Planktonic *Aeromonas* spp. from South African patients were observed to have MICs ranging from 1 – 64 $\mu\text{g/ml}$ for tetracycline, azithromycin, ceftazidime, ciprofloxacin, and gentamicin (Ramalivhana *et al.*, 2009).

Abraham *et al.* (2007) described that differences in the antimicrobial resistance profiles observed for *Aeromonas* spp. may be well related to the source of the *Aeromonas* isolates. Son *et al.* (1997) also observed that the differences in the frequency of resistance could well be related to the source of the *Aeromonas* isolates, and the frequency and type of antimicrobial agents prescribed for treating infections in different geographical areas. High antimicrobial resistance rates were observed among aeromonads isolated from fish (Abraham *et al.*, 2007). This could be explained by the prophylactic use of antimicrobial agents to prevent fish diseases in fish farms (Abraham *et al.*, 2008; Abraham *et al.*, 2007; Baquero *et al.*, 2008; Son *et al.*, 1997). A.

salmonicida and *A. hydrophila*, common fish pathogens, were observed to be highly resistant to a great variety of antimicrobial agents, such as β -lactams, tetracycline, quinolones, as well as second- and third-generation cephalosporins (Austin, 1997; Farmer *et al.*, 2006; L’Abee-Lund and Sorum, 2001; Jacobs and Chenia, 2007). High rates of resistance have also been observed in *Aeromonas* spp. isolates from clinical environmental sources (Abraham *et al.*, 2007). Studies of clinical and environmental isolates of *Aeromonas* have reported high level of resistance to ampicillin (Huddleston *et al.*, 2007). Clinical isolates of *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria*, which are also pathogens of fish (Wahli *et al.*, 2005) displayed very similar susceptibility patterns (Farmer *et al.*, 2006) and were more resistant to fluoroquinolones, ciprofloxacin, ofloxacin and nalidixic acid (Castro-Escarpulli *et al.*, 2003; Farmer *et al.*, 2006; Jacobs and Chenia, 2007). Chien *et al.* (1996) observed that in Taiwan, clinical isolates of *Aeromonas* were found to be susceptible to moxalactam, ceftazidime, cefepime, aztreonam, imipenem, amikacin, and fluoroquinolones and were resistance to tetracycline, trimethoprim-sulfamethoxazole and some cephalosporins.

Many studies have focused on biofilm formation due to their increased resistance to antimicrobial agents, which causes problems in treatment of biofilm-associated diseases (Hoiby *et al.*, 2010). It has been observed that the antimicrobial agents MICs and MBCs of biofilm-growing bacteria (minimum biofilm inhibitory concentration, MBICs) may be up to 100- to 1000-fold higher compared with planktonic bacteria (Hoiby *et al.*, 2010). Using antimicrobial agents to treat biofilms only causes cells on the outer surface may be eliminated, leaving cells in the middle to continue to disseminate and spread within the biofilm when therapy is terminated and thus gain resistance against antimicrobial agents (Davey and O’Toole, 2000; Del Pozo and Patel, 2007). These cells may require higher concentrations of antimicrobial agents (supra-

inhibitory concentrations; supra-MICs), to be effective in removing these cells (Cogan, 2006). Waste accumulation and a distorted microenvironment due to the increased bacterial density within biofilm microcolonies may also have an effect on antimicrobial agent action deep within the biofilm (Del Pozo and Patel, 2007). Cells within biofilms prevent an antimicrobial agent from binding to the target, which leads to the increase in the minimum inhibitory concentration (MIC) (Lewis, 2008).

Unlike planktonic bacteria, biofilms are hard to eradicate due to the fact that they are characterized by the presence of the slimy EPS, which protects microbes from their environment; conferring protection against phagocytosis, interference with the cellular immune response, and reduction of antimicrobial potency (Behlau and Gilmore, 2008; Chmielewski and Frank, 2003; Decho, 2000; Donlan, 2002; Lindsay and von Holy, 2006; Hoiby *et al.*, 2010; Vu *et al.*, 2009). Due to this durability, biofilms are responsible for a wide variety of industrial and health problems (Hoiby *et al.*, 2010; Vu *et al.*, 2009). Targeting the first stage of biofilm formation, i.e., initial attachment of cells to the surface, where the cells are still accessible to elimination by antimicrobial agents is a wise strategy because when cells have already attached to the surface, it is usually not easy to eliminate them due to the presence of EPS (Takahashi *et al.*, 2007). Many antimicrobial agents work differently on various microorganisms because there is a variation among strains and their ability to resist the effects of antimicrobial agents; and are able to efficiently inhibit the adherence of biofilms post- and pre-formation (Dal Sasso *et al.*, 2003).

Antimicrobial agent concentrations within many tissues usually become lower than the MIC, and these are called sub-inhibitory concentrations (sub-MICs) (Pompillo *et al.*, 2010). Depending on the type of the antimicrobial agent used or their modes of action, sub-MIC exposures can eliminate or reduce the ability of pathogens to form biofilms by interfering with

different stages of biofilm formation resulting in the elimination of these pathogens. Microorganisms usually grow in the presence of sub-MICs of antimicrobial agents, which are potentially capable of altering the chemical and physical cell-surface characteristics and consequently the functionality and expression of some virulence properties such as adhesion, biofilm formation, hydrophobicity and motility (Pompillo *et al.*, 2010). Pompillo *et al.* (2010) observed that sub-MICs of moxifloxacin caused a decrease in adhesion and biofilm formation of *Stenotrophomonas maltophilia*. Matjan *et al.* (2007) also observed that sub-inhibitory concentrations of antibiotics affected biofilm formation by clinical strains of *Salmonella enterica* serovar Typhimurium. For some microorganisms being exposed to sub-MIC of antimicrobial agents stimulates biofilm formation, without killing cells within biofilms but inhibit their growth, and this has been observed for cells exposed to cefalexin (Haddadin *et al.*, 2009).

Since *Aeromonas* spp. isolates are important fish and human opportunistic pathogens, it was important to determine their antimicrobial resistance profiles, for comparison to clinical and aquaculture isolates reported in other studies. Evaluating the antimicrobial concentrations required to eradicate biofilms by *Aeromonas* spp. isolates could also facilitate effective treatment of these biofilms using antimicrobial agents, as cells in a biofilm appear to be more resistant to antimicrobial agents. It was, therefore, crucial to determine the effect of varying concentrations (MIC, sub-MIC and supra-MIC) of diverse antimicrobial agents on biofilm cells of *Aeromonas* spp. isolates. Thus, the MICs and MBICs for azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline of planktonic cells and biofilm cells, respectively, were investigated using the broth microdilution and modified microtiter plate assays. To determine the effect of sub-MIC ($0.5 \times \text{MIC}$) and supra-MIC ($2 \times \text{MIC}$) exposures on biofilm-forming cells, microtiter plate assays were utilized.

3.2. Materials and Methods

3.2.1. Antimicrobial agent susceptibility testing

Ninety-nine presumptive *Aeromonas* spp. isolates (Section 2.2.1, Table 2.1) and two type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC^T 7966) were used to determine the antimicrobial resistance profiles of *Aeromonas* spp. isolates. Antimicrobial susceptibility to 26 antimicrobial agents was determined using Oxoid disks (Oxoid, SA), on Mueller-Hinton (MH; Biolab Diagnostics) agar plates following Clinical Laboratory Standards International protocols (CLSI, 2006). The following antimicrobial agent disks were used: amikacin (AK30), amoxicillin (AML10), ampicillin (AMP10), augmentin (AMC30), azithromycin (AZM15), aztreonam (ATM30), cefotaxime (CTX30), cefoxitin (FOX30), cefpodoxime (CPD10), ceftazidime (CAZ30), ceftriaxone (CRO30), cefuroxime (CXM30), chloramphenicol (C30), ciprofloxacin (CIP5), cotrimoxazole (SXT25), erythromycin (E15), gentamicin (CN10), imipenem (IPM10), nalidixic acid (NA30), ofloxacin (OFX5), oxacillin (OX1), piperacillin-tazobactam (TZP110), streptomycin (S10), sulphamethoxazole (RL25), tetracycline (TE30) and trimethoprim (W1.25). Isolates were grown overnight in TSB, washed three times with sterile distilled water and the turbidity of the cell suspensions were adjusted to that equivalent to a 0.5 McFarland standard. These inocula were used to subsequently inoculate MH agar plates using sterile swabs, after which four antimicrobial agent disks were placed on the agar at equal distances from each other and plates were incubated for 24 hours at 30 °C. Bacterial strains *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 29212 were used as antimicrobial susceptibility testing controls, according to CLSI recommendations (2006). Testing was done in duplicate and resistance profiles (resistant, intermediate, or susceptible) were assigned after measuring average zone diameters using CLSI breakpoints.

MAR index values (a/b , where 'a' represents the number of antimicrobial agents the isolate was resistant to and 'b' represents the total number of antimicrobial agents the isolate was tested against) were calculated for all isolates. When isolates exposed to high risk contamination sources originating from humans or animals where antimicrobial agents are often used a MAR index value higher than 0.2 should be observed. When antimicrobial agents are seldom or never used a MAR value less than or equal to 0.2 should be observed (Jacobs and Chenia, 2007).

3.2.2. Broth microdilution and modified microtiter plate assays to determine MICs and MBICs of planktonic and biofilm cells

Twenty-one *Aeromonas* spp. isolates (Table 3.1) as well as the type strains were selected based on their species, biochemical, physiological and biofilm-forming characteristics for the determination of MICs and MBICs of planktonic and biofilm-forming isolates, respectively, for five antimicrobial agents. These isolates were also utilized for the determination of the effect of sub-MIC and supra-MIC exposures on biofilm-forming cells.

Five antimicrobial agents [azithromycin (macrolide), ceftazidime (extended-spectrum cephalosporin), ciprofloxacin (fluoroquinolone), gentamicin (aminoglycoside), and tetracycline (tetracycline)] 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 and MBICs of planktonic and biofilm cells were determined using the broth microdilution (Andrews, 2001) and modified microtiter plate assays (Basson *et al.*, 2008), respectively.

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 MH 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 (Andrews, 2001). Microtiter plate wells, each containing 100 μ l of MH broth medium with the required antimicrobial agent concentration, were inoculated with 10 μ l of cell suspension and incubated at 30 °C for 24 hours without shaking. The MIC was the lowest concentration of antimicrobial agent, which inhibited visible growth of organism. This was done in triplicate, on two separate occasions (Andrews, 2001).

MBICs of cells were determined using the modified microtiter plate assay. Biofilms were formed as described by Basson *et al.* (2008) with conditions determined to be effective for optimal biofilm formation in Chapter 2 (Section 2.2.3), using MH broth but omitting the drying and staining steps. 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 μ l of fresh MH broth at the required antimicrobial agent concentrations and transferred to wells to determine MBICs of the biofilm cells. Wells contained 0.008, 0.5, 12, 32, 256, 1024, 2048, and 4096 μ g/ml of the antimicrobial agents to be tested. Plates were incubated for further 24 hours at 30 °C (Basson *et al.*, 2008). For both assays (broth microdilution and MBIC assay), the negative control wells contained broth only and the positive control wells contained the respective cell suspensions with no antimicrobial agents added.

Contents of each well were aspirated, washed three times with 250 μ l of sterile distilled water and the remaining cells were fixed with 200 μ l of methanol for 15 min. After air-drying, wells were stained with 150 μ l 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 μ l of 33% (v/v) glacial acetic acid and the optical density (OD) of each

well was obtained at 595 nm using Fluoroskan reader (Ascent F1, Thermolabsystems). Tests were done in triplicate, on two separate occasions and the results averaged. The cut-off OD (OD_c) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control (Basson *et al.*, 2008). Isolates were classified as follows: $OD \leq OD_c$ = non-adherent, $OD_c < OD \leq (2 \times OD_c)$ = weakly adherent, $(2 \times OD_c) < OD \leq (4 \times OD_c)$ = moderately adherent, and $(4 \times OD_c) < OD$ = strongly adherent (Basson *et al.*, 2008).

3.2.3. Effect of varying antimicrobial concentrations on biofilm formation

The effect of the sub-MIC, MIC and supra-MIC exposures of selected antimicrobial agents (azithromycin, ceftazidime, ciprofloxacin, gentamicin, and tetracycline) on initial attachment and/or biofilm formation was determined using modified microtiter assays. Two treatments were investigated using the plate assays, i.e., exposure of cultures at the time of inoculation (initial attachment) and exposure after 24 hours biofilm formation (mature biofilm/detachment).

For effect of the sub-MIC, MIC and supra-MIC exposure on initial attachment, bacterial cultures were grown overnight at 30 °C for 18 hours, and microtiter plate assays were set up as described previously by Basson *et al.* (2008). Concentrations equivalent to sub-MIC, MIC and supra-MIC of individual isolates were added to wells at the time of inoculation. These sub-MIC and supra-MIC concentrations were determined based on individual MIC results of isolates (Section 3.2.2).

Table 3.1. *Aeromonas* spp. isolates selected for the determination of effect of MIC, sub-MIC and supra-MIC concentrations of diverse antimicrobial agents on planktonic and biofilm cells

Isolate	Source	Species identification	Casein hydrolysis*	Gelatin hydrolysis*	Motility	A-layer detection*	Biofilm-forming ability [#]	Hydrophobicity [†]	Autoaggregation [‡]
M3	Catfish	<i>A. allosaccharophila</i>	+	+	+	+	S	MHPB	SA
M4	Catfish	<i>A. jandaei</i>	+	-	+	+	S	HPL	SA
M10	Catfish	<i>A. culicicola/jandaei</i>	+	-	+	+	S	HPL	SA
M11	Catfish	<i>Aeromonas</i> spp.	-	+	+	+	S	HPL	SA
M12	Catfish	<i>A. bestiarum</i>	+	+	+	+	S	HPB	SA
M16	Tilapia	<i>A. jandaei</i>	+	+	+	+	S	HPL	SA
M20	Tilapia	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M21	Tilapia	<i>A. allosaccharophila</i>	+	+	+	+	S	MHPB	SA
M27	Sea water	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M36	Sea water	<i>A. jandaei</i>	+	+	+	+	S	HPL	SA
M37	Sea water	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M40	Sea water	<i>A. culicicola</i>	+	+	+	+	S	MHPB	SA
M44	Tilapia	<i>A. ichthiosmia</i>	+	+	+	+	S	HPL	SA
M48	Tilapia	<i>A. ichthiosmia</i>	+	+	+	+	S	HPL	SA
M54	Tilapia	<i>A. ichthiosmia</i>	+	+	+	+	S	HPL	SA
M56	Tilapia	<i>A. culicicola</i>	+	+	+	+	S	HPB	MA
M75	Koi carp	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M78	Koi carp	<i>A. bestiarum</i>	-	+	+	+	S	HPL	MA
M82	Koi carp	<i>A. culicicola</i>	+	+	+	+	S	HPL	SA
M87	Koi carp	<i>A. bestiarum</i>	+	+	+	+	S	MHPB	SA
M89	Koi carp	<i>A. allosaccharophila</i>	+	-	+	-	M	HPL	SA
ATCC 15468 ¹		<i>A. caviae</i>	+	+	+	+	M	HPL	SA
ATCC 7966 ¹		<i>A. hydrophila</i>	+	+	+	+	S	HPL	MA

+: positive for test, -: negative for test; *S=strong biofilm-former, *M=moderate biofilm-former, *HPL=hydrophilic, *MHPB=moderately hydrophobic, *HPB=hydrophobic, *SA=strong autoaggregation ability, *MA=moderate autoaggregation ability.

Staining and determination of OD values were done as previously described above according to Basson *et al.* (2008). Optical density (OD_{595nm}) in the presence of sub-MICs, MICs and supra-MICs of each antimicrobial agent was compared to that of control wells without antimicrobial agent exposure, to determine the effect of exposure of cultures at the time of inoculation in the presence of varying antimicrobial agent concentrations.

The effect of antimicrobial agent exposure on mature biofilm was done by allowing cells to form biofilms over a 24 hour period, after which the sub-MIC, MIC and supra-MIC concentrations of antimicrobial agent were added to the 24 hour mature biofilms and incubated for a further 24 hours. For both assays, the negative control contained broth only and the positive control contained the respective cell suspensions with no antimicrobial agents added. Staining and determination of OD values was done as previously described in Section 3.2.2, according to Basson *et al.* (2008). All experiments were done in triplicate, on two separate occasions. Optical density (OD_{595nm}) in the presence of sub-MIC, MIC or supra-MIC of each antimicrobial agent were compared to that of control wells without antimicrobial agent exposure, to determine the increase or decrease in biofilm formation as a result of antimicrobial agent exposure.

Biofilm persistence in the presence of antimicrobial agents was calculated using the equation or formula: percent of biofilm persistence = $(OD_{595\ x} - OD_{595\ \text{negative control}}) / (OD_{595\ \text{positive control}} - OD_{595\ \text{negative control}}) \times 100\%$, where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

3.2.4. Statistical analyses

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 $p < 0.05$.

3.3. Results

3.3.1. Antimicrobial agent susceptibility testing

Varying levels of susceptibility/resistance to the different antimicrobial agents were displayed by the *Aeromonas* spp. isolates (Table 3.2). Study isolates displayed high levels of resistance to β -lactams (>90%), although majority were susceptible to augmentin (53.1%), piperacillin-tazobactam (98.2%), aztreonam (89.9%), 2nd and 3rd generation cephalosporins (>80%) as well as carbapenems (100%) as shown in Table 3.2. Low-levels (19.4%) of resistance were observed for tetracycline, while higher levels of resistance were observed to the metabolic inhibitors, trimethoprim (65.2%) and sulphamethoxazole (81.1%). Isolates also displayed high levels of susceptibility to macrolides, erythromycin (47.6%) and azithromycin (94.4%). High levels of susceptibility to the quinolones [ciprofloxacin (100%), nalidixic acid (85.5%) and ofloxacin (98.2%)], aminoglycosides [amikacin (100%), gentamicin (100%), and streptomycin (96.2%)] as well as to metabolic inhibitor, cotrimoxazole (96.1%) were observed (Table 3.2). The resistance profiles for all 99 isolates were summarized and listed in Table A5. The resistance profiles, based on disk diffusion assays, for the 21 isolates selected for further study are listed in Table 3.3. Similar antimicrobial resistance profiles were observed for the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T). MAR indices ranged from 0.04 – 0.4. A high

percentage of isolates (89.9 %, 89/99) had MAR indices ≥ 0.2 , of which 32.3% (32/99) of isolates had MAR indices ≥ 0.3 .

Table 3.2. Overall antimicrobial susceptibility patterns displayed by *Aeromonas* spp. isolates obtained from seawater and South African fish

Antimicrobial agents	Antimicrobial agent class	% of isolates		
		Resistance	Intermediate	Susceptible
Amikacin (AK30)	Aminoglycoside	0	0	100
Ampicillin (AMP10)	Penicillin	92.9	0	7.1
Amoxycillin (AML10)	Penicillin	91.6	2	6.4
Augmentin (AMC30)	Penicillin	26.9	20.1	53.1
Azithromycin (AZM15)	Macrolide	0	5.7	94.4
Aztreonam (ATM30)	Monobactam	5.3	4.9	89.9
Ceftazidime (CAZ30)	3rd generation cephalosporin	0	0	100
Cefotaxime (CTX30)	3rd generation cephalosporin	0.8	1.5	97.7
Cefoxitin (FOX30)	2nd generation cephalosporin	8.5	3.4	88.2
Cefpodoxime (CPD10)	3rd generation cephalosporin	15.7	0.8	83.6
Ceftriaxone (CRO30)	3rd generation cephalosporin	0	2.3	97.7
Cefuroxime (CXM30)	2nd generation cephalosporin	6.4	12.1	81.5
Chloramphenicol (C30)	Chloramphenicol	3.1	4.6	92.3
Ciprofloxacin (CIP5)	Quinolone	0	0.0	100
Erythromycin (E15)	Macrolide	18.6	33.8	47.6
Gentamicin (CN10)	Aminoglycoside	0	0.0	100
Imipenem (IPM10)	Carbapenem	0	0.0	100
Nalidixic acid (NA30)	Quinolone	11.2	3.9	85.0
Ofloxacin (OFX5)	Quinolone	0	1.8	98.2
Oxacillin (OX1)	Penicillin	100	0.0	0.0
Tetracycline (TE30)	Tetracycline	19.4	3.9	76.8
Piperacillin-tazobactam (TZP110)	Penicillin	0	1.8	98.2
Trimethoprim (W1.25)	Metabolic inhibitor	65.2	2.0	32.8
Streptomycin (S10)	Aminoglycoside	2.3	1.5	96.2
Sulphamethoxazole (RL25)	Metabolic inhibitor	81.1	1.6	17.4
Sulphamethoxazole / trimethoprim (SXT25)	Metabolic inhibitor	1.5	2.4	96.1

3.3.2. Broth microdilution and modified microtiter plate assays to determine MICs and MBICs of planktonic and biofilm cells

The MICs for azithromycin against *Aeromonas* spp. isolates ranged from 12-32 µg/ml, while ceftazidime MICs ranged from 32-256 µg/ml. Ciprofloxacin MICs ranged from 4-32 µg/ml, and gentamicin and tetracycline MICs ranged from 2-32 µg/ml (Table 3.3). *Aeromonas* spp. isolates and the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) displayed higher levels of resistance towards ceftazidime (MIC > 32 µg/ml), while for majority of the isolates (100%), levels of resistance towards remaining antimicrobial agents tested (tetracycline, azithromycin, ciprofloxacin, and gentamicin) were ≤ 32 µg/ml (Table 3.3).

For the MBIC assay, *Aeromonas* spp. isolates and the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) displayed varying levels of detachment in the presence of all antimicrobial agents at the concentrations tested (Figs. 3.1–3.21). Azithromycin (AZM) had an inhibitory effect on pre-formed biofilms of 100% of *Aeromonas* spp. isolates and the MBICs were higher than the azithromycin MICs, as majority (100%) of the isolates displayed detachment only at the highest concentration of azithromycin (4096 µg/ml) (Figs. 3.1–3.21). The same trend was observed for ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN) and tetracycline (TET), with majority of isolates (100%) displaying detachment at the highest concentration (4096 µg/ml) of these antimicrobial agents (Figs. 3.1–3.21).

Although all isolates displayed increased detachment at the highest concentration (4096 µg/ml) of these antimicrobial agents, with some antimicrobial agents there was increased adhesion rather than increased detachment. In the present study, an increase in adherence upon exposure to azithromycin (0.5 µg/ml), ceftazidime (0.5 µg/ml, 12 µg/ml and 32 µg/ml) and tetracycline (0.008 µg/ml and 0.5 µg/ml) was observed for isolate M3 (Fig. 3.1). This increase in

adherence was also observed for isolate M4 (Fig. 3.2) upon exposure to azithromycin (0.008 µg/ml and 0.5 µg/ml), ceftazidime (0.008 µg/ml, 0.5 µg/ml, 12 µg/ml and 32 µg/ml) and tetracycline (0.008 µg/ml, 0.5 µg/ml, 12 µg/ml, 1024 µg/ml and 4096 µg/ml). For isolate M11 (Fig. 3.4), an increase in adherence was observed upon exposure to tetracycline (0.008 µg/ml), and gentamicin (0.008 µg/ml) for isolate M20 (Fig. 3.7). This increase in adherence upon exposure to azithromycin (0.5 µg/ml), ceftazidime (0.008 µg/ml), ciprofloxacin (0.008 µg/ml, 0.5 µg/ml and 12 µg/ml), gentamicin (0.008 µg/ml, 0.5 µg/ml and 12 µg/ml) and tetracycline (0.008 µg/ml) was also observed for isolate M27 (Fig. 3.9). For isolate M36 (Fig. 3.10), an increase in adherence was observed upon exposure to ceftazidime (0.008 µg/ml and 0.5 µg/ml), and exposure to ciprofloxacin (0.008 µg/ml) for isolate M40 (Fig. 3.12) and upon exposure of gentamicin (0.008 µg/ml) for isolate M48 (Fig. 3.14). An increase in adherence upon exposure to ceftazidime (12 µg/ml), ciprofloxacin (12 µg/ml and 32 µg/ml), gentamicin (2048 µg/ml) and tetracycline (0.008 µg/ml, 32 µg/ml, 256 µg/ml, 1024 µg/ml, 2048 µg/ml and 4096 µg/ml) was also observed for isolate M78 (Fig. 3.18). Lastly, for isolate M82 (Fig. 3.19), an increase in adherence was observed upon exposure to ciprofloxacin (12 µg/ml), and gentamicin (0.008 µg/ml and 0.5 µg/ml), and upon exposure to ceftazidime (256 µg/ml) for isolate M89 (Fig. 3.21). Since majority of the isolates displayed MIC levels ranging from 2-256 µg/ml, there was a ≥ 16 -fold increase in MBICs (4096 µg/ml) compared to the MICs for all the antimicrobial agents.

Table 3.3. Antimicrobial resistance profiles and MICs of *Aeromonas* spp. isolates

Isolate	Source	Species identification	MIC ($\mu\text{g/ml}$)					Resistance profiles
			AZM	CAZ	CIP	GN	TET	
M3	Catfish	<i>A. allosaccharophila</i>	12	32	4	2	2	AMP,AML,OX
M4	Catfish	<i>A. jandaei</i>	32	256	12	12	12	AMP,AML,AMC,OX,W,RL
M10	Catfish	<i>A. culicicola/jandaei</i>	32	256	32	32	32	AMP,AML,CPD,OX,W,RL
M11	Catfish	<i>Aeromonas</i> spp.	32	256	32	4	32	AMP,AML,AMC,E,OX,W
M12	Catfish	<i>A. bestiarum</i>	32	256	12	12	32	AMP,AML,OX,W,RL
M16	Tilapia	<i>A. jandaei</i>	32	256	4	4	32	AMP,AML,CPD,CXM,FOX,OX,W,RL
M20	Tilapia	<i>Aeromonas</i> spp.	32	256	12	32	32	AMP,AML,FOX,CPD,CXM,OX,W,RL,SXT
M21	Tilapia	<i>A. allosaccharophila</i>	32	256	12	32	12	AMP,AML,AMC,FOX,CPD,CXM,OX,W,S
M27	Sea water	<i>Aeromonas</i> spp.	32	256	12	4	12	AMP,E, OX, W, RL
M36	Sea water	<i>A. jandaei</i>	32	256	4	4	12	AMP,AMLE,OX,RL
M37	Sea water	<i>Aeromonas</i> spp.	32	256	32	32	32	AMP,AML,OX,TE,W,RL
M40	Sea water	<i>A. culicicola</i>	32	128	32	32	32	AMP,AML,ATM,OX,RL
M44	Tilapia	<i>A. ichthiosmia</i>	32	256	4	12	12	AMP,AML,OX,RL
M48	Tilapia	<i>A. ichthiosmia</i>	12	256	12	32	2	AMP,AML,OX,W,RL
M54	Tilapia	<i>A. ichthiosmia</i>	32	256	12	12	32	AMP,AML,AMC,E,OX,TE,W,RL
M56	Tilapia	<i>A. culicicola</i>	32	256	12	12	32	AMP,AMLE,NA,OX,W,RL
M75	Koi carp	<i>Aeromonas</i> spp.	32	256	12	4	32	AMP,AML,CPD,CXM,C,E,OX,W
M78	Koi carp	<i>A. bestiarum</i>	32	256	12	12	12	AMP,AML,AMC,CPD,CXM,OX,W,RL
M82	Koi carp	<i>A. culicicola</i>	32	256	12	4	12	AMP,AML,AMC,ATM,CPD,CXM,E,FOX,OX, W,RL
M87	Koi carp	<i>A. bestiarum</i>	32	256	12	32	12	AMP,AML,AMC,OX,W,RL
M89	Koi carp	<i>A. allosaccharophila</i>	32	256	4	12	12	AMP,AML,AMC,CPD,CXM,OX
ATCC 15468 ^T		<i>A. caviae</i>	32	256	12	4	32	AMP,AML,AMC,E,OX,TE,W,RL
ATCC 7966 ^T		<i>A. hydrophila</i>	32	256	32	12	12	AMP,AML,AMC,CPD,OX,W,RL

AMP=ampicillin, AML=amoxicillin, AMC=augmentin, AZM=azithromycin, ATM=aztreonam, CAZ=ceftazidime, FOX=cefoxitin, CPD=cefepodoxime, CXM=cefuroxime, CIP=ciprofloxacin, E=erythromycin, GN=gentamicin, NA=nalidixic acid, OX=oxacillin, TE=tetracycline, W=trimethoprim, S=streptomycin, RL=sulphamethoxazole, SXT=cotrimoxazole.

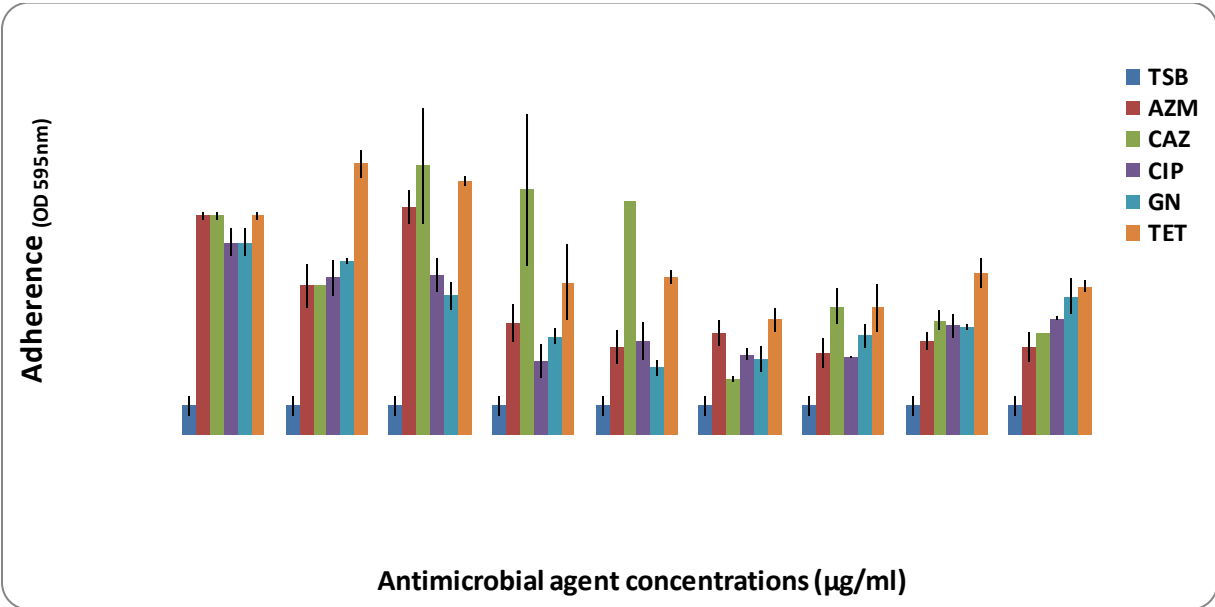


Figure 3.1. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M3 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

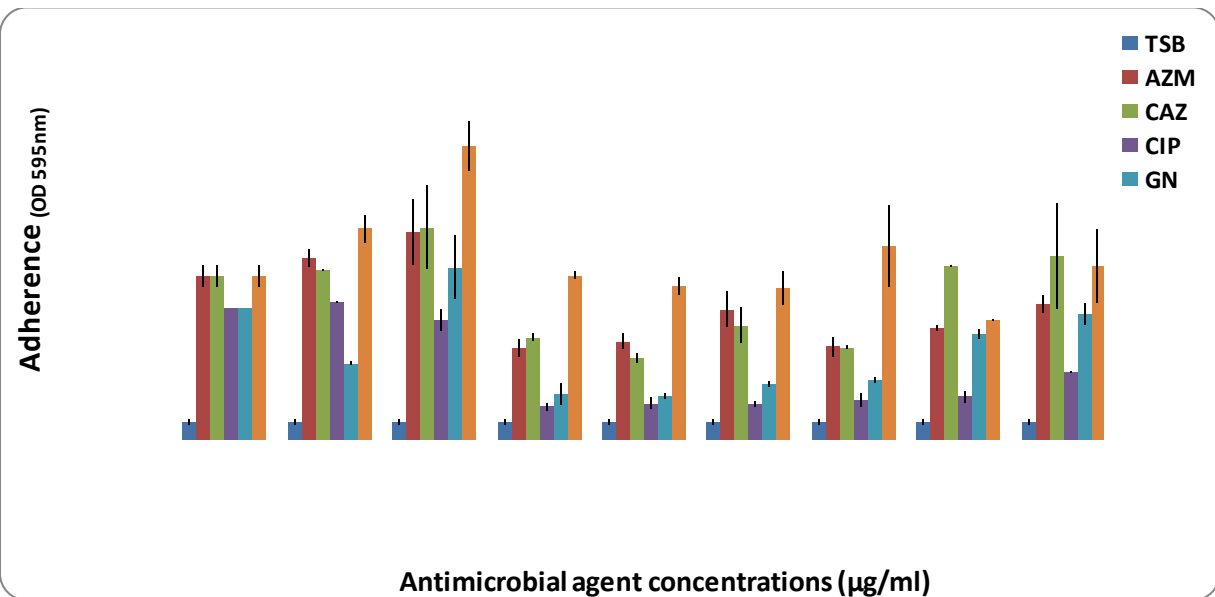


Figure 3.2. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M4 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

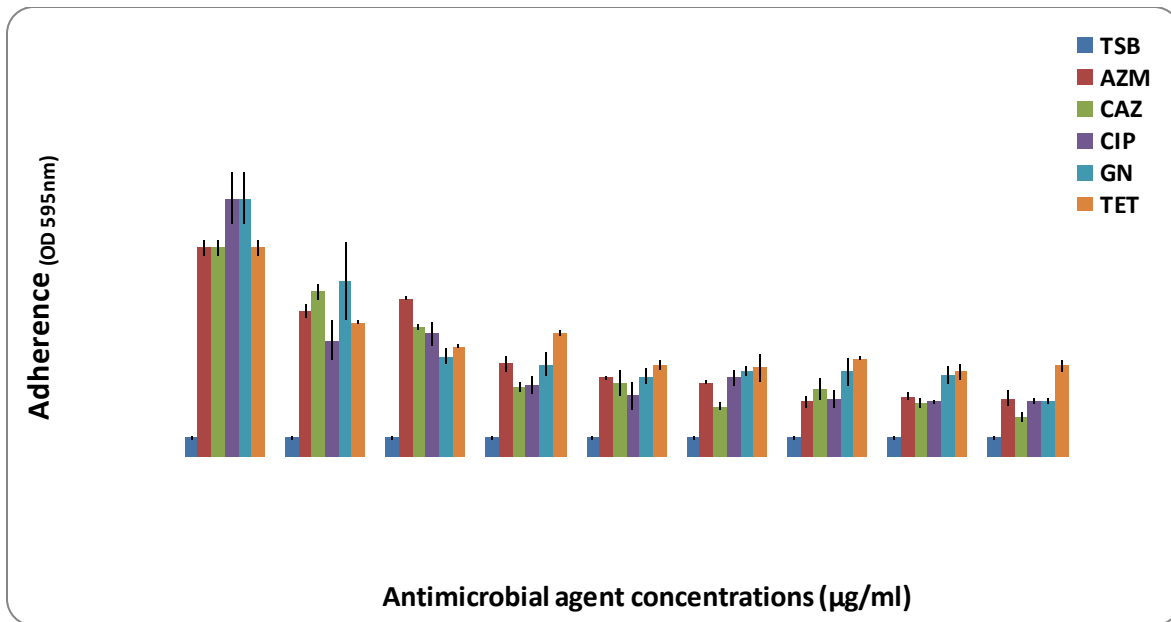


Figure 3.3. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M10 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

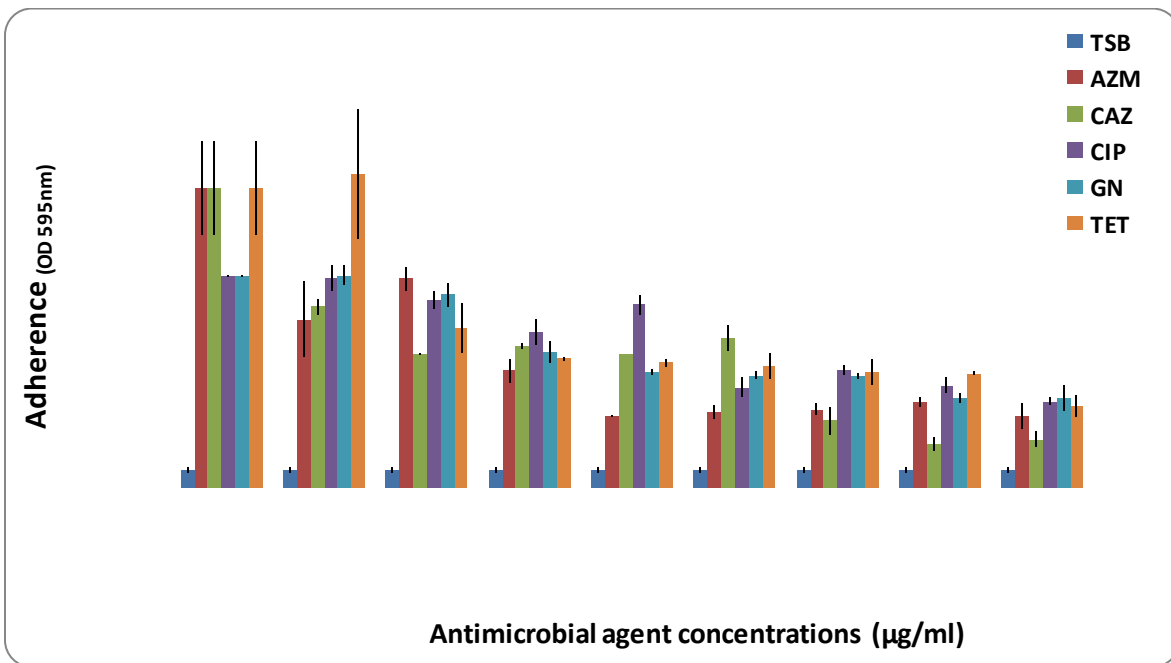


Figure 3.4. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M11 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

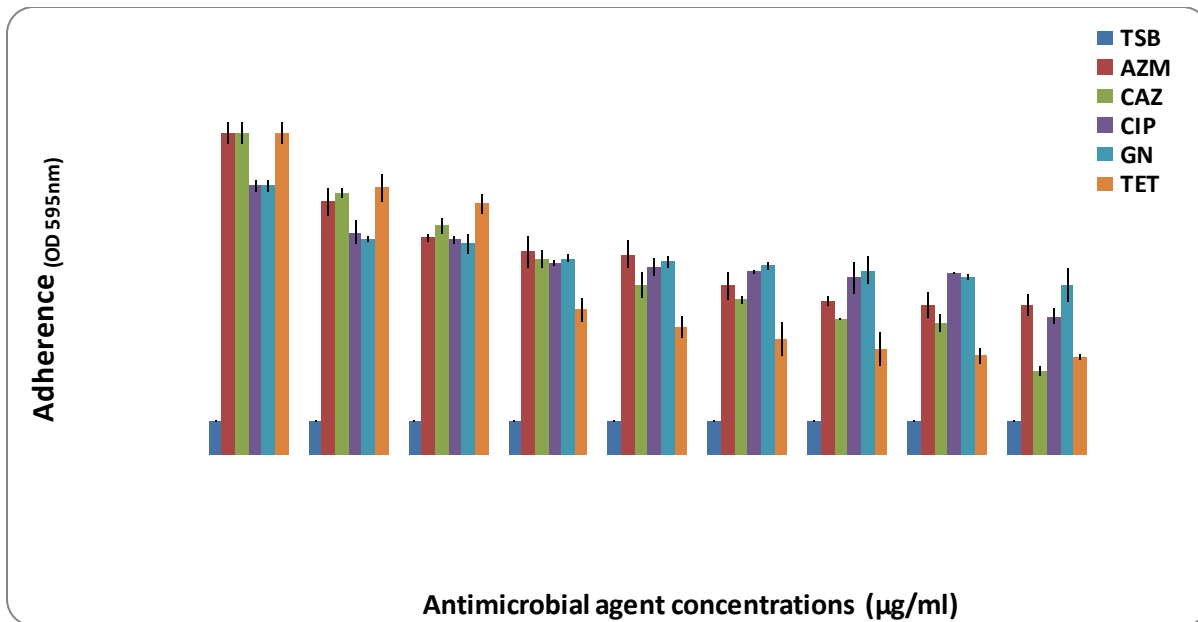


Figure 3.5. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M12 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

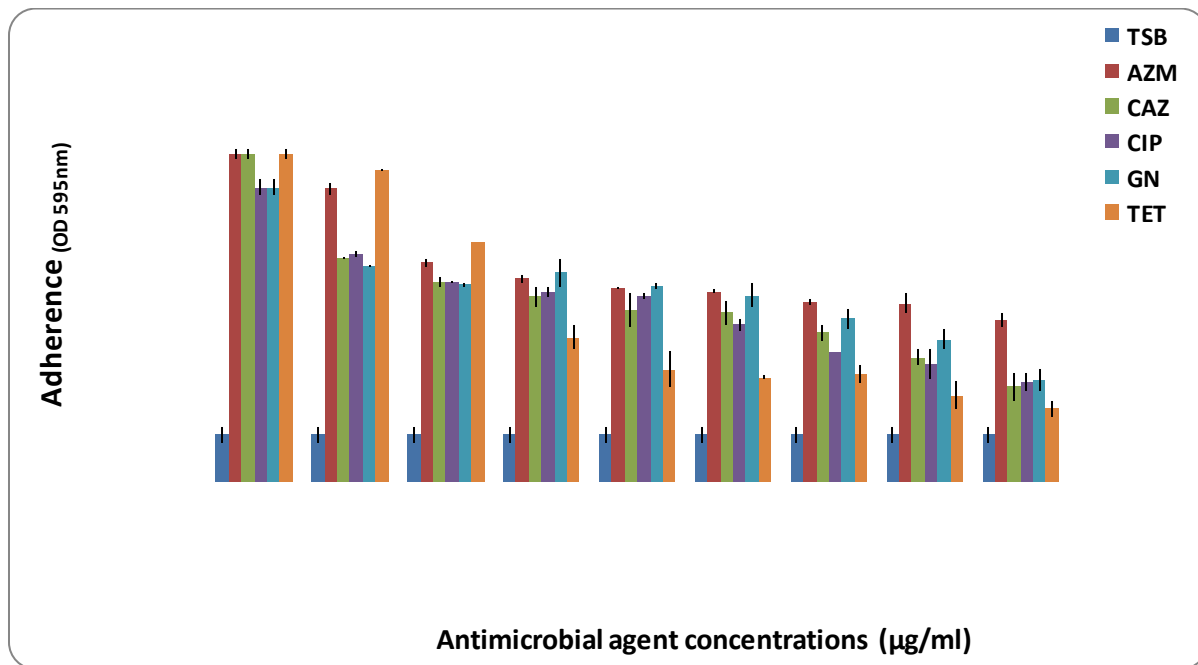


Figure 3.6. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M16 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

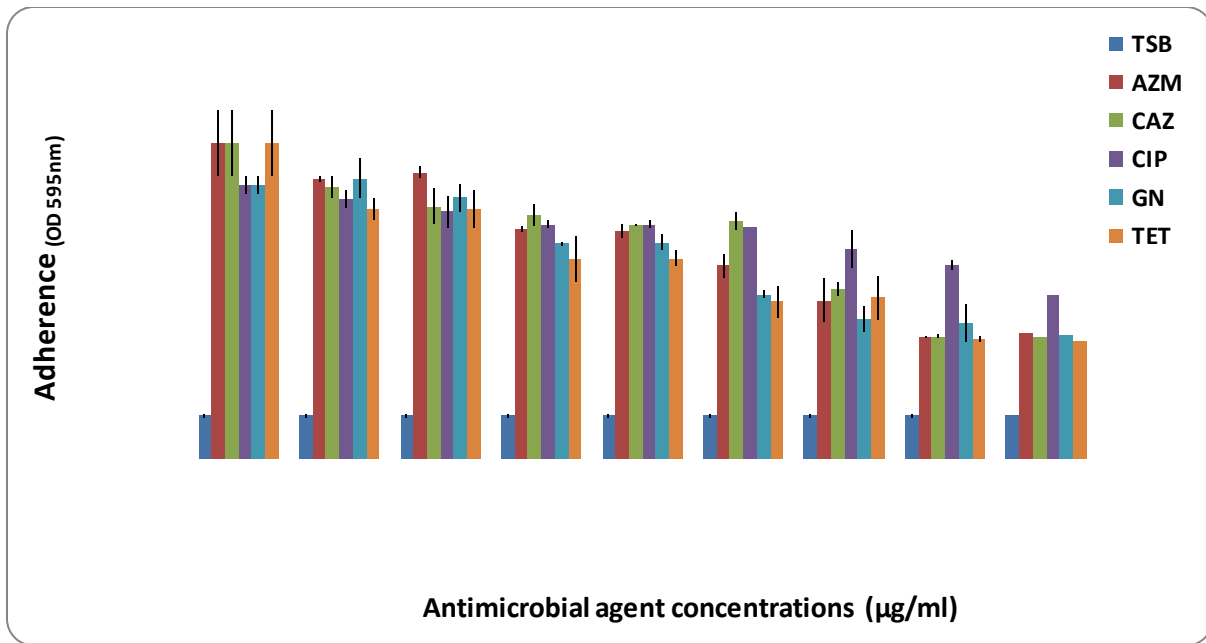


Figure 3.7. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M20 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

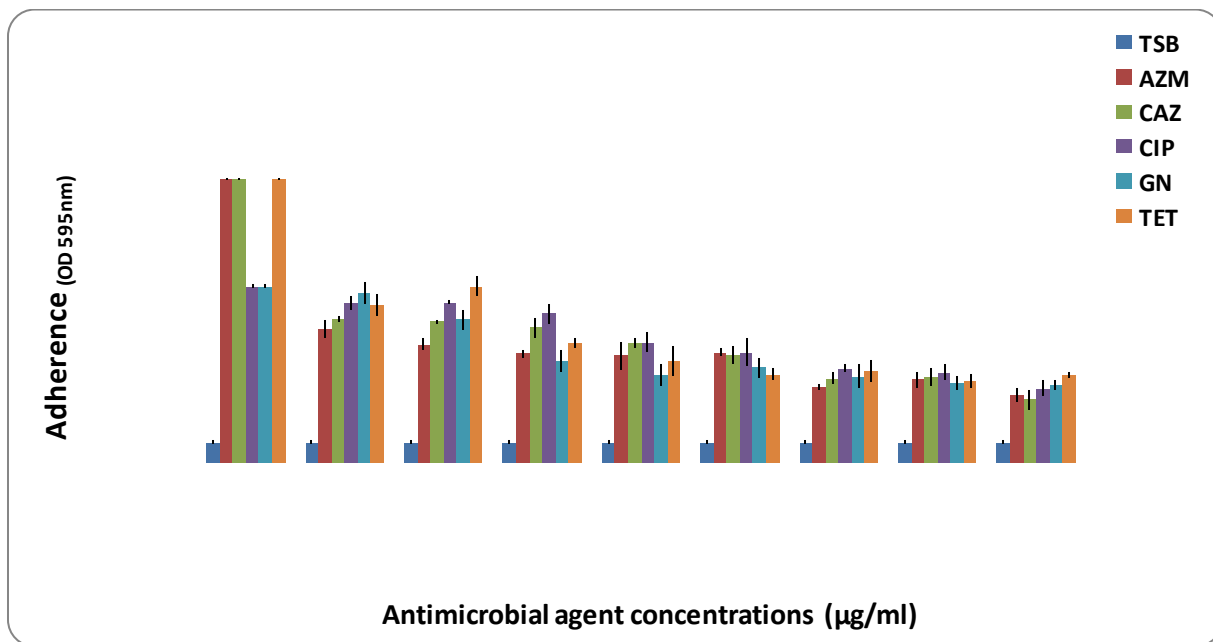


Figure 3.8. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M21 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

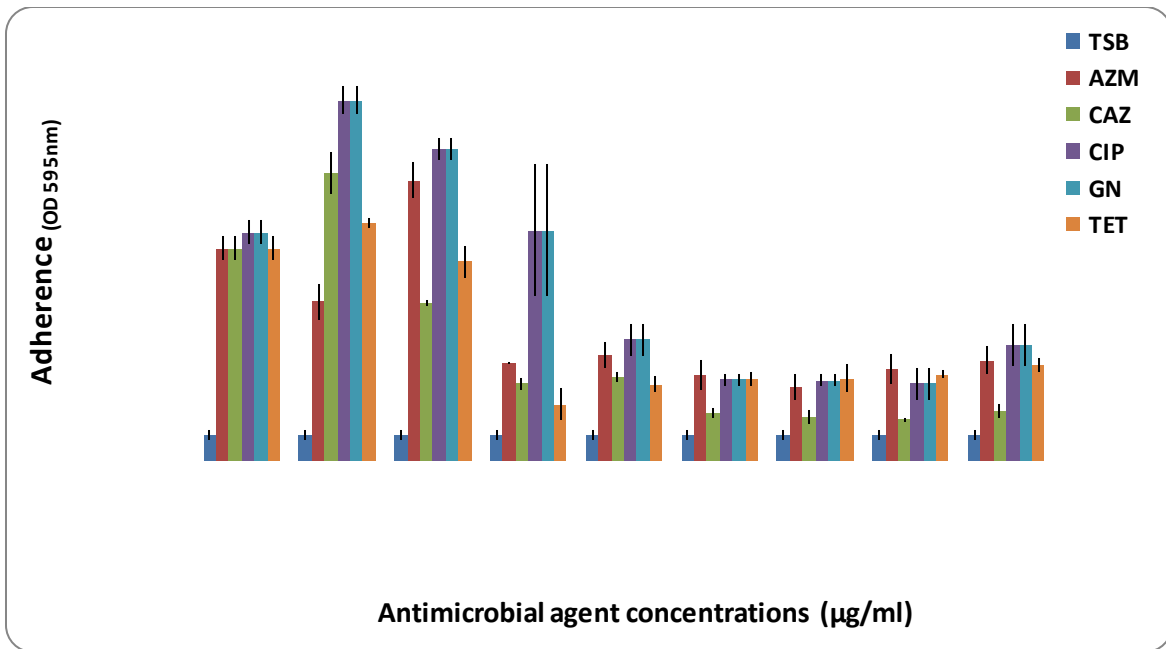


Figure 3.9. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M27 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

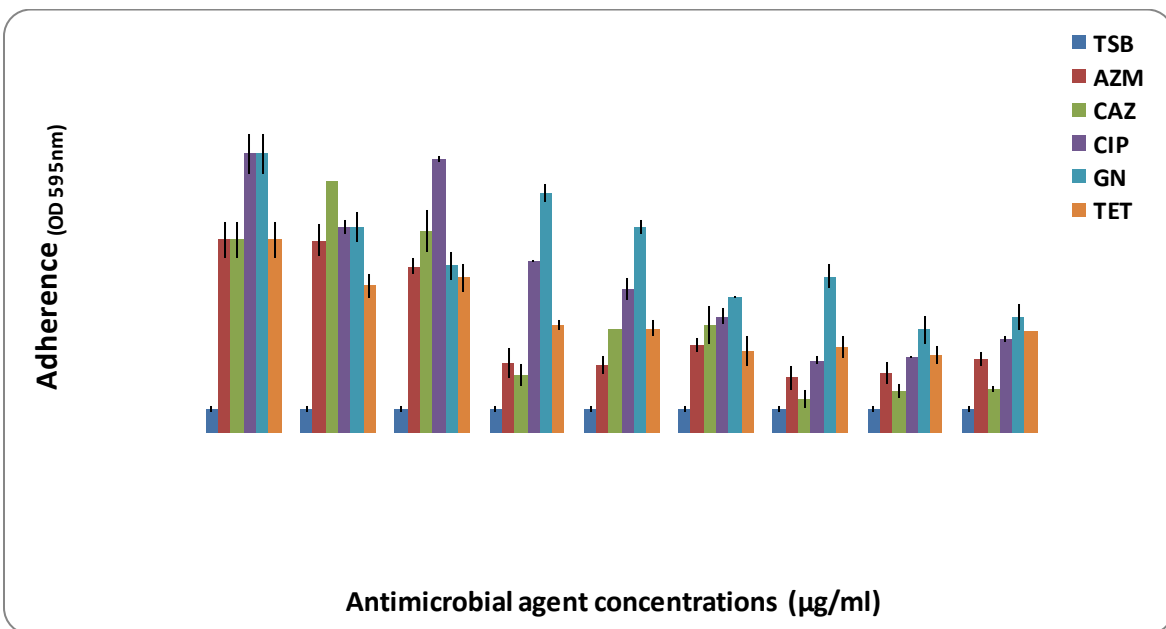


Figure 3.10. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M36 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

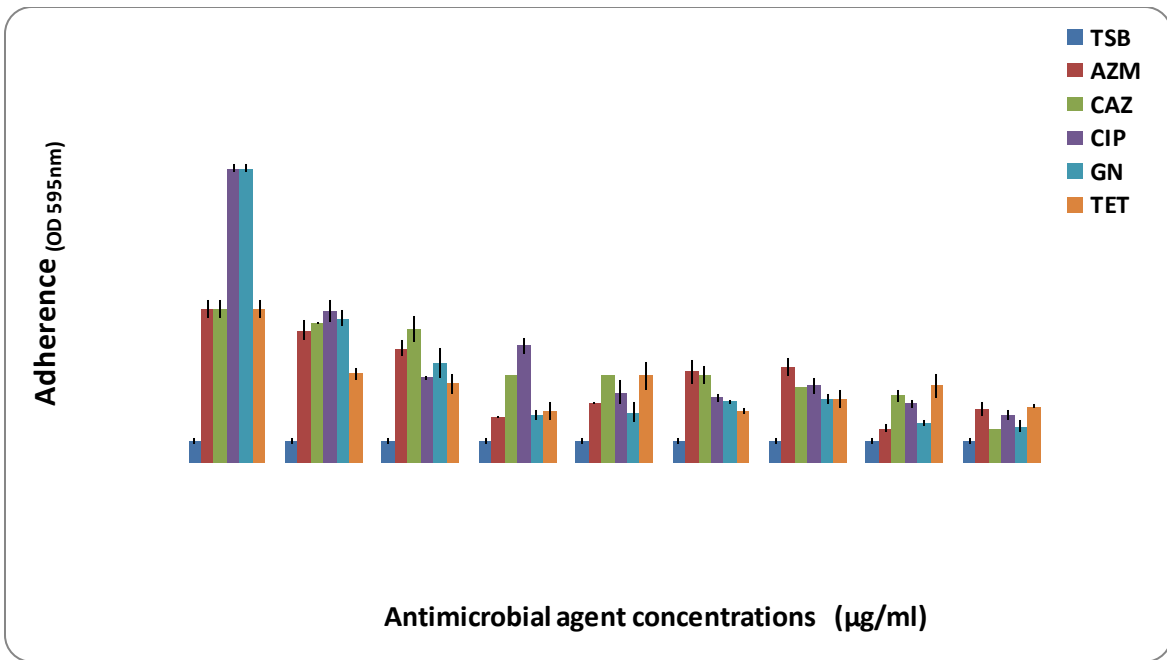


Figure 3.11. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M37 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

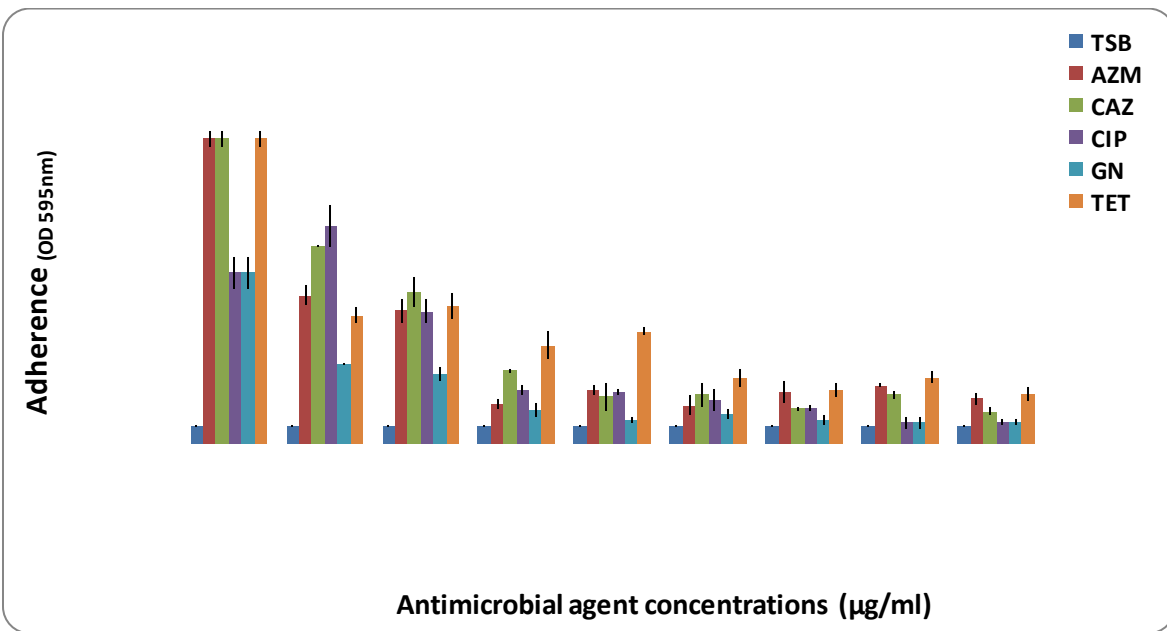


Figure 3.12. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M40 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

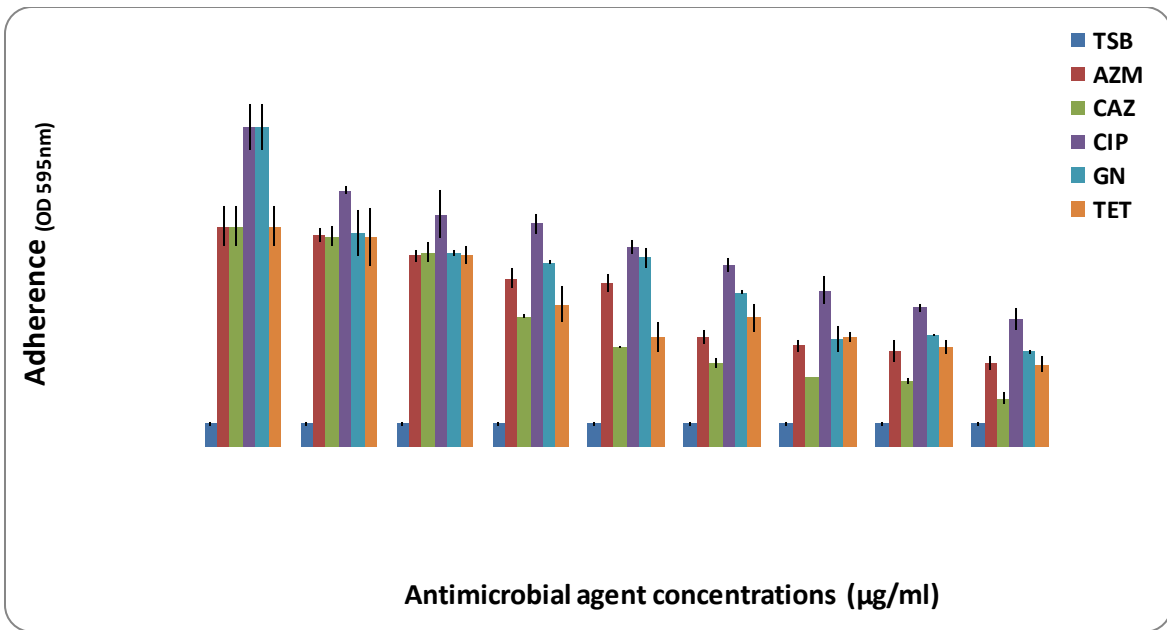


Figure 3.13. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M44 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

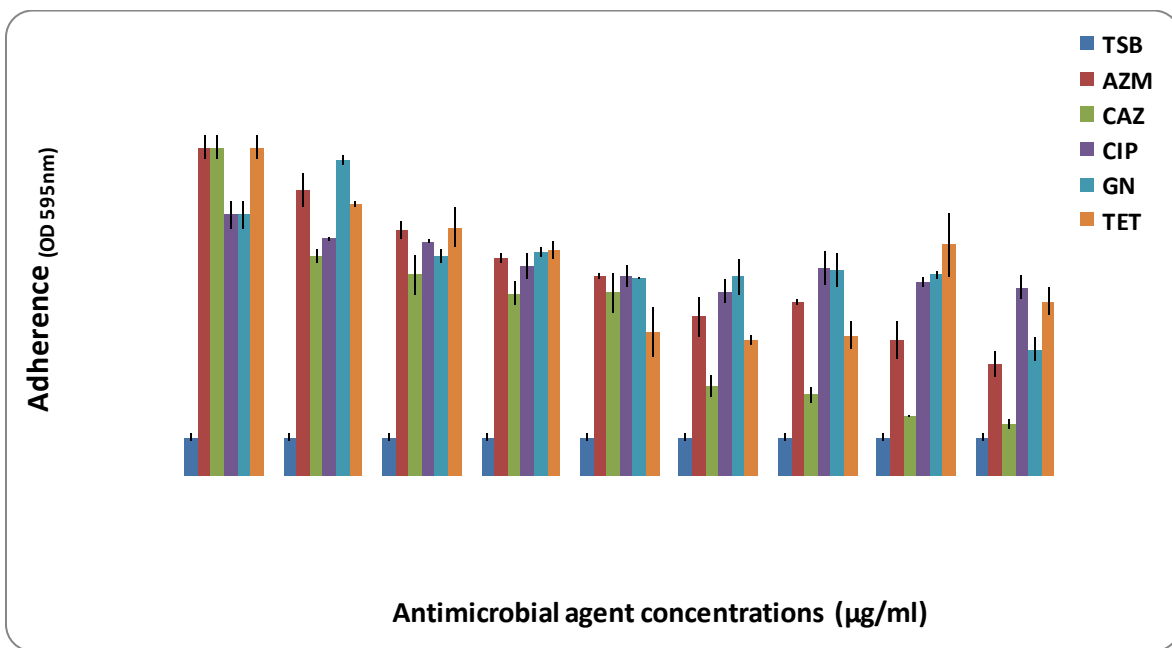


Figure 3.14. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M48 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

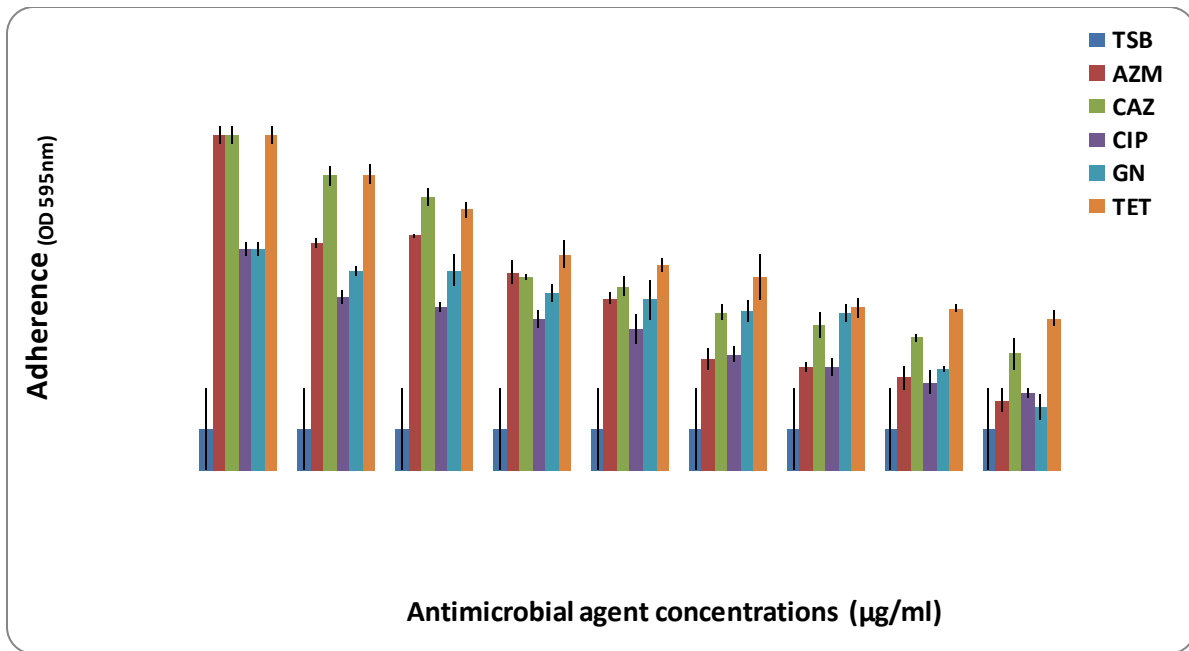


Figure 3.15. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M54 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

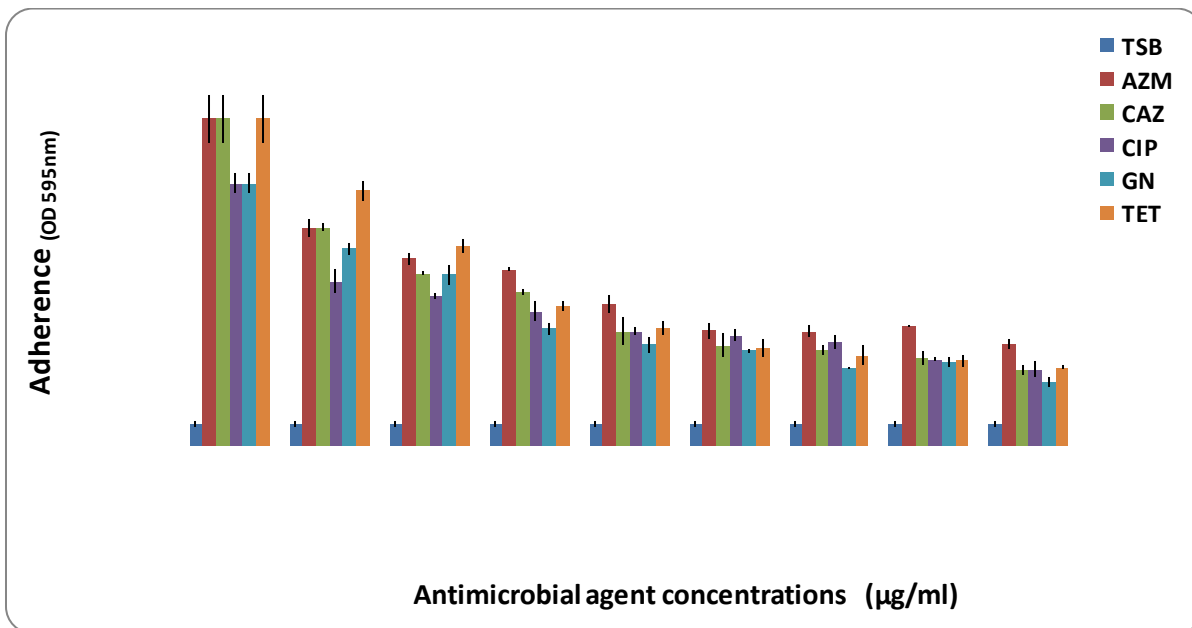


Figure 3.16. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M56 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

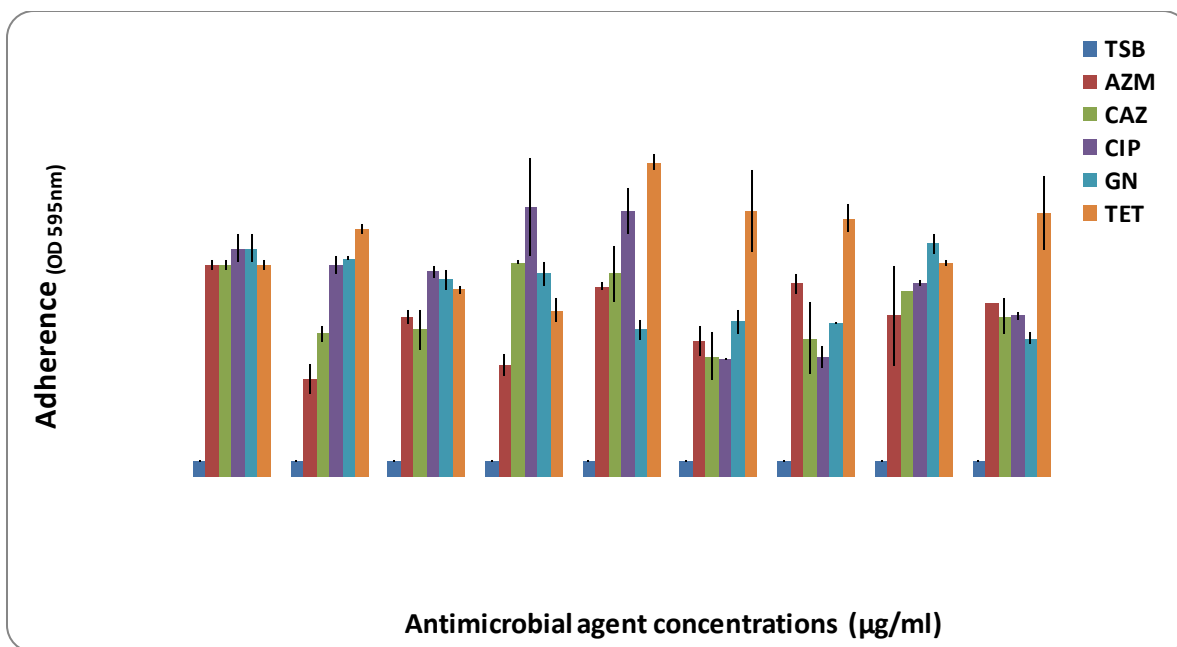


Figure 3.17. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M75 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

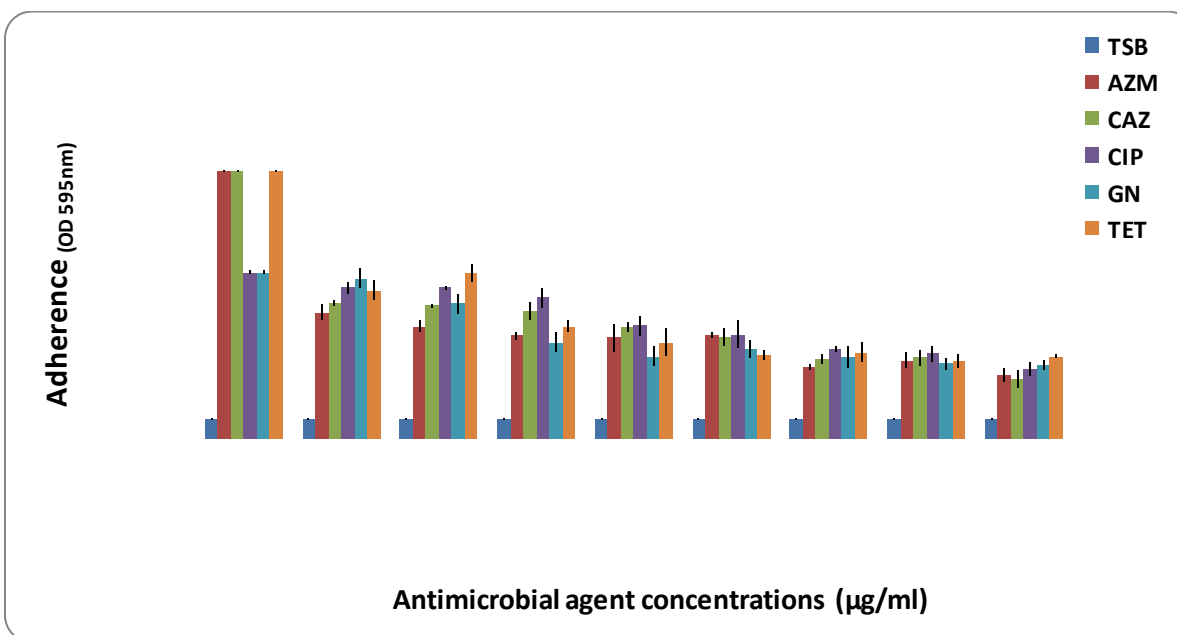


Figure 3.18. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M78 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

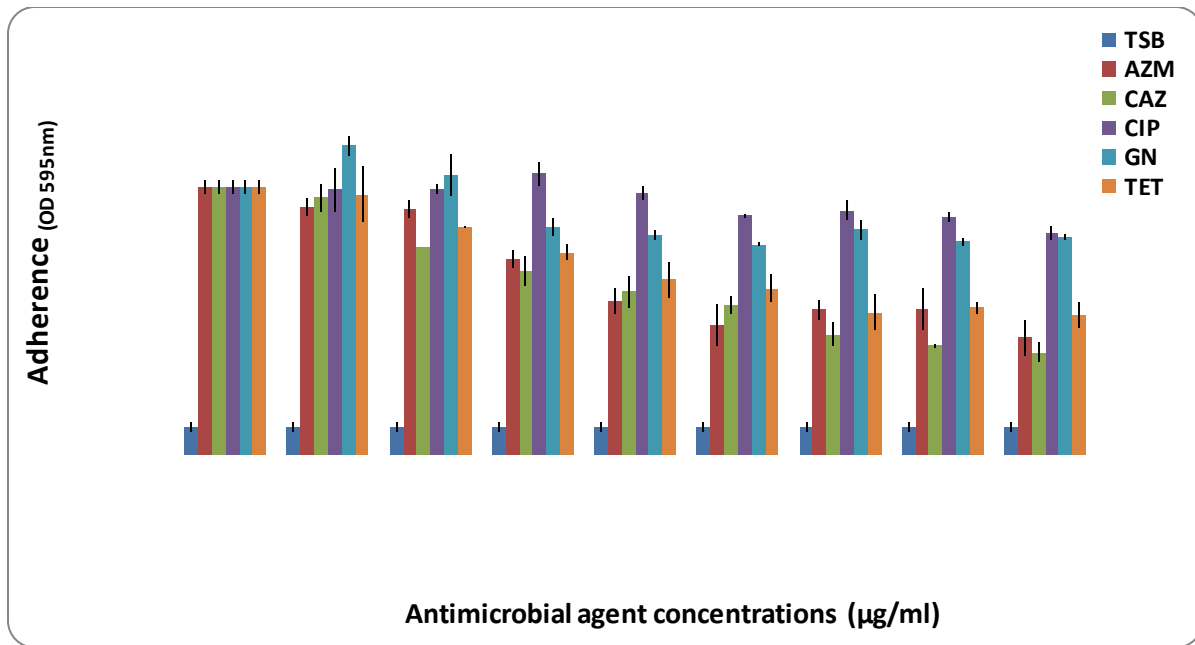


Figure 3.19. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M82 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

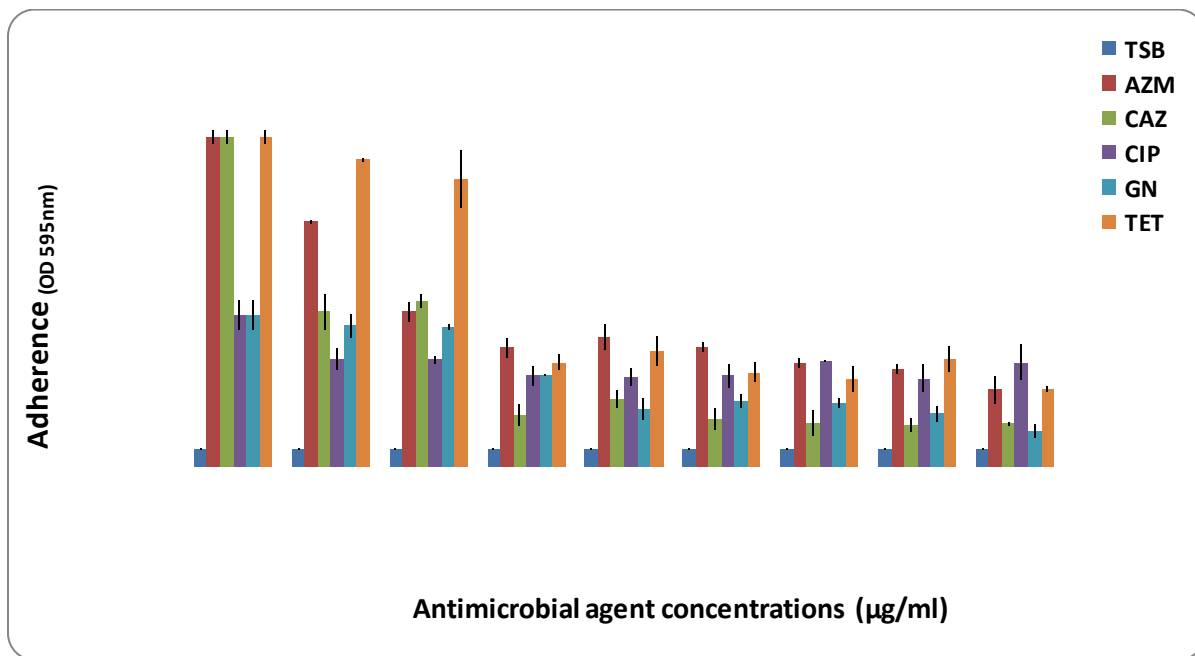


Figure 3.20. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M87 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

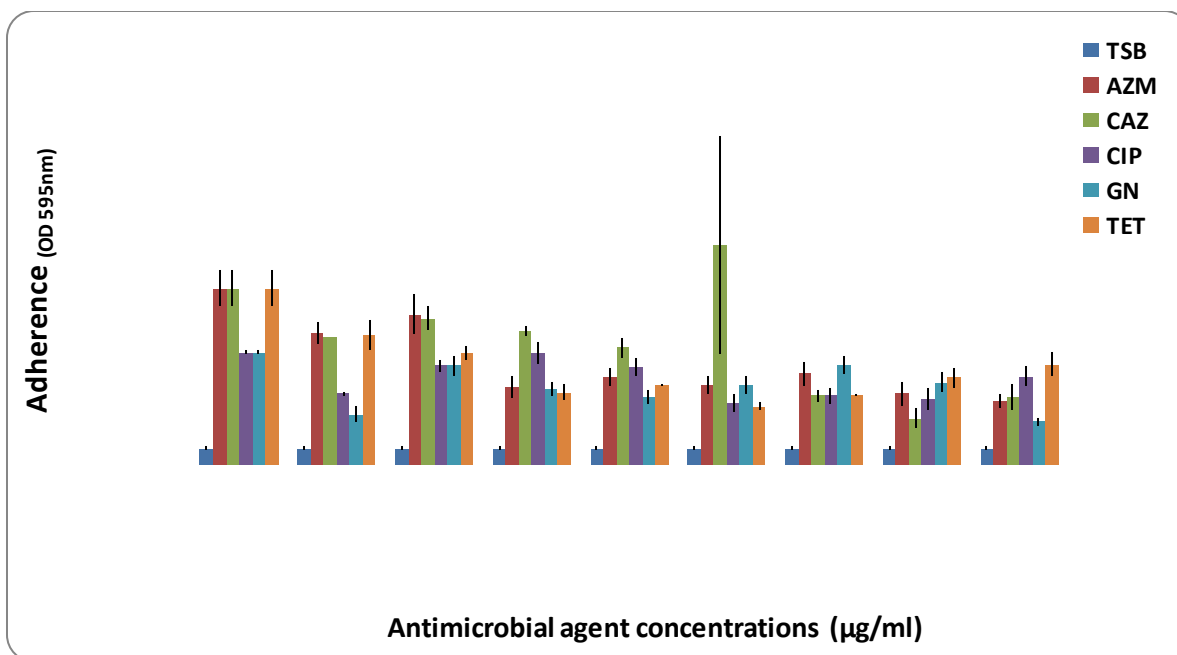


Figure 3.21. Minimum biofilm inhibitory concentrations of *Aeromonas* spp. isolate M89 biofilm cells following exposure to increasing concentrations of azithromycin, ceftazidime, ciprofloxacin, gentamicin and tetracycline, as determined by the microtiter plate assays.

3.3.3. Effect of varying antimicrobial concentrations on biofilm formation

The effect of sub-MIC, MIC and supra-MIC exposures of the selected antimicrobial agents [azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN) and tetracycline (TET)] on initial attachment and/or biofilm formation was determined using microtiter plate assays. For the effect of sub-MIC, MIC and supra-MIC exposures of antimicrobial agents on initial attachment, varying levels of adherence were observed. All (100%, 21/21) of the isolates displayed lower levels of adherence when exposed to azithromycin (AZM) compared to the untreated isolates. This was observed for sub-MIC, MIC and supra-MIC exposures of azithromycin on adherence of *Aeromonas* spp. isolates (Fig. 3.22). The same trend

was also observed for sub-MIC, MIC and supra-MIC exposures of ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN) and tetracycline (TET), when added at the time of inoculation (Figs. 3.23 –3.26). In the present study, an increase in adherence upon sub-MIC exposure to ciprofloxacin was observed for isolates M27 and M75 (Fig. 3.24) and for isolate M27 (Fig. 3.25) upon sub-MIC exposures to gentamicin during the initial attachment assay. A similar trend was observed for the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T), where lower levels of adherence were observed with sub-MIC, MIC and supra-MIC exposures of AZM, CIP, CAZ, GN and TET when these were added at the time of inoculation.

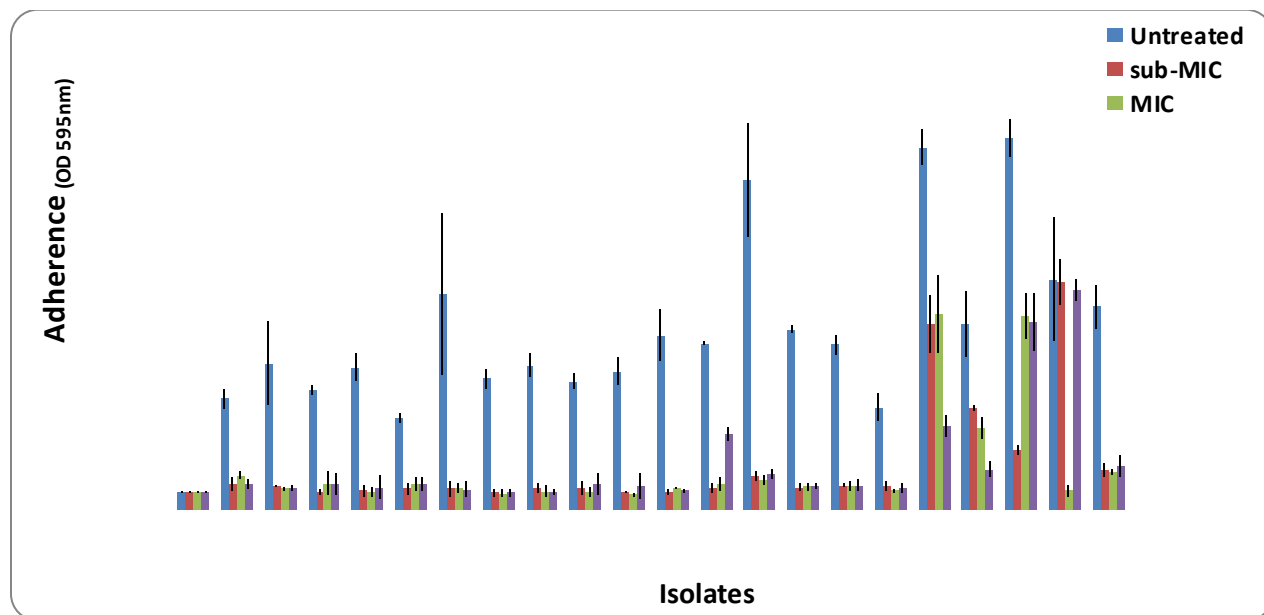


Figure 3.22. Effect of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM) on initial attachment by *Aeromonas* spp. isolates using microtiter plate assays.

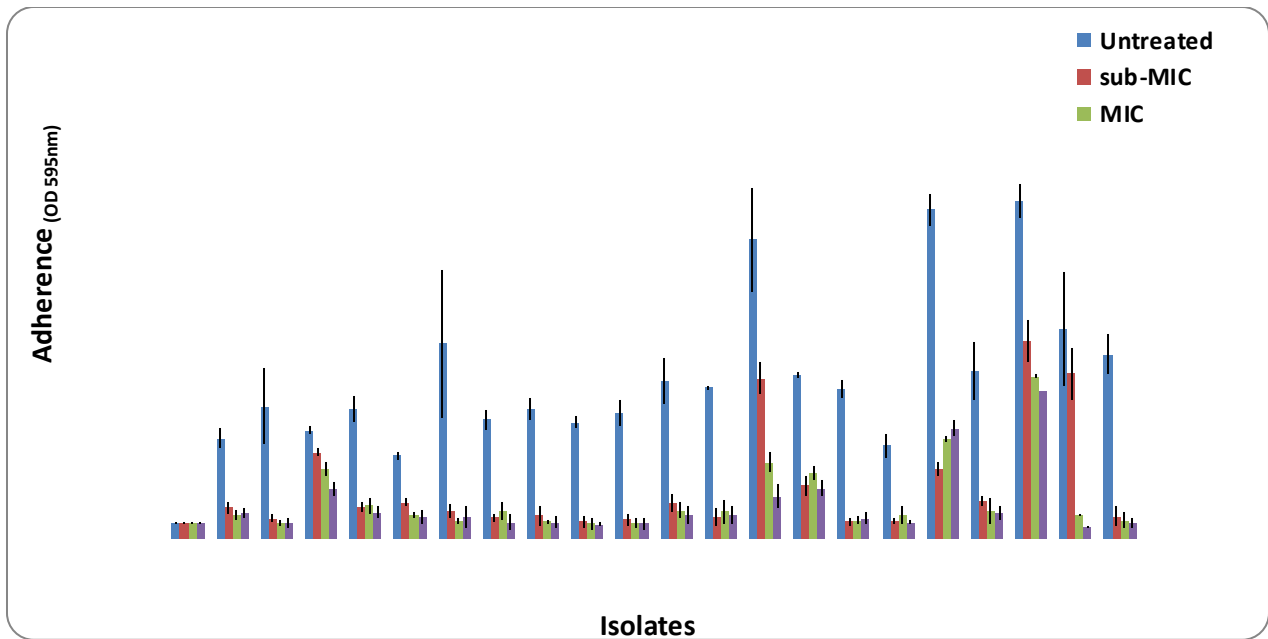


Figure 3.23. Effect of sub-MIC, MIC and supra-MIC exposures of ceftazidime (CAZ) on initial attachment by *Aeromonas* spp. isolates using microtiter plate assays.

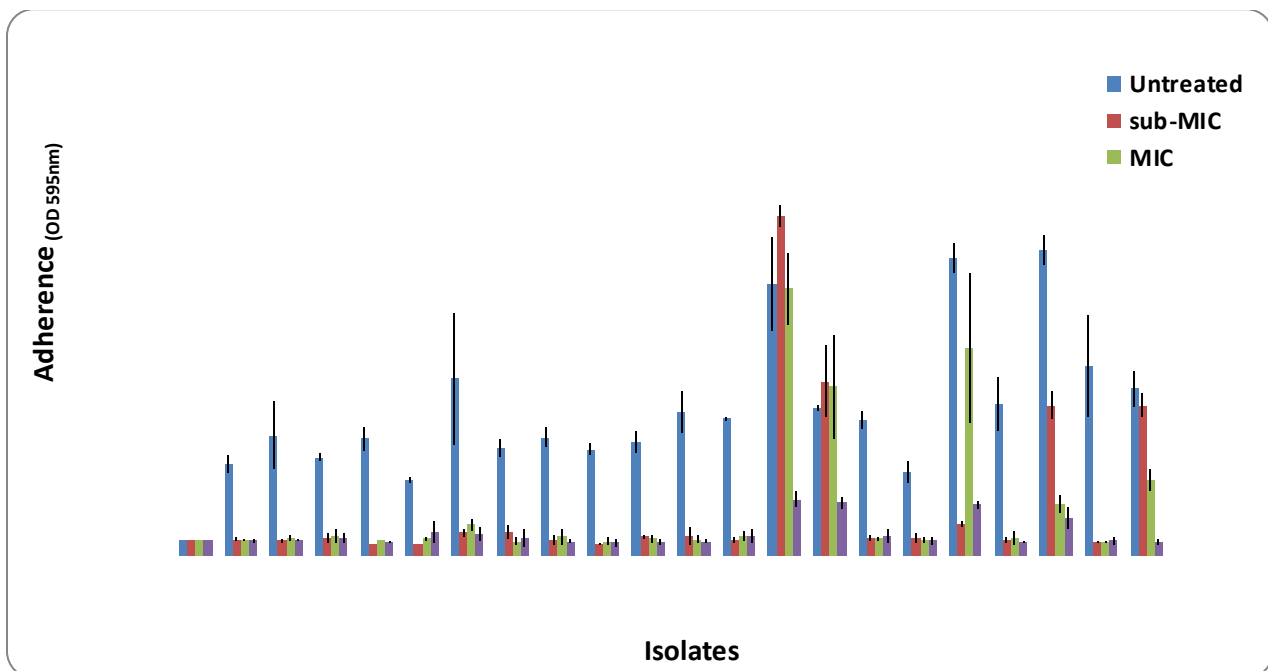


Figure 3.24. Effect of sub-MIC, MIC and supra-MIC exposures of ciprofloxacin (CIP) on initial attachment by *Aeromonas* spp. isolates using microtiter plate assays.

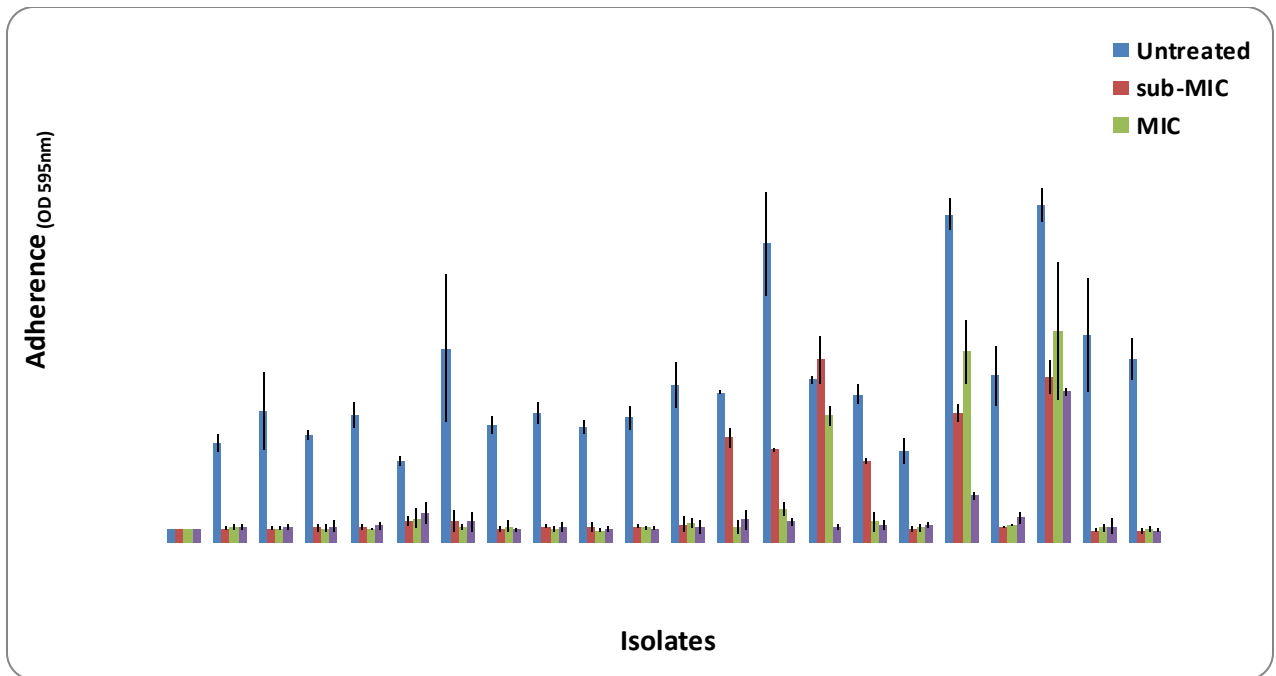


Figure 3.25. Effect of sub-MIC, MIC and supra-MIC exposures of gentamicin (GN) on initial attachment by *Aeromonas* spp. isolates using microtiter plate assays.

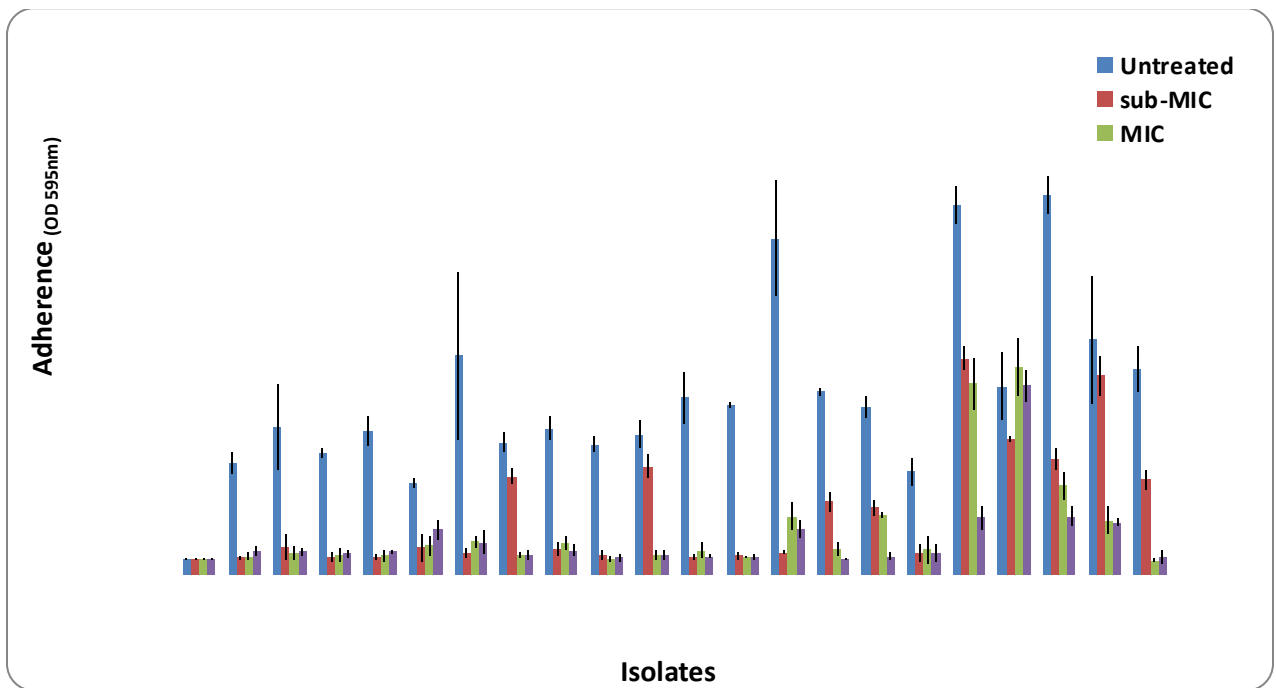


Figure 3.26. Effect of sub-MIC, MIC and supra-MIC exposures of tetracycline (TET) on initial attachment by *Aeromonas* spp. isolates using microtiter plate assays.

For the effect of sub-MIC, MIC and supra-MIC exposures on pre-formed biofilms of *Aeromonas* spp isolates and type strains, varying levels of detachment were also observed. Majority of the isolates displayed increased detachment when exposed to azithromycin compared to when untreated. This was observed for both sub-MIC (90.5%, 19/21) and supra-MIC (95.2%, 20/21) exposures of azithromycin (Fig. 3.27). The same trend was also observed for sub-MIC and supra-MIC exposures to tetracycline (TET), ciprofloxacin (CIP), ceftazidime (CAZ) and gentamicin (GN) of pre-formed *Aeromonas* spp. biofilms (Figs. 3.28–3.31). An increase in adherence upon sub-MIC exposure of ceftazidime was observed for 14.3% (3/21) of isolates (M12, M36, M48; Fig. 3.28), as well as for 33.3% (7/21) of isolates (M3, M10, M16, M36, M48, M54 and M82; Fig. 3.29) upon sub-MIC exposure to ciprofloxacin and for 9.5% (2/21) of isolates (M48, M89; Fig. 3.31) upon sub-MIC exposure to tetracycline, respectively.

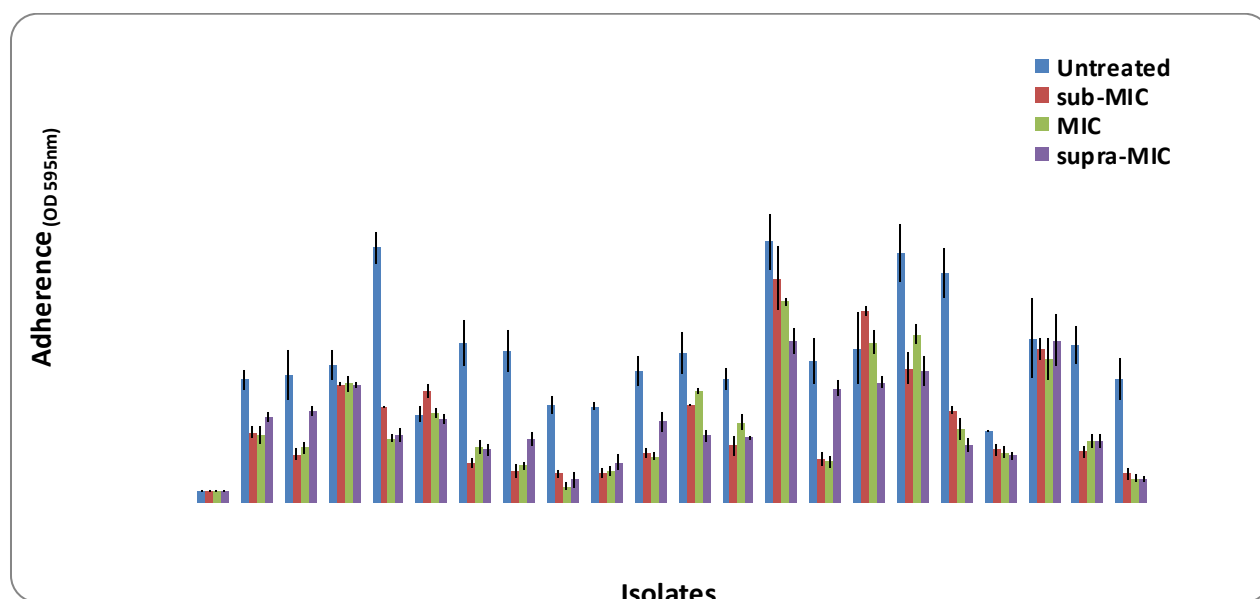


Figure 3.27. Effect of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

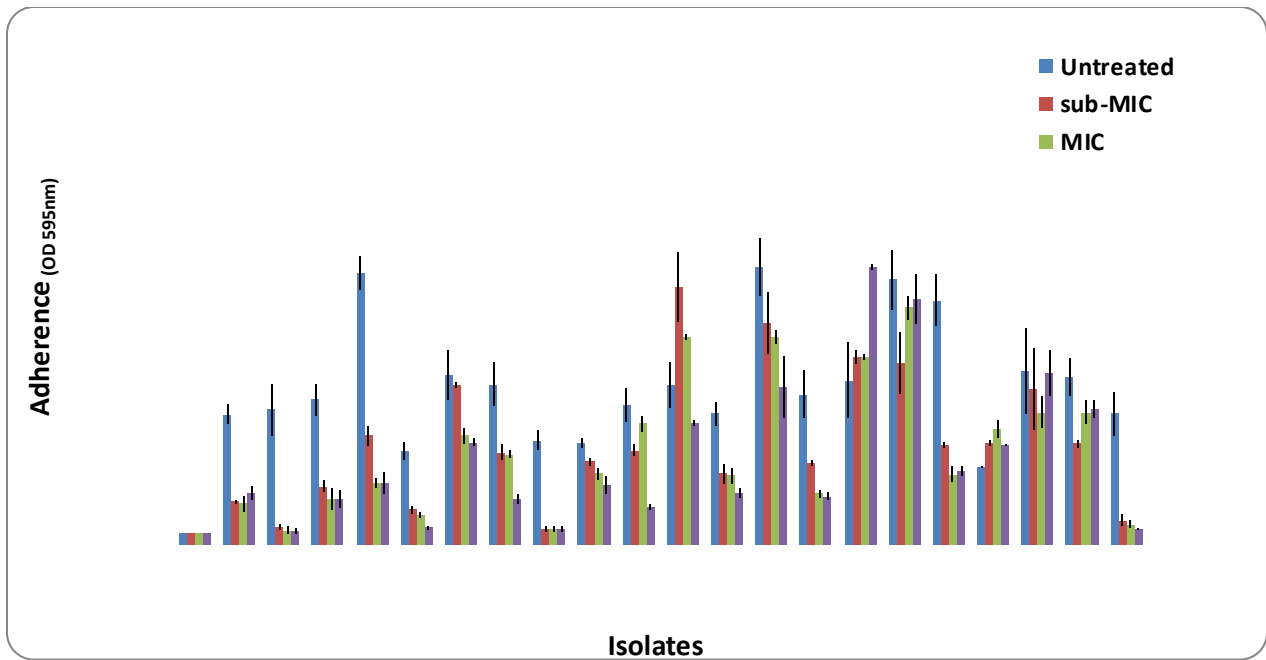


Figure 3.28. Effect of sub-MIC, MIC and supra-MIC exposures of ceftazidime (CAZ) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

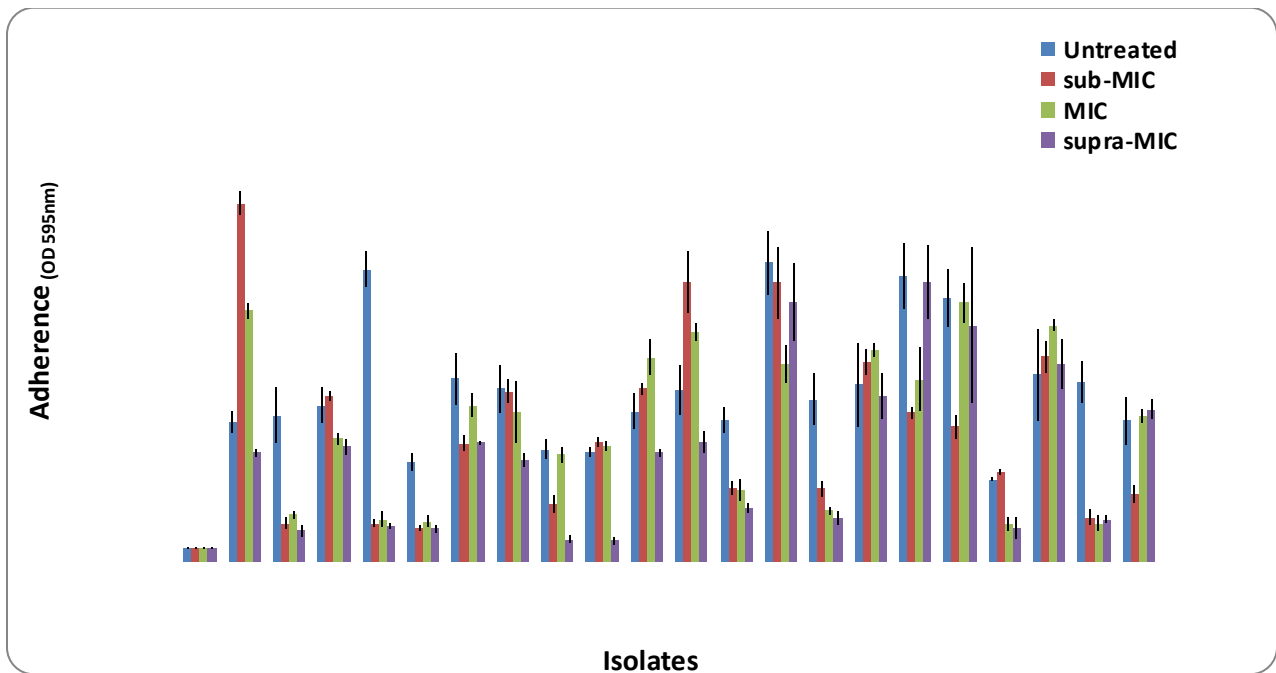


Figure 3.29. Effect of sub-MIC, MIC and supra-MIC exposures of ciprofloxacin (CIP) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

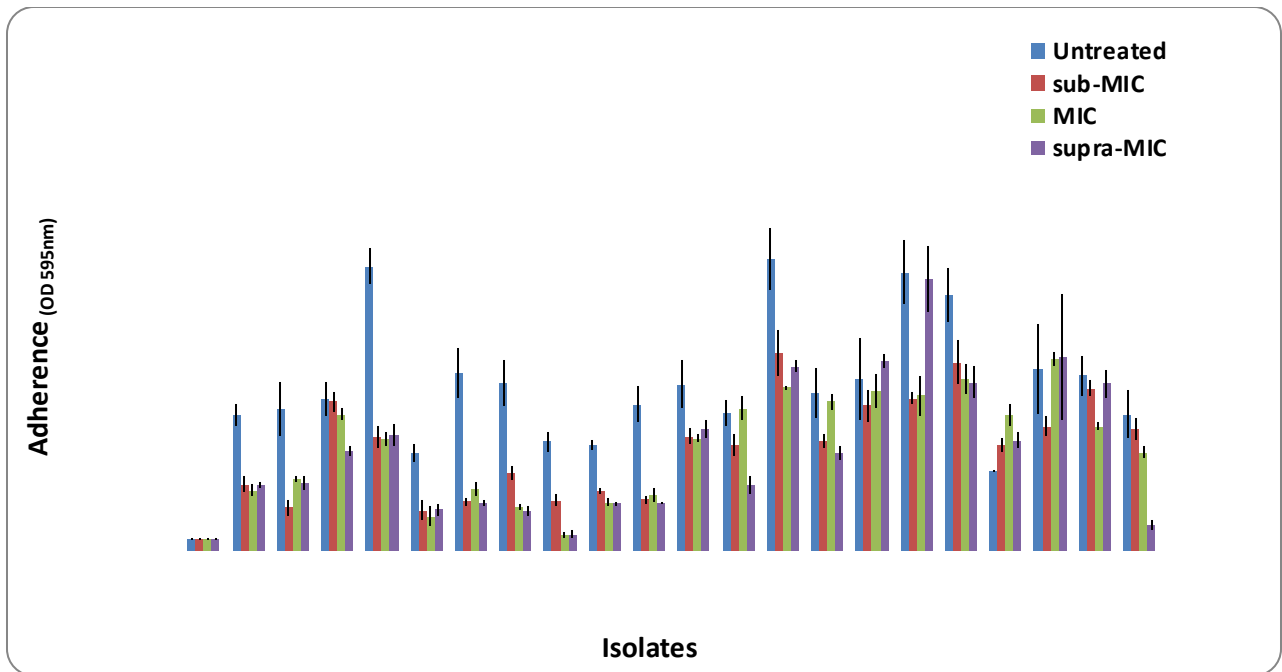


Figure 3.30. Effect of sub-MIC, MIC and supra-MIC exposures of gentamicin (GN) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

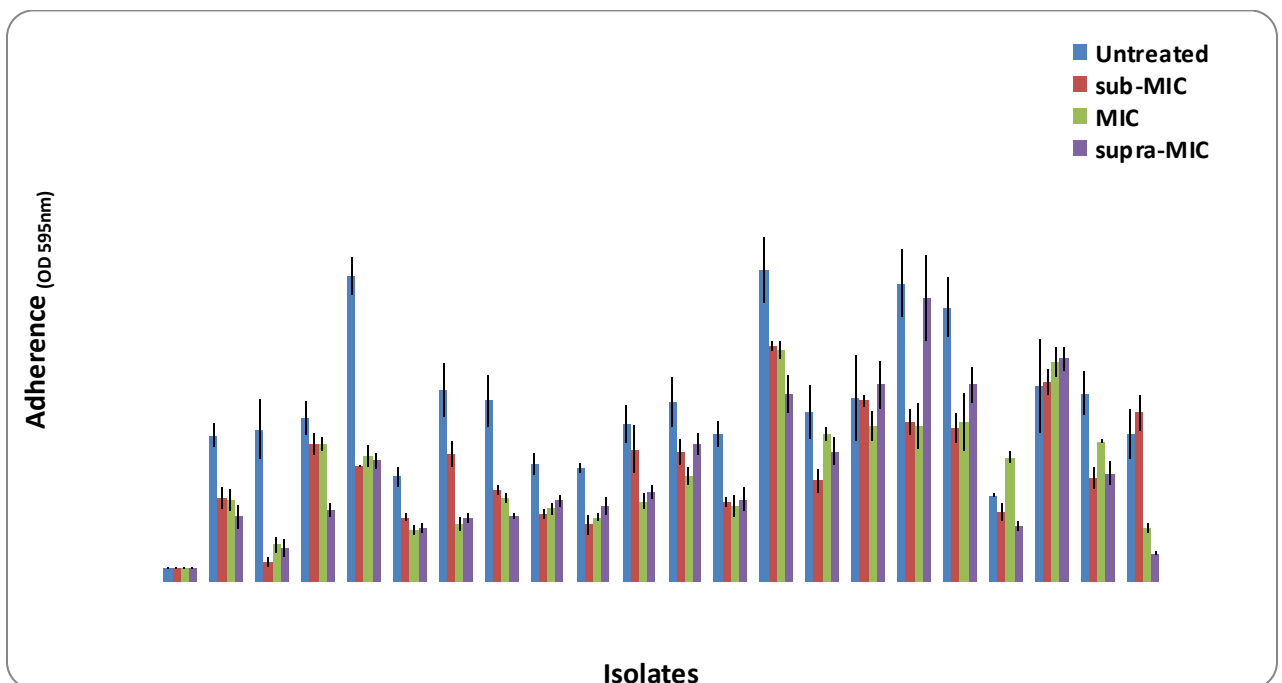


Figure 3.31. Effect of sub-MIC, MIC and supra-MIC exposures of tetracycline (TET) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

When examining biofilm persistence data (Table 3.4), for 76.2% (16/21), 71.4% (15/21) and 66.7% (14/21) of the isolates up to 90% of initial attachment was inhibited by sub-MIC, MIC and supra-MIC exposures of AZM, respectively. For 47.6% (10/21), 61.9% (13/21) and 76.2% (16/21) of the isolates up to 90% of initial attachment was inhibited by sub-MIC, MIC and supra-MIC exposures of CAZ (Table 3.4), respectively. For 80.9% (17/21), 76.2% (16/21) and 80.9% (17/21) of the isolates up to 90% of initial attachment was inhibited by sub-MIC, MIC and supra-MIC exposures of CIP (Table 3.4), respectively. For 66.7% (14/21), 80.9% (17/21) and 90.4% (19/21) of the isolates up to 90% of initial attachment was inhibited by sub-MIC, MIC and supra-MIC exposures of GN (Table 3.4), respectively. Finally, for 52.3% (11/21), 61.9% (13/21) and 76.2% (16/21) of the isolates up to 90% of initial attachment was inhibited by sub-MIC, MIC and supra-MIC exposures of TET (Table 3.4), respectively.

Table 3.4. Biofilm persistence in the presence of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), and tetracycline (TET) on initial attachment of *Aeromonas* spp. isolates

Isolates	% Biofilm persistence*														
	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
M3	3	0	8	1	-1	-1	-4	0	-2	1	-2	0	3	-1	0
M4	0	-1	-1	6	11	1	10	0	3	-1	2	-2	71	3	3
M10	9	18	8	18	10	12	2	1	0	0	1	2	0	2	7
M11	2	6	39	5	8	6	0	4	4	66	1	6	2	1	1
M12	0	2	1	14	9	5	4	1	-1	3	4	1	1	5	1
M16	4	0	0	7	1	1	0	4	0	2	-1	1	8	12	6
M20	0	9	8	76	58	37	3	6	3	1	0	2	1	3	4
M21	7	2	5	4	10	1	5	1	1	-1	1	5	7	10	6
M27	3	3	4	25	33	24	119	115	28	114	76	1	34	6	0
M36	5	4	5	1	2	3	3	2	4	50	5	2	34	29	1
M37	5	2	4	4	0	0	1	3	1	0	0	1	8	4	5
M40	4	11	11	31	13	9	-3	3	13	11	15	23	14	16	38
M44	49	52	19	17	27	30	6	68	13	37	56	10	56	50	11
M48	51	38	14	15	8	7	1	2	0	1	2	7	69	112	101
M54	12	50	48	57	45	41	47	13	8	47	61	42	27	20	12
M56	1	0	4	15	15	9	-2	0	-1	1	-1	3	1	2	5
M75	5	4	6	51	21	9	127	98	16	28	7	2	2	13	9
M78	2	2	1	7	1	3	5	10	4	4	1	4	3	8	8
M82	0	-2	5	3	0	0	4	2	-1	2	0	0	74	3	2
M87	98	1	95	77	4	-2	0	-1	0	-1	0	1	84	18	17
M89	12	11	14	4	1	0	89	40	-1	-1	-1	-1	41	-1	1
ATCC 15468	5	10	6	45	15	12	25	7	-6	5	20	8	45	4	1
ATCC 7966	6	15	2	35	50	9	10	4	1	2	10	3	30	7	1

*Biofilm persistence = (OD₅₉₅ x - OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100,% where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

On examination of biofilm persistence data (Table 3.5), 23.8% (5/21), 19.1% (4/21) and 19.1% (4/21) of the isolates, ≥ 75 of pre-formed biofilm was detached by sub-MIC, MIC and supra-MIC exposures of AZM, respectively. For 14.3% (3/21), 38.1% (8/21) and 38.1% (8/21) of the isolates, ≥ 75 of pre-formed biofilm was detached by sub-MIC, MIC and supra-MIC exposures of CAZ, respectively. For 19.1% (4/21), 14.3% (3/21) and 14.3% (3/21) of the isolates, ≥ 75 of pre-formed biofilm was detached by sub-MIC, MIC and supra-MIC exposures of CIP, respectively. For 9.5% (2/21), 9.5% (2/21) and 19.4% (4/21) of the isolates, ≥ 75 of pre-formed biofilm was detached by sub-MIC, MIC and supra-MIC exposures of GN, respectively. Finally, for 4.8% (1/21), 9.5% (2/21), and 9.5% (2/21) of the isolates, ≥ 75 of pre-formed biofilm was detached by sub-MIC, MIC and supra-MIC exposures of TET, respectively.

Table 3.5. Biofilm persistence in the presence of sub-MIC, MIC and supra-MIC exposures of azithromycin (AZM), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), and tetracycline (TET) on pre-formed biofilms of *Aeromonas* spp. isolates

Isolates	% Biofilm persistence*														
	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
M3	20	23	34	79	66	53	110	106	8	51	39	37	43	50	61
M4	14	18	37	54	52	22	98	86	55	42	21	18	47	42	31
M10	53	50	67	26	24	33	271	187	76	44	39	43	52	51	38
M11	40	61	47	49	47	33	47	45	32	75	104	43	48	46	51
M12	62	72	40	167	133	75	168	136	67	66	65	71	70	55	75
M16	20	5	12	3	3	4	44	95	9	40	4	4	52	57	64
M20	85	85	85	35	25	25	106	77	71	98	89	62	83	82	39
M21	51	66	51	67	89	92	50	62	98	53	54	98	51	50	95
M27	25	22	79	50	28	26	40	25	21	67	94	59	56	86	75
M36	126	104	76	116	116	175	114	121	93	83	93	111	98	83	107
M37	31	37	69	4	2	1	19	26	13	24	46	43	4	16	14
M40	131	103	94	28	21	6	24	32	23	33	26	34	55	42	43
M44	37	28	21	38	25	26	49	98	89	72	65	70	53	56	70
M48	68	63	58	138	160	135	111	35	30	138	182	164	77	152	58
M54	94	86	99	88	74	99	110	128	106	66	105	114	102	113	115
M56	35	22	23	37	19	19	9	11	8	37	36	38	35	38	37
M75	85	76	60	79	74	55	93	65	86	66	54	62	74	73	58
M78	19	29	28	93	61	57	62	84	63	22	30	22	64	24	28
M82	32	28	58	65	86	19	116	139	70	29	33	27	83	46	53
M87	27	34	34	57	77	79	19	15	18	92	69	95	51	72	54
M89	15	11	10	10	7	3	42	104	109	88	70	11	116	30	10
ATCC 15468	35	47	15	45	15	12	55	77	23	56	87	33	22	66	45
ATCC 7966	40	56	20	88	67	20	46	100	45	34	76	23	56	76	34

*Biofilm persistence = $(OD_{595} x - OD_{595} \text{ negative control}) / (OD_{595} \text{ positive control} - OD_{595} \text{ negative control}) \times 100$, where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

3.4. Discussion

Given the importance of *Aeromonas* spp. isolates in causing fish diseases and as human opportunistic pathogens (Davies *et al.*, 2001; Farmer *et al.*, 2006; Kirov *et al.*, 2002; Koksal *et al.*, 2007; Tacao *et al.*, 2005). Since *Aeromonas* spp. cause significant economic losses in farmed fish, it was, therefore, crucial to determine the antimicrobial resistance profiles of *Aeromonas* spp. isolates, and to determine similarity of antimicrobial susceptibility profiles to other clinical and aquacultural isolates reported in the literature. Additionally, it was critical to understand the effect of varying concentrations of antimicrobial agents on aeromonad biofilm formation.

In aquaculture, diagnoses are often presumptive and therapeutic measures in general are administered in the absence of reliable antimicrobial resistance data for the relevant pathogenic organism (Jacobs and Chenia, 2007). Antimicrobial resistance data from previous studies suggest that the indiscriminate use of tetracycline has resulted in high a percentage of resistant strains (Jacobs and Chenia, 2007). Although high levels of aeromonad tetracycline resistance have been reported previously in other studies (Jacobs and Chenia, 2007; Rhodes *et al.*, 2000, 2004; Schmidt *et al.*, 2001), a lower prevalence (19.4%) of tetracycline resistance was observed (Table 3.2) in the present study, and it could due to the source of isolation of the isolates (Jacobs and Chenia, 2007). A high prevalence of 53.3% of tetracycline resistance was observed for *Aeromonas* spp. isolated from farm-raised fresh water fish (Vivekanandhan *et al.*, 2002), while 44.1% of isolates obtained by from frozen fish intended for human consumption were tetracycline-resistant (Castro-Escarpulli *et al.*, 2003). A high prevalence of 78.3% tetracycline resistance was also was observed for *Aeromonas* spp. from South African aquaculture systems

(Jacobs and Chenia, 2007) and 100% tetracycline resistance was observed for *A. veronii* isolates from catfish (Nawaz *et al.*, 2006).

Although majority of these *Aeromonas* isolates spp. showed high resistance to β -lactams (penicillins), majority were susceptible to augmentin, piperacillin-tazobactam, aztreonam, 2nd and 3rd generation cephalosporins, carbapenems, macrolides, fluoroquinolones and aminoglycosides (Table 3.2), as has been reported previously in other studies (Castro-Escarpulli *et al.*, 2003; Jacobs and Chenia, 2007; Koksai *et al.*, 2007). Isolates in the present study also displayed trimethoprim and sulphamethoxazole resistance and this as well as β -lactam resistance could potentially be associated with plasmids and integrons (Casas *et al.*, 2005; Chang *et al.*, 2007; Jacobs and Chenia, 2007; L'Abée-Lund and Sorum, 2000, 2001; Niumsup *et al.*, 2003; Rhodes *et al.*, 2004; Sorum *et al.*, 2003). Similar results were obtained for the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T).

All study isolates in the present study appeared to have susceptibility profiles similar to environmental, fish and clinical isolates and to the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) which displayed susceptibility to amikacin, gentamicin, chloramphenicol, 2nd and 3rd generation cephalosporins, quinolones, carbapenems and cotrimoxazole (Abraham *et al.*, 2007; Castro-Escarpulli *et al.*, 2003; Chien *et al.*, 1996; Farmer *et al.*, 2006; Jacobs and Chenia, 2007; Wahli *et al.*, 2005). As previously described, the newer generation fluoroquinolones, ciprofloxacin and ofloxacin appeared to be highly bactericidal (Table 3.2) (Alcaide *et al.*, 2010; Castro-Escarpulli *et al.*, 2003; Koksai *et al.*, 2007; Jacobs and Chenia, 2007). According to Alcaide *et al.* (2010) and Jacobs and Chenia (2007), fluoroquinolones are the drugs of choice to treat bacterial fish diseases and clinical human *Aeromonas* infections (Castro-Escarpulli *et al.*, 2003; Koksai *et al.*, 2007; Jacobs and Chenia,

2007). The MAR index values were not as high as those reported by Jacobs and Chenia (2007), although 32.3% had MAR index values $\geq 0.3\%$, indicating high risk contamination originating from humans or animals where antimicrobial agents are often used (Jacob and Chenia, 2007) .

Using the broth microdilution assay to determine the MIC of planktonic cells, majority of *Aeromonas* spp. isolates and the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) displayed higher levels of resistance towards ceftazidime (MIC > 32 $\mu\text{g/ml}$, Table 3.3) although they appeared to be highly susceptible to this drug using the disk diffusion assay (Table 3.2). This might have been due to the difference in the sensitivity of both methods, and also because the broth microdilution microtiter assay is quantitative, while the disk diffusion method is qualitative (Arikan *et al.*, 2002). *Aeromonas* spp. isolates displayed higher levels of resistance towards ceftazidime (MIC > 32 $\mu\text{g/ml}$), while levels of resistance towards ciprofloxacin (MIC range of 4 – 32 $\mu\text{g/ml}$), were not as high as described by Cattoir *et al.* (2008) who obtained MICs > 32 mg/ml for environmental *Aeromonas* spp. isolates. Alcaide *et al.* (2010) also observed lower levels of resistance towards ciprofloxacin, with majority of quinolone-resistant *Aeromonas* spp. isolates having MIC ranging of 0.004–8 mg/ml. Picao *et al.* (2008) also obtained MIC levels ≥ 1 mg/l for ciprofloxacin in *A. allosaccharophila* recovered from a Swiss lake. This explains why fluoroquinolones are the drugs of choice to treat bacterial fish diseases and clinical human *Aeromonas* infections (Alcaide *et al.*, 2010; Jacobs and Chenia, 2007). For most isolates in this study, the MICs were at the highest concentration of antimicrobial agents tested (32 $\mu\text{g/ml}$). This is similar to MIC ranges of 1 - 64 $\mu\text{g/ml}$, which were observed in the literature by Ramalivhana *et al.* (2009) for all the tested antimicrobial agents against *Aeromonas* spp. from South African patients.

In medicine it has been estimated that 65% of infections are biofilm-associated, costing the health care system billions of dollars. These biofilm infections are 10 to 1000 times more resistant to the effects of antimicrobial agents (Olson *et al.*, 2002). *Aeromonas* spp. isolates and the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) displayed varying levels of detachment in the presence of all antimicrobial agents at a concentration ranging from 0.008, 0.5, 12, 32, 256, 1024, 2048, to 4096 µg/ml (Figs. 3.1–3.21). All antimicrobial agents used had an effect on pre-formed biofilms by *Aeromonas* spp. isolates, with detachment being observed at all concentrations. However, as expected, majority of isolates displayed maximum detachment at the highest concentration (4096 µg/ml) of these antimicrobial agents. There was a ≥ 16 -fold increase in MBICs (4096 µg/ml) compared to the MICs for all the antimicrobial agents, since majority of the isolates displayed MIC levels ranging from 2-256 µg/ml, and detachment or destruction of the pre-formed biofilms was observed after exposure of antimicrobial agents. Sandoe *et al.* (2006) also observed that MBICs of ampicillin, vancomycin and linezolid for *E. faecalis* isolates were 8192, 4096 and 4096 mg/l, respectively, a similar trend to what was observed in the present study. This was also observed for *Streptococcus pneumoniae* isolates from cystic fibrosis and blood samples, where the MBICs were higher than the MICs of penicillin, tetracycline and rifampicin (Garcia-Castillo *et al.*, 2007).

Sub-MIC exposure can either eliminate or reduce the ability of pathogens to form biofilms by interfering with different stages of biofilm formation resulting in the elimination of these pathogens, depending on the mode of action of the drug and type of bacterial strain (Dal Sasso *et al.*, 2003). Although sub-MICs are not able to inactivate the microorganism, they are potentially capable of altering the chemical and physical cell-surface characteristics and consequently the functionality and expression of some virulence properties such as adhesion,

biofilm formation, hydrophobicity and motility (Pompillo *et al.*, 2010). In the present study, all antimicrobial agents were able to efficiently inhibit initial attachment and increase detachment of pre-formed biofilms of *Aeromonas* spp. isolates and type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) [Figs. 3.22 - 3.31]. This, however, was at varying levels due to their varied modes of action and also perhaps the type of isolates tested. Sub-MICs have been shown to inhibit biofilm formation causing a decrease in adherence by many bacteria including *Stenotrophomonas maltophilia*, *Salmonella enterica* serovar Typhimurium (Matjan *et al.*, 2007; Pompillo *et al.*, 2010). However, Braga and Ricci (1998) observed that sub-MIC and supra-MICs of cefodizime (β -lactam) induced the death of *E. coli*. Hoffman *et al.* (2005) observed that sub-MICs of aminoglycoside antimicrobial agents induced biofilm formation in *P. aeruginosa* and *E. coli*. This was also observed by Ahmed *et al.* (2009) where the sub-MICs and supra-MICs of ampicillin, tetracycline and ciprofloxacin increased biofilm formation in *Streptococcus intermedius*. This was also observed in the present study, although most isolates displayed decreased adherence on exposure of different concentrations of antimicrobial agents. With some antimicrobial agents there was increased adhesion in the initial attachment assay, and increased adhesion rather than increased detachment in the pre-formed biofilm assay as well. This increase in adherence was observed upon sub-MIC exposure of ciprofloxacin for isolates M27 and M75 (Fig. 3.24) and upon sub-MIC exposures of gentamicin for isolate M27 (Fig. 3.25) during the initial attachment assay. An increase in adherence upon sub-MIC exposure of ceftazidime was observed for 14.3% (3/21) of isolates (M12, M36, M48; Fig. 3.28), as well as for 33.3% (7/21) of isolates (M3, M10, M16, M36, M48, M54 and M82; Fig. 3.29) upon sub-MIC exposure to ciprofloxacin and for 9.5% (2/21) of isolates (M48, M89; Fig. 3.31) upon sub-MIC exposure to tetracycline, respectively, in the pre-formed biofilm assay.

With supra-MIC exposures of antimicrobial agents on initial attachment and pre-formed biofilms of *Aeromonas* spp. isolates and type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T), varying levels of adherence were observed, but supra-MIC exposure of all antimicrobial agents had maximum inhibitory effect on majority of the isolates for both the initial attachment and biofilm detachment assays. Tilmicosin and roxithromycin (newer macrolides) completely inhibited the growth of *Pasteurella multocida* after supra-MIC exposures (Lim and Yun, 2001). Similarly, exposure to all antimicrobial agents at supra-MIC caused decreased initial attachment adhesion or increased pre-formed biofilm detachment.

The fluoroquinolones which are the drugs of choice to treat bacterial fish diseases and clinical human *Aeromonas* infections were highly effective in the present study, as could be observed from the disk diffusion assay results and MIC levels for these drugs. Evaluating the antimicrobial concentration required to eradicate biofilms by *Aeromonas* spp. isolates could facilitate effective treatment of these biofilms using antimicrobial agents, as cells in a biofilm are to be more resistant to antimicrobial agents and higher therapeutic doses should be prescribed. In the present study, all antimicrobial agents used had an effect on pre-formed biofilms (MBICs) of *Aeromonas* spp. isolates with majority of isolates displaying maximum detachment at the highest concentration (4096 µg/ml) of these antimicrobial agents. The sub-MIC, MIC, and supra-MIC exposures of all antimicrobial agents had an inhibitory effect on both initial attachment and pre-formed biofilms by *Aeromonas* spp. isolates.

CHAPTER FOUR

Biofilm Control Strategies: Effect of efflux pump inhibition on biofilm formation by

Aeromonas spp.

4.1. Introduction

Most bacteria have evolved over the years, and have developed multidrug resistance mechanisms to cope with the stresses they encounter. One of the mechanisms for multidrug tolerance of biofilms involves the production of persister cells which are produced in small numbers and exhibit multidrug tolerance (Lewis, 2008). Other mechanisms include phenotypic changes in bacteria that occur within biofilm environments that result in resistance, inactivation of antimicrobial agents by extracellular polymers or modifying enzymes (Davey and O'Toole, 2000; Hoiby, 2010) as well as up-regulated efflux pumps (Hoiby *et al.*, 2010; Gilbert *et al.*, 2002). In biofilm environments of most pathogens, multidrug efflux pumps are highly expressed and pose a huge threat for antimicrobial therapy and human health. They are relevant elements in intrinsic and acquired antimicrobial resistance of pathogenic bacteria (Martinez *et al.*, 2009). These proteins extrude chemically unrelated antimicrobial agents from the cell into the environment, thereby decreasing the intracellular concentration of the antimicrobial agent to subtoxic levels which cells can withstand (Borges-Walmsley *et al.*, 2003; Nelson, 2002; Mah and O'Toole, 2001).

Efflux proteins can be specific, facilitating the efflux of only one compound or a class of compounds and/or non-specific exhibiting broad specificity for chemical compounds that are structurally unrelated (Nelson, 2002). Piddock (2006) also described that a single organism can possess multiple multidrug efflux pumps, and may transport different classes of antimicrobial

agents, not within the same class of antimicrobial agents (Van Bambeke *et al.*, 2000). They can extrude various compounds from antimicrobial agents, to disinfectants, dyes, and detergents, however, the substrates for each pump are different depending on the actual pump and on the bacterial species (Piddock, 2006a). In clinical environments, they may efflux virulence factors, and are also involved in the quorum sensing-regulated expression of virulence factors (Martinez *et al.*, 2009), which are important traits required for pathogenicity and virulence in bacteria. In non-clinical environments, they have relevant implications for the environment as they allow for heavy-metal resistance, resistance to organic solvents and resistance to antimicrobial agents produced by plants (Fernandes *et al.*, 2003; Martinez *et al.*, 2009).

A wide variety of efflux pump systems exhibiting drug specificity and multidrug resistance have been identified in many bacteria, including *Aeromonas* spp. isolates. These include efflux pump systems (AcrA, AcrB) of the RND family commonly dominant in Gram-negative bacteria such *E. coli*, *Enterobacter aerogenes*, and *Citrobacter freundii*, causing resistance to β -lactams, quinolones, tetracycline and chloramphenicol (Blair and Piddock, 2009; Bornet *et al.*, 2003; Martinez *et al.*, 2009; Nikaido and Zgurskaya, 2001; Piddock, 2006a, 2006b; Sanchez-Cespedes and Vila, 2007). Increased expression of the AdeABC efflux pump causing decreased susceptibility to carbapenems among *Acinetobacter baumannii* isolates in a Chinese hospital was also reported by Huang *et al.* (2008). A multidrug efflux pump, AheABC belonging to the RND family has been identified in *Aeromonas* spp. isolates, specifically *A. hydrophila* and *A. salmonicida*. This pump was involved in resistance to the antimicrobial agents erythromycin, trimethoprim, fusidic acid, rifampicin as well as to the quinolones and fluoroquinolones nalidixic acid, oxolinic acid, ofloxacin and ciprofloxacin (Giraud *et al.*, 2004; Hernould *et al.*, 2008).

Efflux pump inhibition represents one of the promising strategies to control bacterial biofilms due to the fact that cells in biofilm over-express these efflux pump systems compared to planktonic cells. This has been observed in most bacteria such as *E. coli* and *Klebsiella* spp. as a result of the AaeAB efflux pump (Kvist *et al.*, 2008). Efflux pump inhibitors (EPIs) have been used to inhibit different efflux pump systems used by different bacterial species thus preventing elimination of antimicrobial agents within the cells. There are different efflux pump systems and different inhibitors inhibit specific efflux pumps depending on the type of pump and on their spectrum of activity (Kvist *et al.*, 2008).

EPIs are added together with other antimicrobial compounds, and this causes a reduction in the MIC values of different antimicrobial agents because the cells become more susceptible to these antimicrobials (Kvist *et al.*, 2008). Both PA β N and NMP inhibit RND pumps that are mostly abundant in Gram-negative bacteria including *Vibrio cholerae* (Hernould *et al.*, 2008). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is potential inhibitor used to control bacterial infections as it interferes with proton motive force (PMF)-dependent transporters abundant in Gram-negative bacteria, and contributes to the efflux of antimicrobial agents and other compounds (Ikonomidis *et al.*, 2008; Ramon-Garcia *et al.*, 2006). PA β N, NMP and CCCP are promising candidates to be used in managing bacterial associated diseases (Van Bambeke *et al.*, 2006).

Since efflux pumps are highly expressed in biofilms and play a huge role in antimicrobial resistance in bacteria such as *Aeromonas* spp. isolates, it was, therefore, important to determine the prevalence and diversity of these efflux pumps in *Aeromonas* spp. and additionally, determine the substrate specificity of these efflux pumps. This was determined using disk diffusion assays incorporating efflux pump inhibitors. The study also investigated the effect of

CCCP, PA β N and NMP on initial attachment and detachment of pre-formed biofilms of *Aeromonas* spp. isolates, using modified microtitre plate assays. This was to determine the point at which efflux pump inhibitors are effective for biofilm control and could facilitate effective removal of these biofilms, thus providing a solution to the infections caused by these aeromonad biofilms.

4.2. Materials and Methods

4.2.1. Identification of efflux pump-associated antimicrobial resistance in *Aeromonas* spp.

Forty-five presumptive *Aeromonas* spp. isolates (Table 4.1) were selected based on their antimicrobial resistance profiles (Chapter 3), biofilm phenotypes and associated phenotypic characteristics (motility, casein hydrolysis, A-layer, hydrophobicity, autoaggregation) for the identification of efflux pump-mediated resistance. The two type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T were also included in the assays. Bacterial colonies were grown on TSB agar plates.

To determine the presence of an efflux mechanism, Mueller-Hinton (MH) agar plates were prepared with or without EPIs [(carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma, SA), phenylalanine arginine β -naphthylamide (PA β N) or 1-(1-naphthylmethyl)-piperazine (NMP)] (Magnet *et al.*, 2001; Shi *et al.*, 2005). The final concentration of the efflux inhibitors in the MH agar was 20 μ g/ml. MH agar with or without efflux inhibitors was inoculated with standardized cell suspensions equivalent to a 0.5 MacFarland standard, and 16 Oxoid antimicrobial agent disks {aminoglycosides [amikacin (AK30), gentamicin (CN10), streptomycin (S10)], macrolides [azithromycin (AZM15), erythromycin (E15)], cephalosporins

[cefepodoxamine (CPD10)], chloramphenicols [chloramphenicol (C30)], metabolic inhibitors [sulphamethoxazole (RL25), trimethoprim (W1.25)], penicillins [ampicillin (AMP10)], quinolones [enrofloxacin Baytril (ENR5), ciprofloxacin (CIP5), nalidixic acid (NA30), norfloxacin (NOR10), ofloxacin (OFX5)], and tetracyclines [tetracycline (TE30)] were placed onto the inoculated plates. The plates were incubated at 30 °C for 24 hours.

Inhibition zone diameters were measured and the resistance or susceptibility profiles of the isolates were determined in the presence/absence of EPIs. When the efflux was present in isolates, zone diameters on the EPI-containing plates were greater than corresponding zone diameters on plates without the inhibitor (Magnet *et al.*, 2001). MH agar plates without EPIs inoculated with respective cell suspensions were used as growth controls. *E. coli* ATCC 25922 served as the efflux-negative control and multi-drug resistant *P. aeruginosa* ATCC 27853 was the efflux-positive control.

4.2.2. Effect of efflux pump inhibitors on initial attachment and/or biofilm formation by *Aeromonas* spp.

EPIs (CCCP, PAβN and NMP) were used to determine their effect on initial attachment and pre-formed biofilm using a modified microtiter assay (Basson *et al.*, 2008). Sixteen hour-old cultures were used to prepare cell suspensions, which were standardised equivalent to a 0.5 McFarland standard (Basson *et al.*, 2008). The first assay was to investigate the effect of EPIs on initial attachment of cells. EPIs (20 µg/ml) were added to 90 µl TSB and 10 µl of cell suspension and incubated for 24 hours at 30 °C with agitation.

Table 4.1. *Aeromonas* spp. isolates selected for the identification of efflux pump-mediated resistance

Isolate	Source	Species identification	Casein hydrolysis*	Gelatin hydrolysis*	Motility*	A-layer detection*	Biofilm-forming ability*	Hydrophobicity*	Autoaggregation*
M1	Catfish	<i>A. culicicola</i>	+	+	+	-	S	HPL	SA
M3	Catfish	<i>A. allosaccharophila</i>	+	+	+	+	S	MHPB	SA
M4	Catfish	<i>A. jandaei</i>	+	-	+	+	S	HPL	SA
M7	Catfish	<i>A. culicicola</i>	+	+	+	+	S	MHPB	SA
M10	Catfish	<i>A. culicicola/jandaei</i>	+	-	+	+	S	HPL	SA
M11	Catfish	<i>Aeromonas</i> spp.	-	+	+	+	S	HPL	SA
M12	Catfish	<i>A. bestiarum</i>	+	+	+	+	S	HPB	SA
M15	Tilapia	<i>Aeromonas</i> spp.	+	+	+	+	M	HPL	SA
M16	Tilapia	<i>A. jandaei</i>	+	+	+	+	S	HPL	SA
M19	Tilapia	<i>A. jandaei</i>	+	+	+	+	M	HPL	MA
M20	Tilapia	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M21	Tilapia	<i>A. allosaccharophila</i>	+	+	+	+	S	MHPB	SA
M24	Sea water	<i>A. jandaei</i>	+	+	+	+	S	MHPB	SA
M27	Sea water	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M29	Sea water	<i>A. culicicola</i>	+	+	+	+	S	MHPB	SA
M30	Sea water	<i>A. jandaei</i>	+	+	+	+	S	HPL	MA
M33	Sea water	<i>Aeromonas</i> spp.	+	+	+	+	S	MHPB	SA
M35	Sea water	<i>A. culicicola</i>	+	+	+	+	S	MHPB	MA
M36	Sea water	<i>A. jandaei</i>	+	+	+	+	S	HPL	SA
M37	Sea water	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M40	Sea water	<i>A. culicicola</i>	+	+	+	+	S	MHPB	SA
M42	Tilapia	<i>A. culicicola</i>	+	+	+	-	S	HPL	SA
M43	Tilapia	<i>Aeromonas</i> spp.	+	+	+	+	S	MHPB	SA
M44	Tilapia	<i>A. ichtiosmia</i>	+	+	+	+	S	HPL	SA
M48	Tilapia	<i>A. ichtiosmia</i>	+	+	+	+	S	HPL	SA
M54	Tilapia	<i>A. ichtiosmia</i>	+	+	+	+	S	HPL	SA
M56	Tilapia	<i>A. culicicola</i>	+	+	+	+	S	HPB	MA
M61	Tilapia	<i>A. culicicola</i>	+	+	+	+	S	HPL	SA
M69	Koi carp	<i>A. bestiarum</i>	+	-	+	+	S	HPL	SA
M71	Koi carp	<i>A. bestiarum</i>	-	-	+	+	M	MHPB	SA
M73	Koi carp	<i>A. bestiarum</i>	+	+	+	+	S	HPL	SA
M74	Koi carp	<i>A. allosaccharophila</i>	+	-	+	+	S	HPB	SA
M75	Koi carp	<i>Aeromonas</i> spp.	+	+	+	+	S	HPL	SA
M78	Koi carp	<i>A. bestiarum</i>	-	+	+	+	S	HPL	MA
M79	Koi carp	<i>A. bestiarum</i>	+	+	+	-	S	MHPB	SA
M82	Koi carp	<i>A. culicicola</i>	+	+	+	+	S	HPL	SA
M83	Koi carp	<i>A. bestiarum</i>	+	+	+	+	M	HPB	SA
M84	Koi carp	<i>A. bestiarum</i>	+	+	+	-	S	HPL	SA
M85	Koi carp	<i>A. allosaccharophila</i>	+	+	+	-	S	HPL	SA
M87	Koi carp	<i>A. bestiarum</i>	+	+	+	+	S	MHPB	SA
M89	Koi carp	<i>A. allosaccharophila</i>	+	-	+	-	M	HPL	SA
M91	Koi carp	<i>A. bestiarum</i>	+	-	+	+	S	MHPB	SA
M93	Koi carp	<i>A. allosaccharophila</i>	+	+	+	+	S	HPL	SA
M97	Koi carp	<i>A. bestiarum</i>	+	+	+	-	S	HPL	SA
M98	Koi carp	<i>Aeromonas</i> spp	+	+	+	+	S	HPL	SA
ATCC 15468 ^T		<i>A. caviae</i>	+	+	+	+	M	HPL	SA
ATCC 7966 ^T		<i>A. hydrophila</i>	+	+	+	+	S	HPL	MA

+: positive for test, -: negative for test; *S=strong biofilm-former, *M=moderate biofilm-former, *HPL= hydrophilic, *MHPB= moderately hydrophobic, *HPB= hydrophobic, *SA= strong autoaggregation ability, *MA= moderate autoaggregation ability

For the second assay, pre-formed 24 hour biofilms were washed three times with sterile deionised water, and allowed to air-dry. Following the addition of fresh 90 µl TSB and EPIs (20 µg/ml), microtiter plates were incubated for a further 24 hours with agitation at 30 °C. For both assays, the negative control contained broth only and the positive control contained the respective cell suspension with no EPIs added. Staining and determination of OD values (OD₅₉₅) was done as described previously in Section 2.2.3 (Basson *et al.*, 2008). OD_{595nm} of isolates with EPIs were compared with OD_{595nm} of positive control, without EPIs to determine the change in biofilm formation as a result of EPI exposure.

Biofilm persistence in the presence of EPIs was calculated using the equation or formula: percent of biofilm persistence = (OD₅₉₅ x – OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100%, where x corresponds to the tested EPI (Tre-Hardy *et al.*, 2008).

4.2.3. Statistical analyses

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. Identification of efflux pump-associated antimicrobial resistance in *Aeromonas* spp.

Varying levels of susceptibility/resistance to the different antimicrobial agents were displayed by the different *Aeromonas* spp. isolates, in the presence and absence of EPIs (Table 4.2). A diameter difference of ≥ 5 mm was used as a cut-off, together with susceptibility/resistance

patterns. For majority of isolates in the presence of CCCP, susceptibility to norfloxacin (53%), azithromycin (63%), and tetracycline (58%) was increased (Table 4.2). Furthermore, for majority of isolates in the presence of NMP, susceptibility to norfloxacin (56%), tetracycline (62%), azithromycin (42%) and erythromycin (51%) was increased (Table 4.2). Lastly, for majority of isolates in the presence of PA β N, susceptibility to erythromycin (47%) was increased (Table 4.2). The type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, also demonstrated increased susceptibility to norfloxacin, azithromycin, tetracycline and erythromycin, following exposure to all EPIs (CCCP, NMP, PA β N).

There were three different categories of EPI-induced changes observed (Table 4.3). Some isolates which were initially susceptible in the absence of the respective EPIs, displayed increased susceptibility. Some isolates which were intermediately susceptible became fully susceptible. Some isolates which were resistant displayed intermediate susceptibility or full susceptibility. Some isolates displayed changes in susceptibility following exposure to all three EPIs simultaneously, whereas others were only affected by 1 or more EPIs simultaneously (Table 4.3).

Table 4.2. Antimicrobial agent susceptibility of *Aeromonas* spp. isolates in the presence and absence of efflux pump inhibitors

Antimicrobial agent	No. (%) susceptible without inhibitor (n=19)	No. (%) susceptible with CCCP* (n=19)	No. (%) susceptible without inhibitor (n=45)	No. (%) susceptible with NMP* (n=45)	No. (%) susceptible without inhibitor (n=45)	No. (%) susceptible with PAβN* (n=45)
<i>Aminoglycosides</i>						
Amikacin (AK30)	17 (89)	2 (11)	39 (87)	6 (13)	36 (80)	9 (20)
Gentamicin (CN10)	15 (79)	4 (21)	25 (56)	20 (44)	29 (64)	16 (36)
Streptomycin (S10)	12 (63)	7 (37)	27 (60)	18 (40)	35 (78)	10 (22)
<i>Macrolides</i>						
Azithromycin (AZM15)	7 (37)	12 (63)	16 (36)	19 (42)	30 (47)	5 (33)
Erythromycin (E15)	12 (63)	7 (37)	12 (27)	23 (51)	14 (31)	21 (47)
<i>Cephalosporins</i>						
Cefpodoxime (CPD10)	13 (68)	6 (32)	30 (67)	15 (33)	35 (78)	10 (22)
<i>Chloramphenicols</i>						
Chloramphenicol (C30)	11 (58)	8 (42)	29 (64)	16 (36)	27 (60)	18 (40)
<i>Metabolic inhibitors</i>						
Trimethoprim (W1.25)	19 (100)	0 (0)	45 (100)	0 (0)	45 (100)	0 (0)
Sulphamethoxazole (RL25)	19 (100)	0 (0)	45 (100)	0 (0)	45 (100)	0 (0)
<i>Penicillins</i>						
Ampicillin (AMP 10)	15 (79)	4 (21)	32 (82)	8 (18)	40 (89)	5 (11)
<i>Quinolones</i>						
Ciprofloxacin (CIP5)	14 (74)	5 (26)	32 (71)	13 (29)	35 (78)	10 (22)
Enrofloxacin (ENR5)	13 (68)	6 (32)	28 (62)	17 (38)	31 (69)	14 (31)
Nalidixic acid (NA30)	13 (68)	6 (32)	26 (58)	19 (42)	32 (71)	13 (29)
Norfloxacin (NOR10)	9 (47)	10 (53)	20 (44)	25 (56)	27 (60)	18 (40)
Ofloxacin (OFX5)	12 (63)	7 (37)	28 (62)	17 (38)	36 (80)	9 (20)
<i>Tetracyclines</i>						
Tetracycline (TE30)	8 (42)	11 (58)	7 (38)	28 (62)	32 (71)	13 (29)

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

Table 4.3. Three categories of EPI-induced susceptibility observed for *Aeromonas* spp. isolates

	Total no. (%) of isolates susceptible without inhibitor (n=45)	No. (%) susceptible with CCCP* (n=19)	No. (%) susceptible with NMP* (n=45)	No. (%) susceptible with PAβN* (n=45)	Total no. (%) of isolates intermediately susceptible without inhibitor (n=45)	No. (%) susceptible with CCCP* (n=19)	No. (%) susceptible with NMP* (n=45)	No. (%) susceptible with PAβN* (n=45)	Total no. (%) of isolates resistant without inhibitor (n=45)	No. (%) susceptible with CCCP* (n=19)	No. (%) susceptible with NMP* (n=45)	No. (%) susceptible with PAβN* (n=45)
<i><u>Aminoglycosides</u></i>												
Amikacin (AK30)	13 (28.9)	2 (10.5)	6 (13.3)	8 (17.8)								
Gentamicin (CN10)	26 (57.8)	4 (21.1)	20 (44.4)	13 (28.9)								
Streptomycin (S10)	26 (57.8)	5 (26.3)	17 (37.8)	9 (20.0)	1 (2.2)	1 (5.3)	0 (0)	0 (0)				
<i><u>Macrolides</u></i>												
Azithromycin (AZM15)	27 (60.0)	7 (36.8)	18 (40.0)	8 (17.8)	2 (4.4)	1 (5.3)	1 (2.2)	1 (2.2)	1 (2.2)	1 (5.3)	1 (2.2)	1 (2.2)
Erythromycin (E15)	15 (33.3)	1 (5.3)	11 (24.4)	10 (22.2)	7 (15.6)	1 (5.3)	5 (11.1)	4 (8.9)	13 (28.9)	4 (21.1)	6 (13.3)	6 (13.3)
<i><u>Cephalosporins</u></i>												
Cefpodoxime (CPD10)	22 (48.9)	5 (26.3)	14 (31.1)	9 (20.0)					2 (4.4)	1 (5.3)	2 (4.4)	1 (2.2)
<i><u>Chloramphenicols</u></i>												
Chloramphenicol (C30)	20 (44.4)	5 (26.3)	15 (33.3)	12 (26.7)	3 (6.7)	1 (5.3)	2 (4.4)	3 (6.7)	3 (6.7)	0 (0)	2 (4.4)	3 (6.7)
<i><u>Penicillins</u></i>												
Ampicillin (AMP10)	2 (4.4)	0 (0)	2 (4.4)	1 (2.2)					7 (15.6)	4 (8.9)	5 (11.1)	2 (4.4)
<i><u>Quinolones</u></i>												
Ciprofloxacin (CIP5)	13 (28.9)	1 (5.3)	9 (20.0)	1 (2.2)								
Enrofloxacin (ENR5)	22 (48.9)	6 (31.6)	14 (31.1)	14 (31.1)								
Nalidixic acid (NA30)	25 (55.6)	6 (31.6)	19 (42.2)	14 (31.1)					1 (2.2)	0 (0)	1 (2.2)	0 (0)
Norfloxacin (NOR10)	29 (64.4)	12 (63.2)	25 (55.2)	19 (42.2)								
Ofloxacin (OFX5)	11 (24.4)	5 (26.3)	8 (17.8)	8 (17.8)								
<i><u>Tetracyclines</u></i>												
Tetracycline (TE30)	23 (51.1)	4 (21.1)	21 (46.7)	8 (17.8)	2 (4.4)	0 (0)	1 (2.2)	1 (2.2)	10 (22.2)	3 (15.8)	7 (15.6)	4 (8.9)

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

4.3.2. Effect of efflux pump inhibitors on initial attachment and/or biofilm formation by *Aeromonas* spp.

Following CCCP treatment at the time of inoculation (initial attachment), 100% (45/45) of isolates demonstrated decreased adhesion, while none of the isolates demonstrated increased adhesion (Fig. 4.1). Following NMP treatment, 11% (5/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 89% (40/45) of isolates (Fig. 4.2). Following PA β N treatment, 36% (16/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 64% (29/45) of isolates (Fig. 4.3). A similar trend of results was obtained for the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T), where lower levels of adherence were observed for the initial attachment assay. Treatment with EPIs in the initial attachment assay resulted in statistically significant altered adherence ($p = 0.001$).

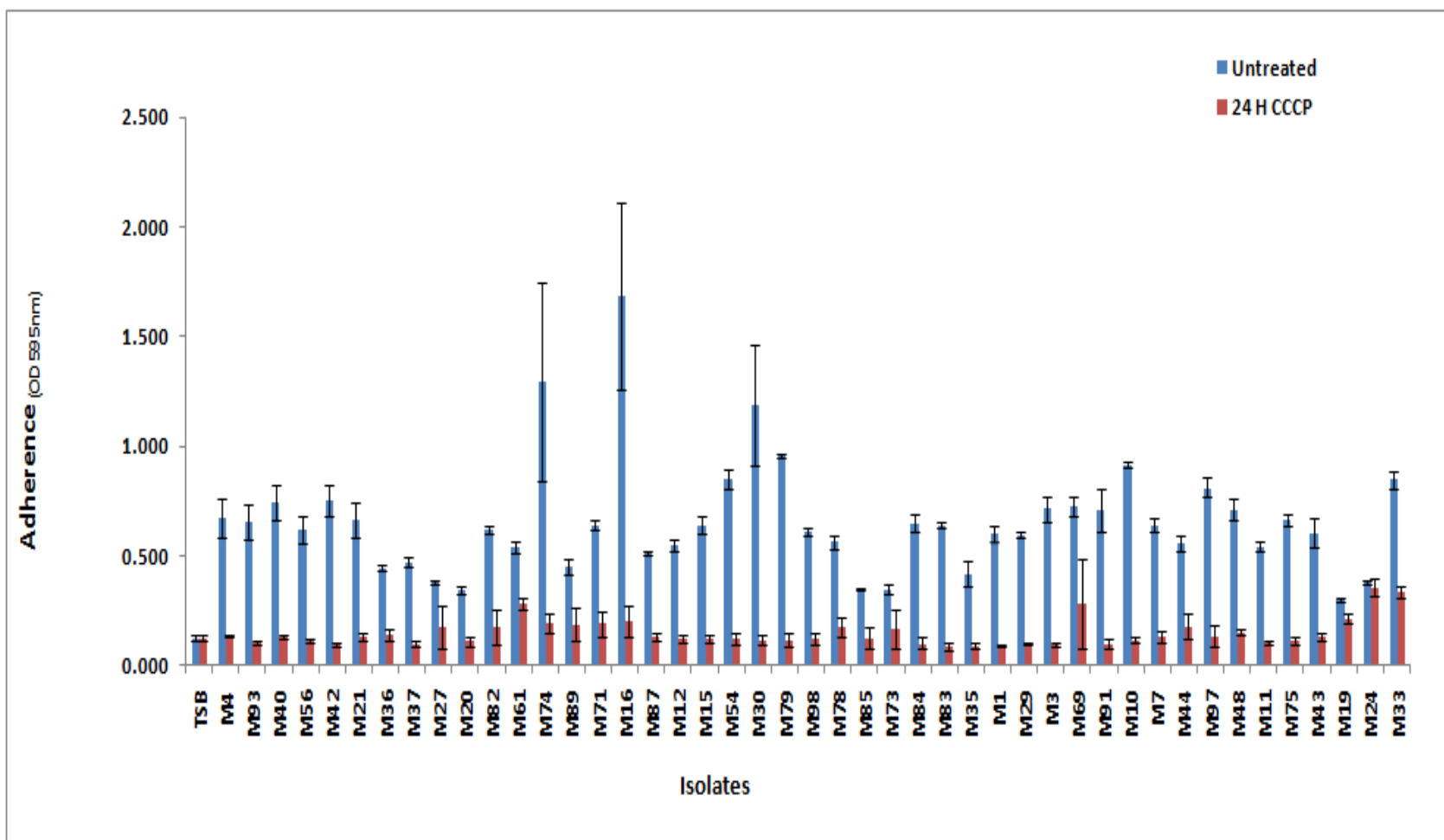


Figure 4.1. Effect of 20 μ g/ml carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

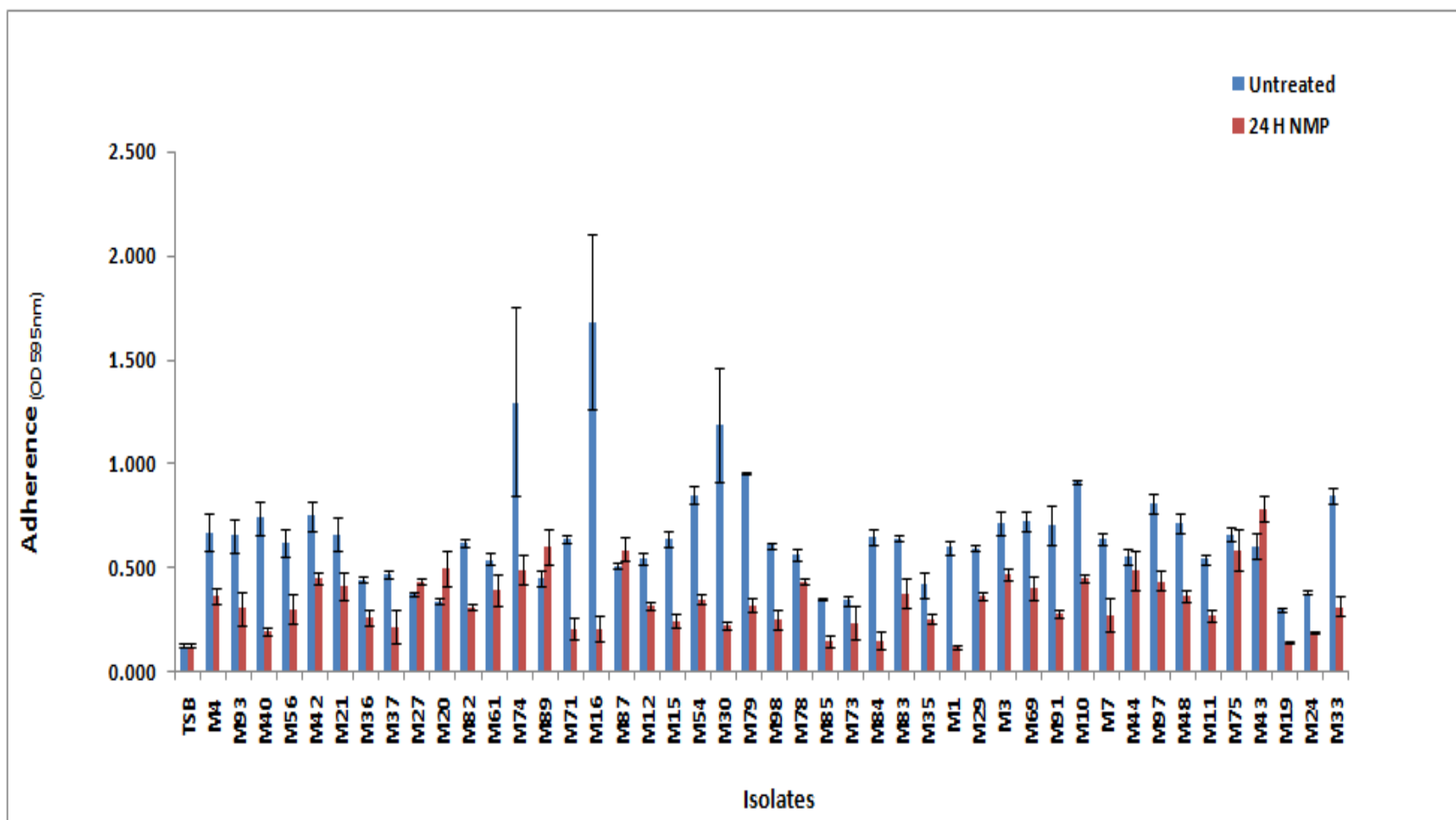


Figure 4.2. Effect of 20 µg/ml 1-(1-naphthylmethyl)-piperazine (NMP) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

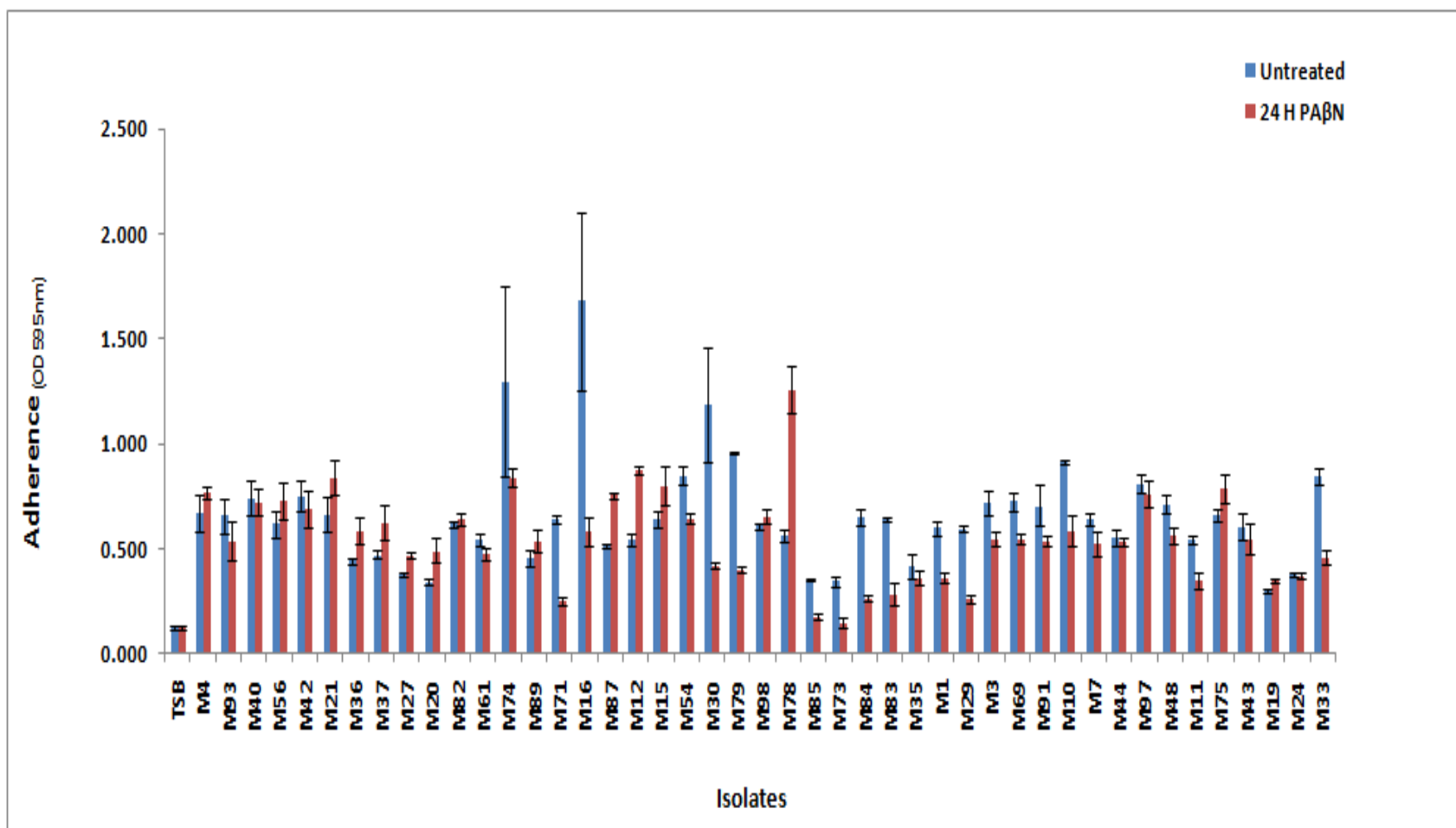


Figure 4.3. Effect of 20 µg/ml phenylalanine arginine β-naphthylamide (PAβN) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

On treatment of pre-formed biofilms with CCCP, 100% (45/45) of isolates demonstrated increased detachment, while none of the isolates demonstrated increased adhesion (Fig. 4.4). On treatment of pre-formed biofilms with NMP, 2% (1/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 98% (44/45) of isolates (Fig. 4.5). On treatment of pre-formed biofilms with PAβN, 20% (9/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 80% (36/45) of isolates (Fig. 4.6). A similar trend of results was obtained for the type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T), where increased detachment was observed in the pre-formed biofilm assay. Treatment with all EPIs in the pre-formed assay resulted in statistically significant altered adherence ($p = 0.001$).

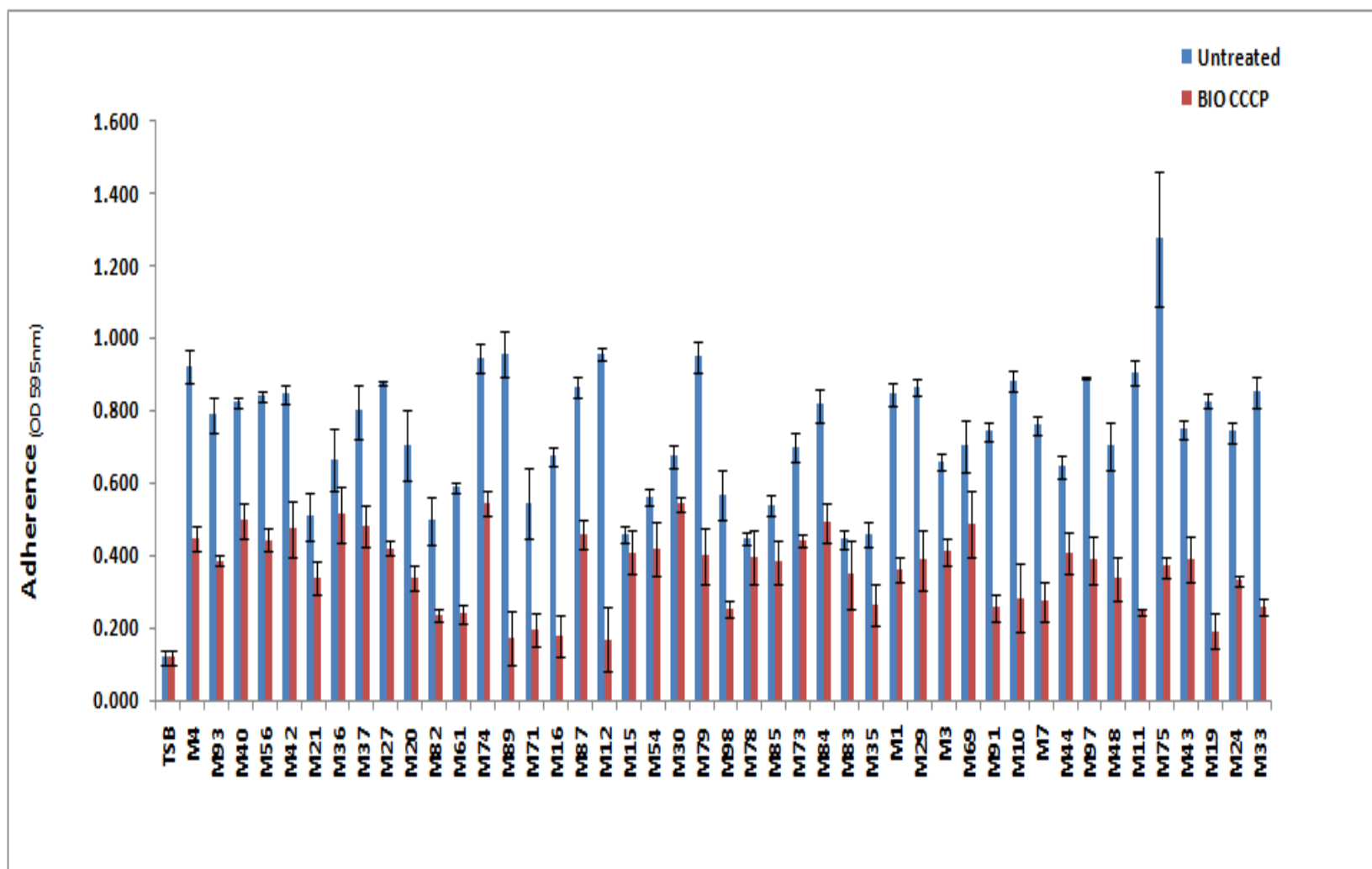


Figure 4.4. Effect of 20 µg/ml carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

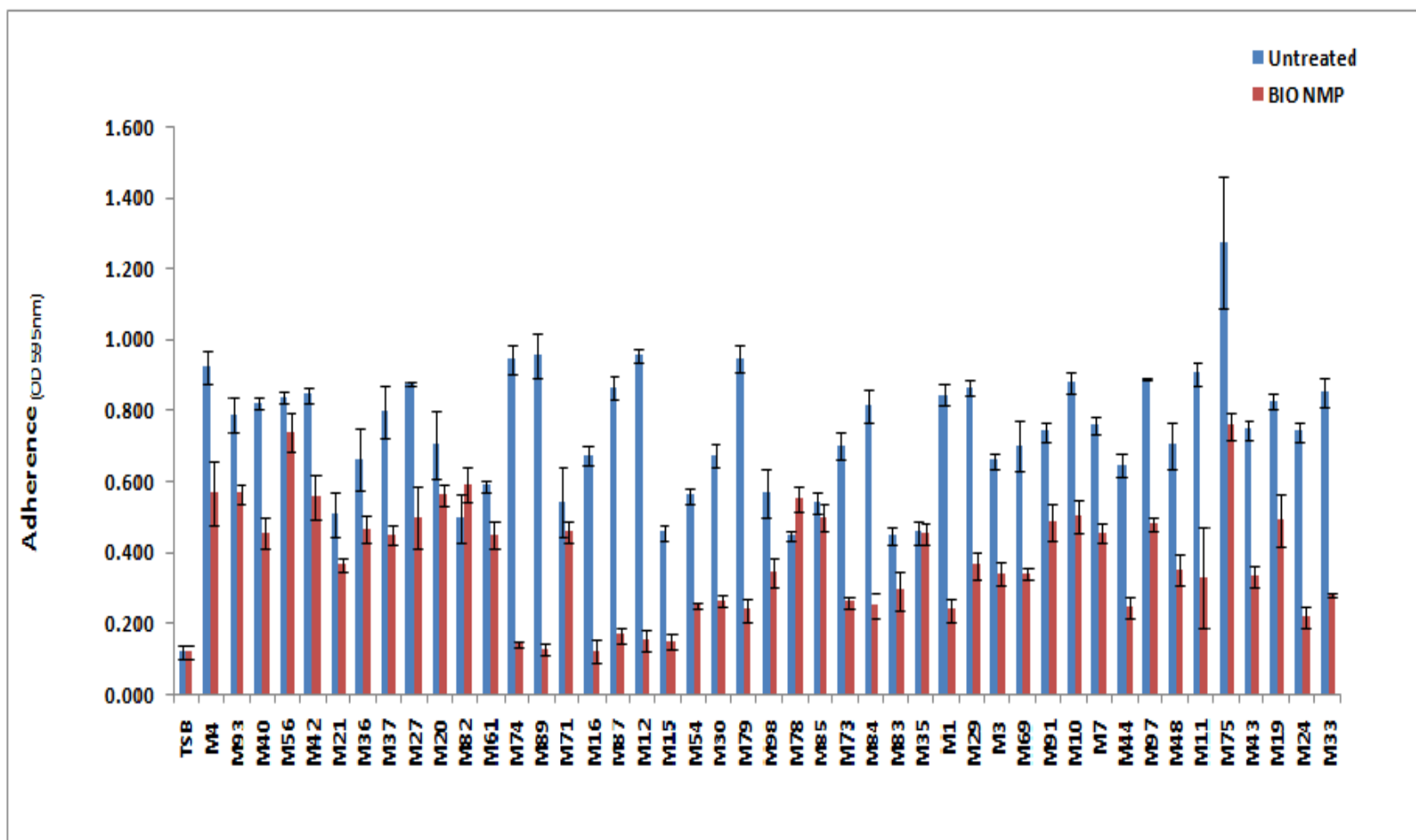


Figure 4.5. Effect of 20 µg/ml 1-(1-naphthylmethyl)-piperazine (NMP) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

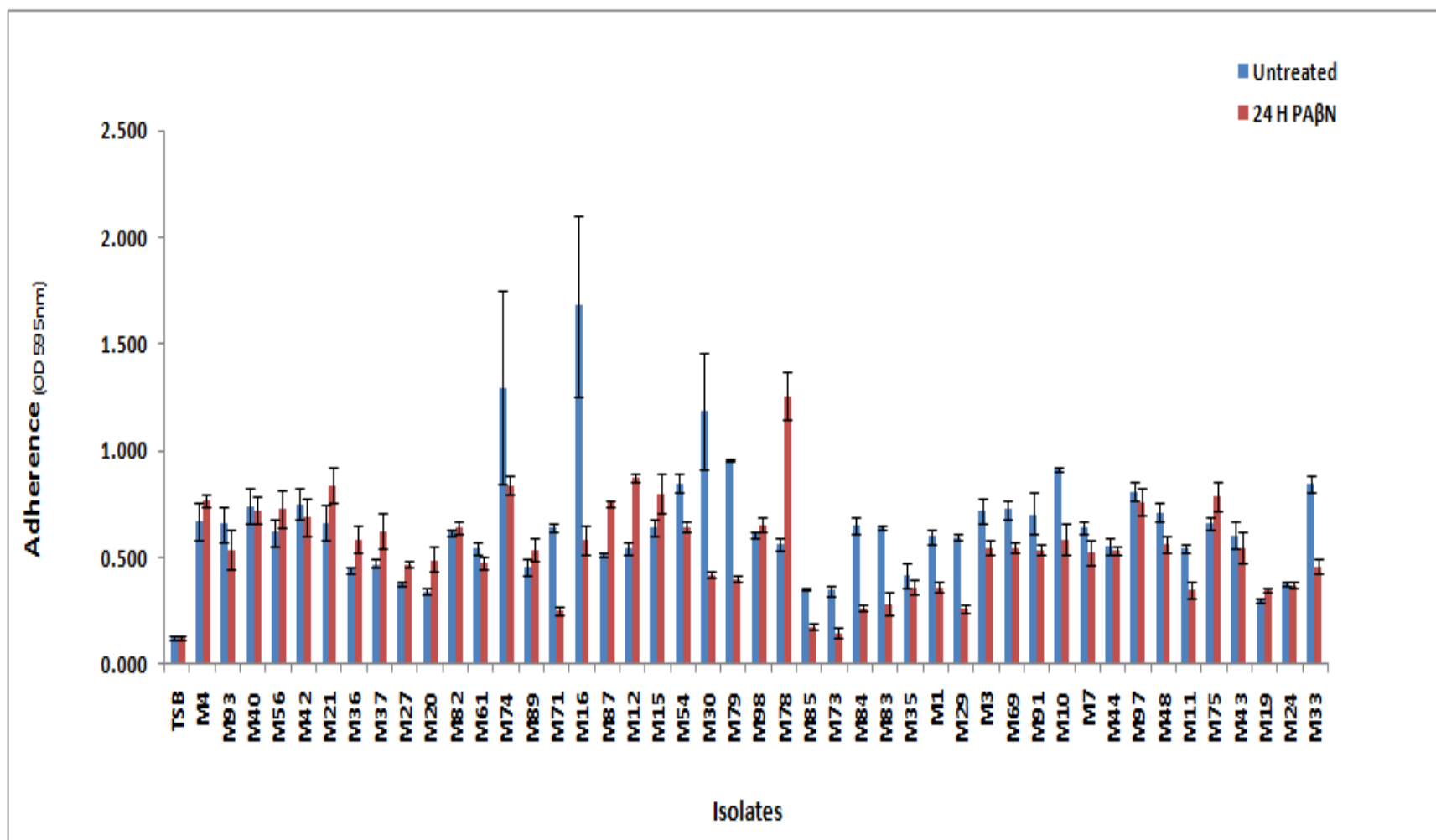


Figure 4.6. Effect of 20 µg/ml phenylalanine arginine β-naphthylamide (PAβN) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

Analysis of biofilm persistence data (Table 4.4) revealed that for 88.9% (40/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by CCCP exposure at the time of inoculation. For 6.7% (3/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by NMP exposure at the time of inoculation (Table 4.4). Finally, for 20% (9/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by PA β N exposure at the time of inoculation (Table 4.4).

Examination of biofilm persistence data (Table 4.4) revealed that for 20% (9/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by CCCP exposure. For 28.9% (13/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by NMP exposure (Table 4.4). Finally, for 4.5% (2/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was PA β N exposure destroyed by PA β N exposure (Table 4.4).

Table 4.4. Biofilm persistence in the presence of CCCP, NMP and PAβN exposure on initial attachment (24 H) and pre-formed biofilms (BIO) of *Aeromonas* spp. isolates

Isolates	% Biofilm persistence*					
	24 H CCCP	24H NMP	24H PAβN	BIO CCCP	BIO NMP	BIO PAβN
M1	-7	-2	49	34	17	77
M3	-5	58	72	54	41	92
M4	2	44	118	41	56	99
M7	1	29	76	24	53	57
M10	-1	41	58	22	51	76
M11	-5	35	54	16	27	78
M12	-1	46	177	6	4	1
M15	-1	24	131	86	9	164
M16	5	5	29	11	1	21
M19	50	9	128	10	53	47
M20	-7	170	168	38	76	108
M21	1	53	132	57	64	142
M24	90	27	97	34	16	72
M27	19	123	136	40	50	35
M29	-5	51	28	36	33	96
M30	-1	9	28	76	26	95
M33	29	26	46	19	22	63
M35	-12	44	79	43	98	169
M36	4	42	144	73	64	115
M37	-7	28	144	53	49	48
M40	1	12	97	54	48	62
M42	-5	52	90	49	60	72
M43	1	137	88	43	34	77
M44	12	84	95	55	24	81
M48	5	41	74	37	40	55
M54	-1	31	71	68	30	116
M56	-2	36	121	45	86	55
M61	37	65	85	26	71	72
M69	26	47	70	63	38	42
M71	13	16	25	19	80	11
M73	18	51	11	56	24	118
M74	6	31	61	51	3	26
M75	-2	85	123	22	55	73
M78	11	71	258	84	131	145
M79	-1	24	33	34	15	70
M82	10	38	105	31	125	62
M83	-7	49	31	70	53	77
M84	-5	4	26	53	19	114
M85	0	11	23	63	90	166
M87	1	120	162	46	7	55
M89	20	145	126	6	1	40
M91	-4	27	71	22	59	78
M93	-4	34	77	40	67	56
M97	1	46	93	35	47	75
M98	-1	26	109	30	50	84
ATCC 15468 ^T	6	56	78	45	44	77
ATCC 7966 ^T	3	34	99	28	56	90

*Biofilm persistence = (OD₅₉₅ x – OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100, where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

Table 4.5 summarises the overall effects of EPI exposures on initial attachment and biofilm detachment, respectively. CCCP was effective in inhibiting initial attachment as well as increasing detachment from pre-formed biofilms. NMP and PA β N were more effective in increasing detachment of pre-formed biofilms compared to inhibiting initial attachment.

Table 4.5. Summary table for the effect of EPIs on initial attachment and pre-formed biofilms of *Aeromonas* spp. isolates

Treatments	Initial attachment			Pre-formed biofilms		
	% Increase (number of isolates)	% Decrease (number of isolates)	% No effect (number of isolates)	% Increase (number of isolates)	% Decrease (number of isolates)	% No effect (number of isolates)
20 μg/ml CCCP	-	100 (45/45)	-	-	100 (45/45)	-
20 μg/ml NMP	11 (5/45)	89 (40/45)	-	2 (1/45)	98 (44/45)	-
20 μg/ml PAβN	36 (16/45)	64 (29/45)	-	20 (9/45)	80 (36/45)	-

4.4. Discussion

Since efflux pumps are highly expressed in bacterial biofilms and play a huge role in antimicrobial resistance in bacteria such as *Aeromonas* spp. isolates, it was therefore important to determine the prevalence and diversity of these efflux pumps in *Aeromonas* spp. and additionally, determine the substrate (antimicrobial agent) specificity of these efflux pumps. Majority of *Aeromonas* spp. isolates in the present study were more susceptible to tetracycline, norfloxacin, and azithromycin due to CCCP and NMP inhibition of the efflux pumps (Table 4.2). This efflux data suggests the presence of broad-substrate range efflux pumps and/or multiple efflux pumps, which are able to extrude different classes of antimicrobial agents. Piddock (2006)

reported that a single organism can possess multiple multi-drug efflux pumps and these may also transport different classes of antimicrobial agents, from different classes (Van Bambeke *et al.*, 2000).

Hernould *et al.* (2008) studied the multidrug efflux system in *Aeromonas* spp., i.e., AheABC efflux pump, belonging to the RND family which was able to extrude non-quinolone antimicrobials [erythromycin, tetracycline, trimethoprim, fusidic acid, and rifampicin] as well as quinolones and fluoroquinolones [nalidixic acid, oxolinic acid, norfloxacin, ofloxacin and ciprofloxacin]. In the present study, majority of isolates (Table 4.2) and type strains were more susceptible to tetracycline, norfloxacin, and azithromycin due to CCCP and NMP inhibition of the efflux pumps. This has also been reported by Hernould *et al.* (2008), and was rather disturbing in the case of norfloxacin, as quinolones are the drugs of choice for treatment of *Aeromonas* infections in humans and in treatment of bacterial fish diseases (Alcaide *et al.*, 2010). This may this may pose a threat in antimicrobial therapy if these bacteria have evolved ways to remove these drugs. Ramon-Garcia *et al.* (2006) observed that tetracycline efflux was inhibited with the use of CCCP in *Mycobacterium fortuitum*. This was rather not surprising as tetracycline is primarily extruded PMF-driven efflux pumps, and thus tetracycline efflux is usually abolished in presence of a PMF inhibitor such as CCCP. Bean and Wareham (2008) observed that 11.3% of *Acinetobacter* spp. isolates showed an increase in susceptibility to tetracycline in the presence of NMP, which was lower than that obtained in the present study for *Aeromonas* spp. isolates. PA β N and NMP have been shown to cause an increase in susceptibility to erythromycin, norfloxacin and tetracycline in other bacteria like *Campylobacter jejuni* spp. (Hannula and Hanninen, 2008), so it is not surprising the same scenario was observed for *Aeromonas* spp.

PA β N and NMP both inhibit the RND pumps that are mostly abundant in Gram-negative bacteria including *V. cholerae*, *F. johnsoniae*, *E. coli* and other important clinical isolates (Bina *et al.*, 2009; Hirakata *et al.*, 2009). CCCP, NMP and PA β N all produced significant reduction of biofilm formation by *Aeromonas* spp. isolates and type strains (both initial attachment and mature biofilm), although CCCP was most effective for all (100%) of the isolates. This may be due to the varied modes of action of these EPIs. For *P. aeruginosa*, CCCP at the beginning of biofilm growth resulted in reduced growth compared to the untreated cells (Ikomomidis *et al.*, 2008). In the present study, NMP had a greater effect on biofilm formation causing a significant reduction in 98% of isolates compared to PA β N. This is similar to what was obtained by Kvist *et al.* (2008), where NMP (20 μ g/ml) was shown to have a significant effect on biofilm formation by *S. aureus* and *P. putida* and resulted in 50% reduction, whereas PA β N had no significant effect on biofilm formation by these two organisms. In summary, in the present study, CCCP was most effective, followed by NMP then lastly PA β N, for both the initial attachment and pre-formed biofilm assays (Table 4.5).

An efflux phenotype was observed for *Aeromonas* spp. isolates, eliminating diverse classes of antimicrobial agents, thus suggesting the presence of broad-substrate range efflux pumps and/or multiple efflux pumps. Since microorganisms have multiple efflux pump systems, EPIs with a broad spectrum of activity need to be used so they can be effective against diverse, multiple efflux pump systems present in different microbial pathogens. This is one of the few studies, on the analysis of multidrug efflux pumps in *Aeromonas* spp. isolates, since there have been limited reports of efflux in *Aeromonas* spp. (Hernould *et al.*, 2008). Further study is required to explore the type of efflux pumps, substrate profile, and regulation mechanisms of these pumps in these *Aeromonas* spp. isolates.

Based on present data, EPIs do inhibit the different efflux pump systems used by *Aeromonas* spp. isolates and thus could prevent elimination of antimicrobial agents within these cells, and thus reduce multidrug resistance. These EPIs also can be used as potential inhibitors of biofilm formation by *Aeromonas* spp. isolates, as they caused reduction of adherence and increased detachment of majority of the isolates. Although more work still needs to be done with these inhibitors and their mechanism, efflux pump inhibition represents a potential control strategy to limit aeromonad biofilms and may not only prevent disease outbreaks but also increase effectiveness of existing therapeutic agents.

CHAPTER FIVE

Biofilm Control Strategies: Effect of Lytic enzymes and Quorum-sensing inhibition on biofilm formation by *Aeromonas* spp.

5.1. Introduction

The EPS is the outermost layer of the biofilm and consists of a wide variety of polysaccharides, proteins, glycoproteins, glycolipids and often, large amounts of extracellular DNA (eDNA) (Kaplan, 2009). The eDNA is not mainly considered to be a remnant of lysed cells but a major structural component of the EPS (Bockelmann *et al.*, 2006). Previous studies show that eDNA may function as a cell-to-surface adhesin and/or cell-to-cell adhesin in the initial phase of biofilm formation in the Gram-negative bacterium, *P. aeruginosa* (Molin and Tolken-Nielsen, 2003; Steinberger and Holden, 2005). Steinberg and Holden (2005) reported that extracellular DNA was present in unsaturated *P. aeruginosa* biofilm and was maximally 50% more abundant than cellular DNA and that it played a role in initial attachment and early biofilm formation. Das *et al.* (2010) also reported that eDNA released by autolysins, acts as an adhesive and strengthens the biofilm, and found that the removal of eDNA from Gram-positive bacteria reduced initial adhesion and bacterial aggregation of cells to surfaces. Inhibition of biofilm formation was observed for *Bacillus cereus*, *Bdellovibrio bacteriovorus*, *Comamonas denitrificans*, *Enterococcus faecalis*, non-typeable *H. influenzae*, *P. aeruginosa*, *Shewanella oneidensis*, *S. aureus*, *Staph. epidermidis*, and *Streptococcus intermedius* when DNase I was added during the initial attachment assay (Kaplan, 2009).

eDNA has also been shown to be an important component of the extracellular matrix of *Neisseria meningitidis* biofilms, as it was shown to stabilize biofilm structures in the late stages

of biofilm formation by *N. meningitidis* (Lappann *et al.*, 2010). Kirkpatrick and Viollier (2010) reported the role of eDNA in *Caulobacter crescentus* biofilm dispersal. Detachment from pre-formed biofilms following DNase I treatment was observed for *A. baumannii*, *A. actinomycetemcomitans*, *B. bacteriovorus*, *C. jejuni*, *E. coli*, non-typeable *H. influenzae*, *K. pneumoniae*, *S. oneidensis*, *Staph. haemolyticus*, *Strep. mutans*, *Strep. pneumoniae*, and *Strep. pyogenes* (Kaplan, 2009). However, on addition of DNase to pre-formed biofilm, some biofilms are resistant or partially resistant to detachment, this has been observed with biofilms of *Comamonas denitrificans*, *E. faecalis*, *P. aeruginosa*, *Staph epidermidis*, and *Strep. intermedius*. This might be explained by cells in mature biofilms being held together by adhesins other than eDNA, or eDNA may be stabilized and resistant to lysis by DNase I (Kaplan, 2009).

DNase can also increase the bactericidal activity of different antimicrobial agents by sensitizing bacterial cells to antimicrobial agents, making them more susceptible to antimicrobial agents. This was observed with *P. aeruginosa* and *S. aureus* where the effectiveness of levofloxacin, rifampin, benzalkonium chloride, cetylpyridinium chloride, bleach, and chlorhexidine gluconate was increased when these bacterial cells were pre-exposed to the DNase prior to the treatment of these bactericidal agents (Kaplan, 2009). The mechanism by which the DNase sensitize the cells is not known, however, it is predicted that DNA acts as a barrier for the diffusion of bactericidal agents, allowing for the entry of these antimicrobial agents to act on bacterial cells and eliminates them (Kaplan, 2009). Another possibility is that DNA that is surface-attached restricts the entry of the antimicrobial agents, so by eliminating this surface-exposed DNA, the antimicrobial agents gains entry to the cell and act on their targets site (Kaplan, 2009).

Quorum sensing (QS) is a regulatory mechanism that allows surface-associated cells to coordinate their communal behavior through the production, release and detection of autoinducing signals. QS regulates functions like conjugation, secretion of virulence factors, antibiotic production and biofilm formation (Bi *et al.*, 2007). QS systems are important in regulating bacterial attachment and for biofilm maturation (Xiong and Liu, 2010). It has been shown in detail that QS regulates biofilm formation in most bacteria including *Aeromonas* spp (Bi *et al.*, 2007; Garde *et al.*, 2010; Lynch *et al.*, 2002; Ponnusamy *et al.*, 2009). Due to the critical role of QS in biofilm formation, three potential QS targets have been identified in Gram-negative bacteria: the signal generator, the signal molecule and the signal receptor (Rasmussen & Givskov, 2006), with quorum sensing inhibitors (QSIs) presenting as potential therapeutic options for the control of biofilms (Francolini and Donelli, 2010). QS may be interrupted in many ways and one of these is to inhibit the signal molecule from binding to the receptor via analogues of the signal molecules. The halogenated furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone and its synthetic derivatives are potent inhibitors of Gram-negative QS, which disrupt AHL- as well as autoinducer 2 (AI-2)-mediated signalling in Gram-negative bacteria, without affecting bacterial growth (Hentzer and Givskov, 2003). Furanones mimic the AHL signal and bind LasR, acting as competitive inhibitors of AHL binding (Hentzer *et al.*, 2002). Furanones can inhibit biofilm formation while not affecting cell growth, and have been shown to reduce *P. aeruginosa* virulence, increase sensitivity to antibiotics and cause increased biofilm detachment (Hentzer *et al.*, 2002). They also disrupt the AI-2 biosynthetic pathway by inactivating LuxS, an enzyme essential for AI-2 production (Xiong and Liu, 2010).

Since AHL signals are highly homologous, so are their synthases. S-adenosylmethionine (SAM) is a critical intermediate used as the amino donor for generation of the homoserine lactone ring moiety. Traditional inhibitors of SAM-utilizing enzymes such as S-adenosylhomocysteine (SAH), sinefungin, and other intermediate mimics are potent inhibitors of AHL synthesis acting on the synthase responsible for producing C4-HSL, the secondary messenger in *P. aeruginosa* (Musk and Hergenrother, 2006). Autoinducer-2 (AI-2) is another SAM-derived QS signal molecule, whose proximate precursor is SAH. S-adenosylhomocysteine is the metabolite that is generated as a by-product of all SAM-utilizing methylation reactions (Musk and Hergenrother, 2006).

trans-cinnamaldehyde (3-phenyl-2-propenal, TC) is a natural flavouring substance and a potent aromatic compound which has a broad spectrum of antimicrobial activity (Nuryastuti *et al.*, 2009; Amalaradjou *et al.*, 2010). Cinnamaldehyde causes inhibition of the PMF, respiratory chain, electron transfer, and substrate oxidation, resulting in uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, proteins, lipids, and polysaccharides. It results in extensive leakage from bacterial cells or the exit of critical molecules and ions leads cell death (Nuryastuti *et al.*, 2009). Cinnamaldehyde has been observed to inhibit biofilm formation of *E. coli* and *P. aeruginosa* (Niu and Gilbert, 2004), as well as *Burkholderia multivorans* and *B. cenocepacia* (Brackmann *et al.*, 2009). It has also been identified as an inhibitor of both the AHL- and AI-2-based QS systems in *V. harveyi* (Niu *et al.*, 2006).

Vanillin is the major component of vanilla beans, also a principal flavoring compound used in numerous foods (20 – 26 mM concentrations), such as ice cream, chocolate, and confectionary products (Ponnusamy *et al.*, 2009). Vanillin has been shown to have inhibitory

effects against many bacteria including *B. subtilis*, *S. enteritidis*, and *E. coli* (Katayama and Nagai, 1960). Ponnusamy *et al.* (2009) also described the potential use of vanillin as a QSI for *A. hydrophila* biofilm formation, where it had an effect on the adherence and detachment of *A. hydrophila*.

Since eDNA and quorum sensing play a huge role in biofilm formation by many bacteria, including *Aeromonas* spp., it was, therefore, important to evaluate the effect of different biofilm inhibitors on biofilm formation by *Aeromonas* spp. isolates. Therefore, the current study aimed at investigating the effect of DNase I, a lytic enzyme; (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, S-adenosylhomocysteine, cinnamaldehyde, and vanillin on initial attachment and detachment of pre-formed biofilms of *Aeromonas* spp. isolates, using modified microtitre plate assays. This was to determine the point at which lytic enzymes and QSIs are effective for biofilm control and was critical as it may facilitate effective removal of these biofilms.

5.2. Materials and Methods

5.2.1. Bacterial isolates and growth conditions

Forty-five presumptive *Aeromonas* spp. isolates (Chapter 4, Table 4.1) and 2 type strains (*A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T) were used for the detection of AHL signalling molecules, for the determination of DNase activity and for investigating the effect of DNase I and QSIs on initial attachment and pre-formed biofilms. Bacterial colonies were grown on tryptic soy broth (TSB) agar plates [Tryptic soy broth (TSB); Merck Chemicals, Gauteng, South Africa] supplemented with 10% bacteriological agar (Bacteriological agar; Merck Chemicals, Gauteng, South Africa).

5.2.2. Investigation of DNase production

DNase production was determined using DNase Agar supplemented with 0.01% bromophenol blue (Merck, SA). Plates were inoculated with five microlitres of cell suspension, standardised equivalent to a 0.5 McFarland standard, in triplicate, and incubated for 4 days at 30 °C. DNase-positive *Streptococcus pyogenes* ATCC 19615 was used as a positive control. Plates were flooded with 0.1% of a 1 M HCl solution, and development or appearance of either a pink halo or zones of clearance around a colony were taken as a positive result (Jeffries *et al.*, 1957). Colony diameters as well as zone diameters were measured and the relative DNase activity (RDA) was determined using the formula: $RDA = \frac{\text{halo diameter (zone)} - \text{bacterial growth diameter}}{\text{bacterial growth diameter}}$ (Zacaria *et al.*, 2010).

5.2.3. Effect of DNase I on initial attachment and/or biofilm formation by *Aeromonas* spp. isolates

DNase I was added at the time of inoculation and to pre-formed biofilms to determine if *Aeromonas* spp. isolates were using eDNA for initial attachment (as an adhesin) or to maintain their biofilm structure in mature biofilms. Sixteen hour TSB cultures were used to prepare cell suspensions, which were standardised equivalent to a 0.5 McFarland standard (Basson *et al.*, 2008). For initial attachment assays, bovine DNase I (Sigma-Aldrich, St Louis, MO, USA) 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 hours at 30 °C with agitation (Basson *et al.*, 2008).

For pre-formed biofilm detachment assays, 24 hour biofilm were established following addition of 90 µl TSB and 10 µl of standardized cell suspension to microtitre plate wells, which

were incubated at 30 °C for 24 hours. After a 24 hour incubation period, microtitre plates were washed three times with sterile deionised water, and allowed to air-dry. Following the addition of fresh 90 µl TSB and DNase I (to a final concentration of 1 mg/ml), microtitre plates were incubated for a further 24 hours with agitation at 30 °C. For both assays, the negative control contained only TSB, while positive controls contained respective cell suspensions without DNase I added. Staining and determination of OD values (OD_{595nm}) was done as described previously in Chapter 2 (Section 2.2.3), according to Basson *et al.* (2008). These were compared with the OD_{595nm} of the control wells without DNase I to determine the effect of DNase I on biofilm formation.

Biofilm persistence in the presence of DNase I was calculated using the equation or formula: percent of biofilm persistence = (OD₅₉₅ x - OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100%, where x corresponds to the DNase I enzyme (Tre-Hardy *et al.*, 2008).

5.2.4. Identification N-acyl homoserine lactone (AHL) quorum sensing signal production by *Aeromonas* spp. isolates using biosensors

In order to identify N-acyl homoserine lactones (AHLs) production by *Aeromonas* spp. isolates, 24 hour TSA cultures were cross-streaked against the 24 hour *C. violaceum* CV026 biosensor on LB agar plates and *A. tumefaciens* A136 biosensor on LB agar plates containing 50 µl of 20 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside [X-gal] (Swift *et al.*, 1997). Plates were incubated at 30 °C for 24 hours. Positive assays were judged as the production of the purple color by the *C. violaceum* CV026 reporter and the production of blue color by *A. tumefaciens* A136 reporter due X-gal hydrolysis (Swift *et al.*, 1997). *C. violaceum* ATCC 31532 (C6-HSL

overproducer) was used as a positive control for the CV026 biosensor assay for AHL detection, while *A. tumefaciens* KYC6 (3-oxo-C8 HSL overproducer) was used as a positive control for the A136 biosensor assay for AHL detection.

5.2.5. Effect of quorum-sensing inhibitors on initial attachment and/or biofilm biofilm formation by *Aeromonas* spp. isolates

QSIs [cinnamaldehyde, (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, S-adenosylhomocysteine (SAHC) and vanillin; Sigma-Aldrich] were used to determine their effect on initial attachment and pre-formed biofilms using modified microtiter assays. Sixteen hour-old cultures were used to prepare cell suspensions, which were standardised equivalent to a 0.5 McFarland standard (Basson *et al.*, 2008). The first assay was to investigate the effect of QSIs on initial attachment of cells. QSIs at a concentration of 5 µg/ml cinnamaldehyde, 5 µg/ml 2(5H)-furanone, 5 µg/ml S-adenosylhomocysteine or 5 µg/ml vanillin, and were added to 90 µl TSB and 10 µl of cell suspension and incubated for 24 hours at 30 °C with agitation.

For the second assay, pre-formed 24 hour biofilms were exposed to QSIs at the respective concentrations, in TSB (90 µl) and incubated for a further 24 hours with agitation at 30 °C. After a 24 h incubation period, microtiter plates were washed three times with sterile deionised water, and allowed to air-dry. The negative control contained broth only and the positive control contained the respective cell suspension only without quorum sensing inhibitors added. Staining and determination of OD values (OD_{595nm}) was done as described previously in Chapter 2 (Section 2.2.3), according to Basson *et al.* (2008). These were compared with the OD_{595nm} of the control wells without QSIs to determine the effect of these QSIs on biofilm formation.

Biofilm persistence in the presence of QSIs was calculated using the equation or formula:
percent of biofilm persistence = $(OD_{595} \text{ x} - OD_{595} \text{ negative control}) / (OD_{595} \text{ positive control} - OD_{595} \text{ negative control}) \times 100\%$, where x corresponds to the QSI (Tre-Hardy *et al.*, 2008).

5.2.6. Statistical analyses

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 $p < 0.05$.

5.3. Results

5.3.1. Investigation of DNase production

Zones of clearing were observed for 97,7% (44/45) of isolates and type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, which were DNase-positive, with zone diameters ranging from 9.7 – 21.7 mm and the relative caseinolytic activity (RDA) ranged from 0 – 1.3 (Table A2). There was also a positive correlation the fish host/origin of isolates and relative DNase activity ($r = 0.225$, $p = 0.0108$). A strong positive correlation was also observed between DNase activity and autoaggregation [Chapter 2, Section 2.3.5] ($r = 0.207$, $p = 0.0399$). A zone of clearing (20 mm) was also observed for the DNase-positive control, *Streptococcus pyogenes* ATCC 19615.

5.3.2. Effect of DNase I on initial attachment and/or biofilm formation by *Aeromonas* spp. isolates

Following DNase I treatment, 47% (21/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 53% (24/45) of isolates in the initial attachment assay (Fig. 5.1). Similar results were obtained for both type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, where lower levels of adherence were observed in the initial attachment assay. Although DNase I treatment at the time of inoculation ($p = 0.223$) resulted in altered adherence of the study isolates, these were not statistically significant. No statistically significant correlations were evident between DNase agar zone diameter, untreated adhesion or adhesion following attachment and biofilm treatments.

On treatment of pre-formed biofilms with DNase I, 40% (18/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 60% (27/45) of isolates (Fig. 5.2). Similar results were obtained for both type strains *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, where increased detachment was observed in pre-formed biofilm assay. Although DNase I treatment of pre-formed *Aeromonas* spp. biofilms resulted in altered adherence of the study isolates, these were not statistically significant ($p = 0.071$). No statistically significant correlations were evident between DNase agar zone diameter, untreated adhesion or adhesion following attachment and biofilm treatments.

Based on biofilm persistence data (Table 5.1), for 4.5% (2/45) of the isolates up to $\geq 75\%$ of the biofilm was destroyed by DNase I added at the time of inoculation. For the pre-formed biofilms, $\geq 75\%$ of the pre-formed biofilm was destroyed by DNase I for 2.2% (1/45) of the isolates (Table 5.1).

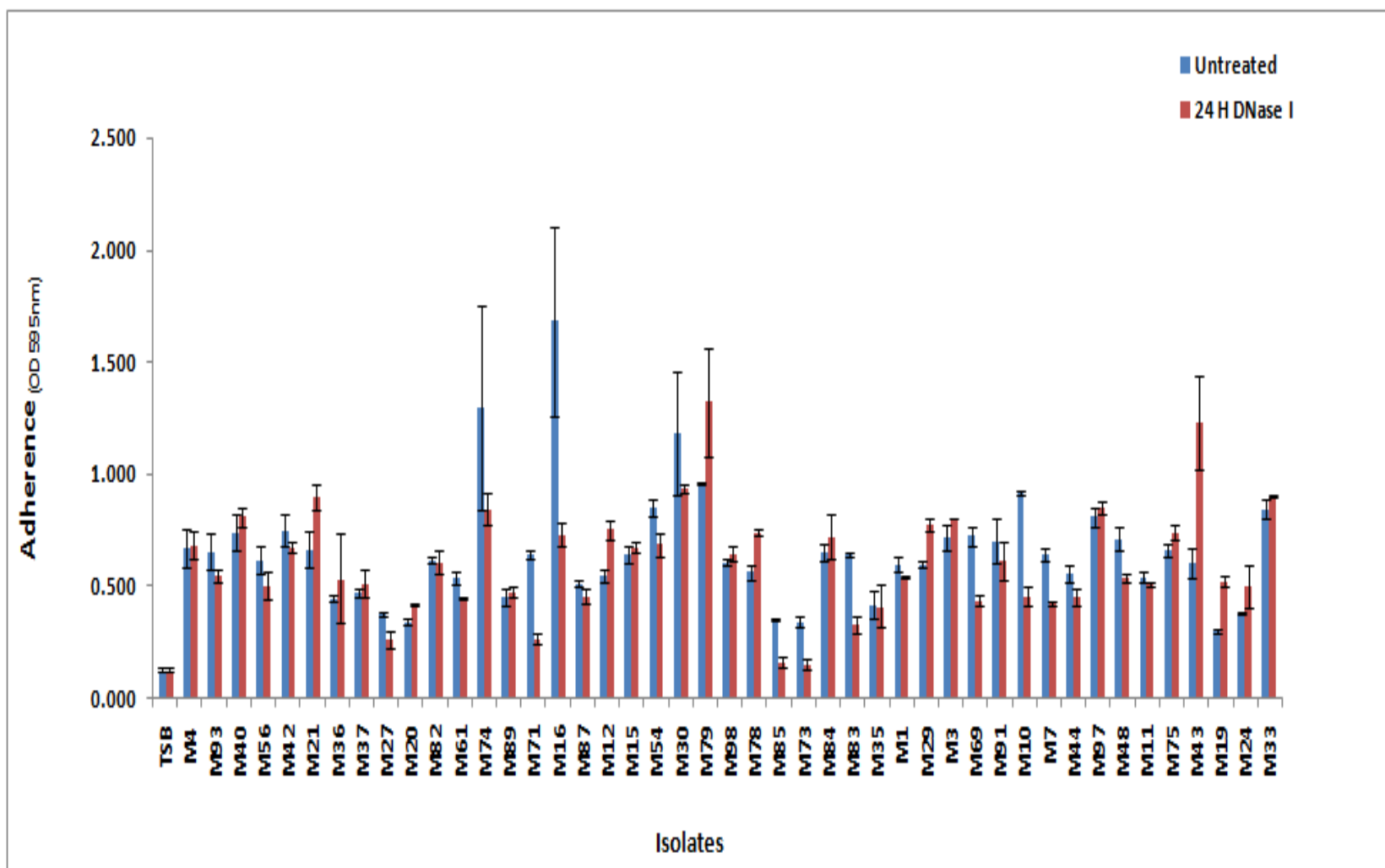


Figure 5.1. Effect of 1 mg/ml DNase I on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

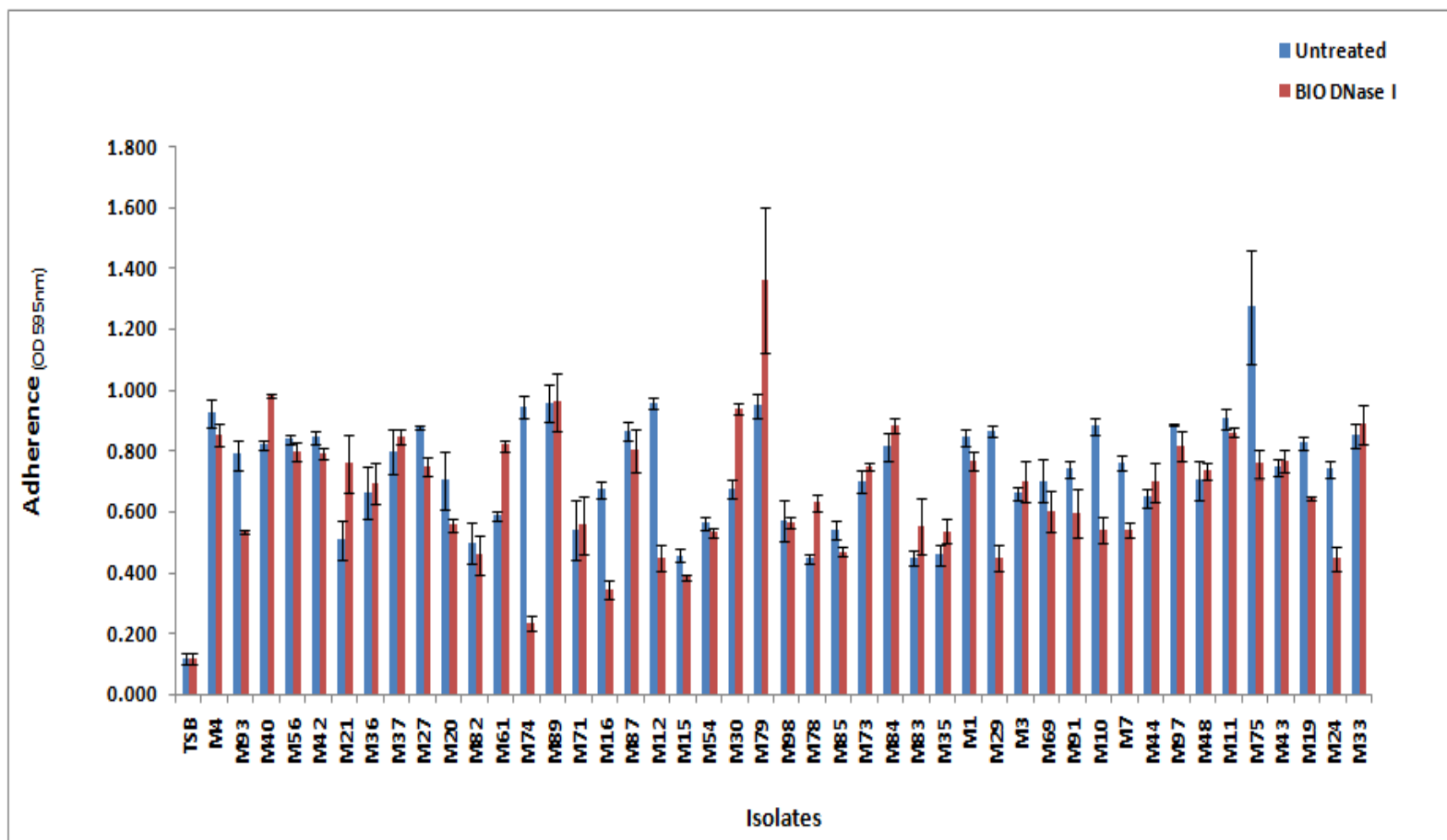


Figure 5.2. Effect of 1 mg/ml DNase I on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

Table 5.1. Biofilm persistence in the presence of DNase I on initial attachment (24 H) and pre-formed biofilms (BIO) of *Aeromonas* spp. isolates

Isolates	% Biofilm persistence*	
	24 H DNase I	BIO DNase I
M1	88	89
M3	115	107
M4	102	91
M10	42	56
M11	92	94
M12	149	40
M15	107	79
M16	39	41
M19	230	74
M20	135	75
M21	143	164
M24	147	53
M27	54	83
M29	138	44
M30	77	148
M33	108	105
M35	97	123
M36	128	105
M37	112	107
M40	111	123
M42	88	93
M43	230	103
M44	77	109
M48	70	105
M54	78	93
M56	76	94
M61	77	149
M69	52	83
M7	58	66
M71	27	104
M73	12	108
M74	62	14
M75	115	56
M78	141	156
M79	144	150
M82	98	89
M83	40	133
M84	114	110
M85	18	83
M87	86	91
M89	107	100
M91	84	77
M93	79	62
M97	106	91
M98	107	99
<i>A. caviae</i> ATCC 15468 ^T	98	109
<i>A. hydrophila</i> ATCC 7966 ^T	86	96

*Biofilm persistence = (OD₅₉₅ x - OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100%, where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

5.3.3. Identification N-acyl homoserine lactone (AHL) quorum sensing signal production by *Aeromonas* spp. isolates using biosensors

The *C. violaceum* CV026 biosensor detects short and medium AHLs (C6, C6-3-oxo C8, C8-3-oxo), and produces purple violacein pigment. *C. violaceum* CV026 reporter responded to 33% (33/99) of isolates by the production of violacein. *Agrobacterium tumefaciens* A136 detects a broad range of AHLs (all 3-oxo, C6, C8, C10, C12, C14, C6-3-hydroxy, C8-3-hydroxy and C10-3-hydroxy) and utilizes X-gal hydrolysis to produce a blue color. In the present study, the *A. tumefaciens* A136 reporter responded to all isolates (100%) producing a signal (blue colour) in the presence of X-gal. *A. tumefaciens* A136 and *C. violaceum* CV026 reporter responded to both the type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T.

5.2.4. Effect of quorum-sensing inhibitors on initial attachment and/or biofilm biofilm formation by *Aeromonas* spp. isolates

Following cinnamaldehyde treatment at the time of inoculation, 11% (5/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 89% (40/45) of isolates (Fig. 5.3). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed lower levels of adherence during the initial attachment assay. Treatment at the time of inoculation resulted in statistically significant altered adherence ($p = 0.001$).

Following furanone treatment, 16% (7/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 84% (38/45) of isolates in the initial attachment assay (Fig. 5.4). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed lower levels of adherence during the initial attachment assay. Treatment at the time of inoculation resulted in statistically significant altered adherence ($p = 0.001$).

Following SAHC treatment at the time of inoculation, 9% (4/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 87% (39/45) of isolates (Fig. 5.5). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed lower levels of adherence during the initial attachment assay. Treatment resulted in statistically significant altered adherence ($p = 0.001$).

Following vanillin treatment at the time of inoculation, 22% (10/45) of isolates demonstrated increased adhesion while decreased adhesion was observed for 78% (35/45) of isolates (Fig. 5.6). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed lower levels of adherence during the initial attachment assay. Treatment at the time of inoculation resulted in statistically significant altered adherence ($p = 0.001$). In the initial attachment assay, treatments with all four compounds resulted in statistically significant alterations in adhesion ($p < 0.05$).

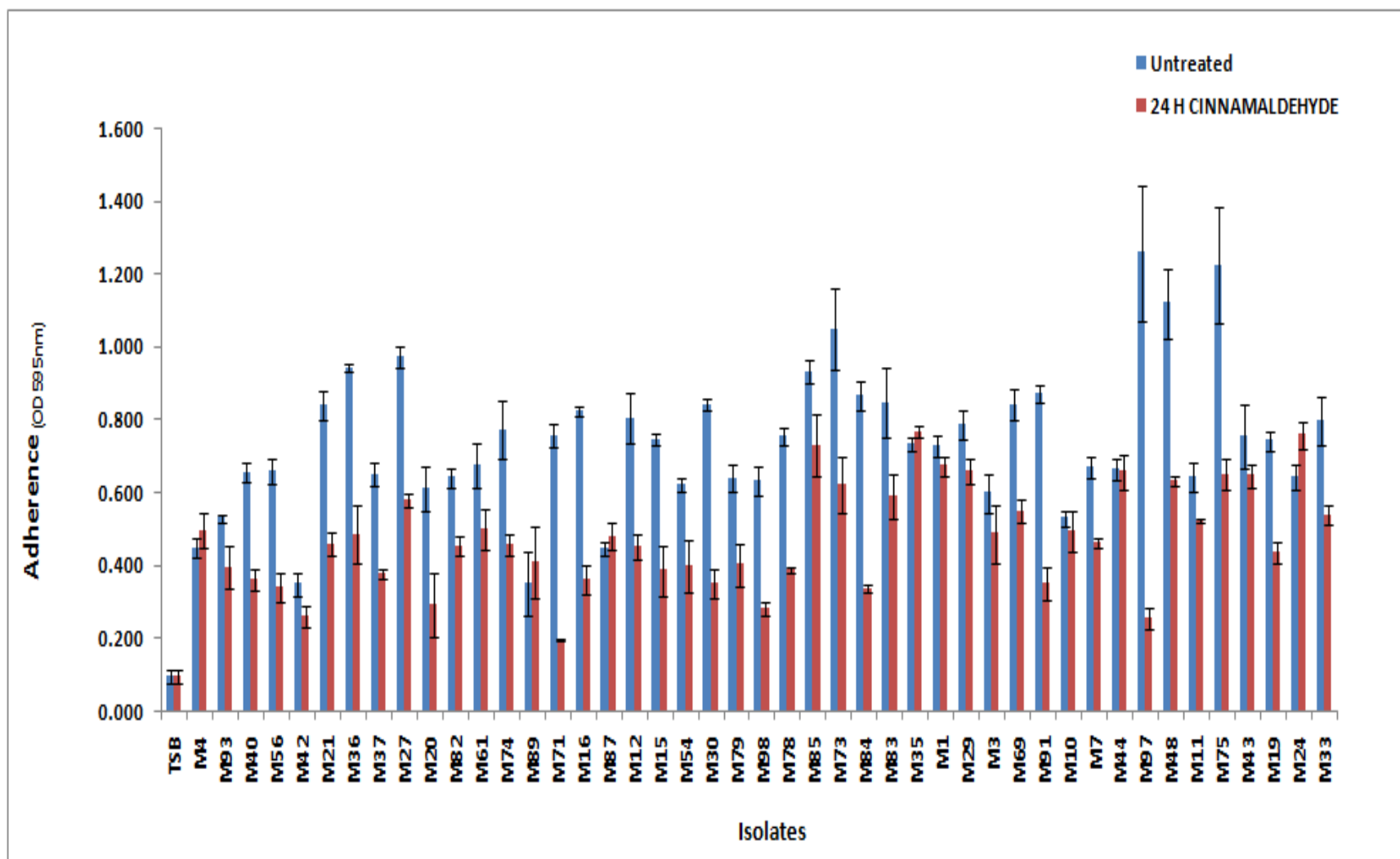


Figure 5.3. Effect of 5 µg/ml cinnamaldehyde on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

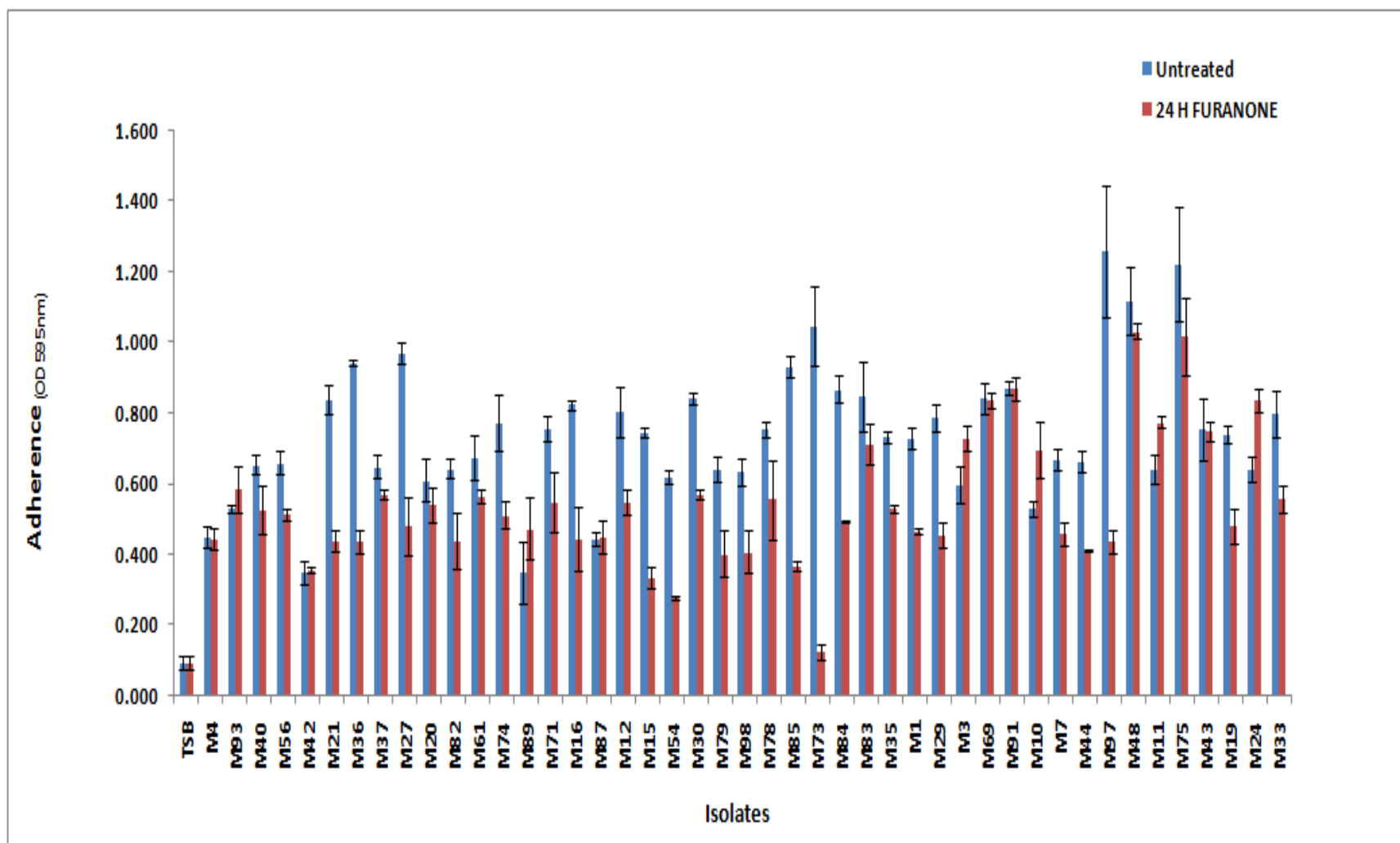


Figure 5.4. Effect of 5 µg/ml 2(5H)-furanone on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

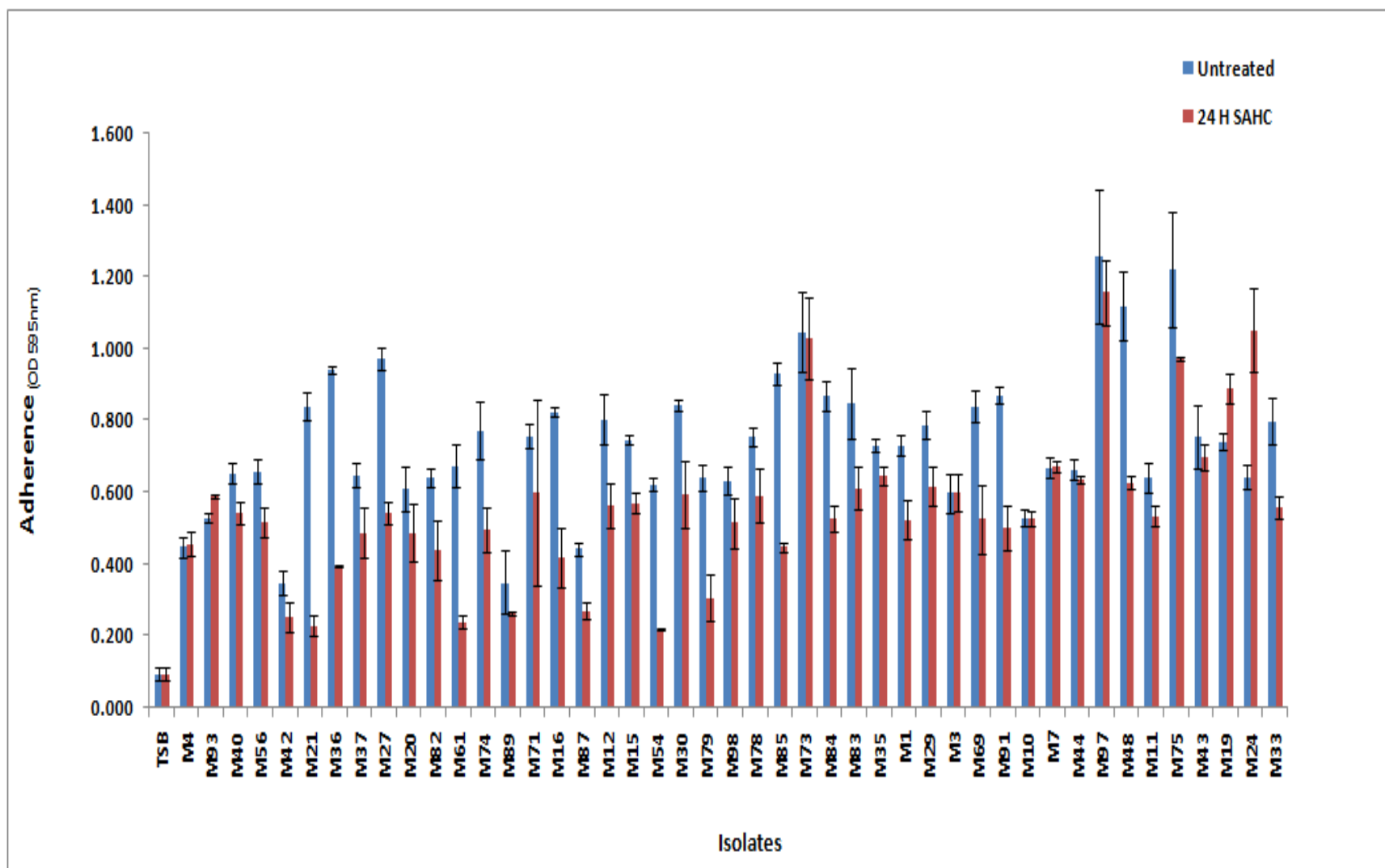


Figure 5.5. Effect of 5 μ g/ml S-adenosylhomocysteine (SAHC) on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

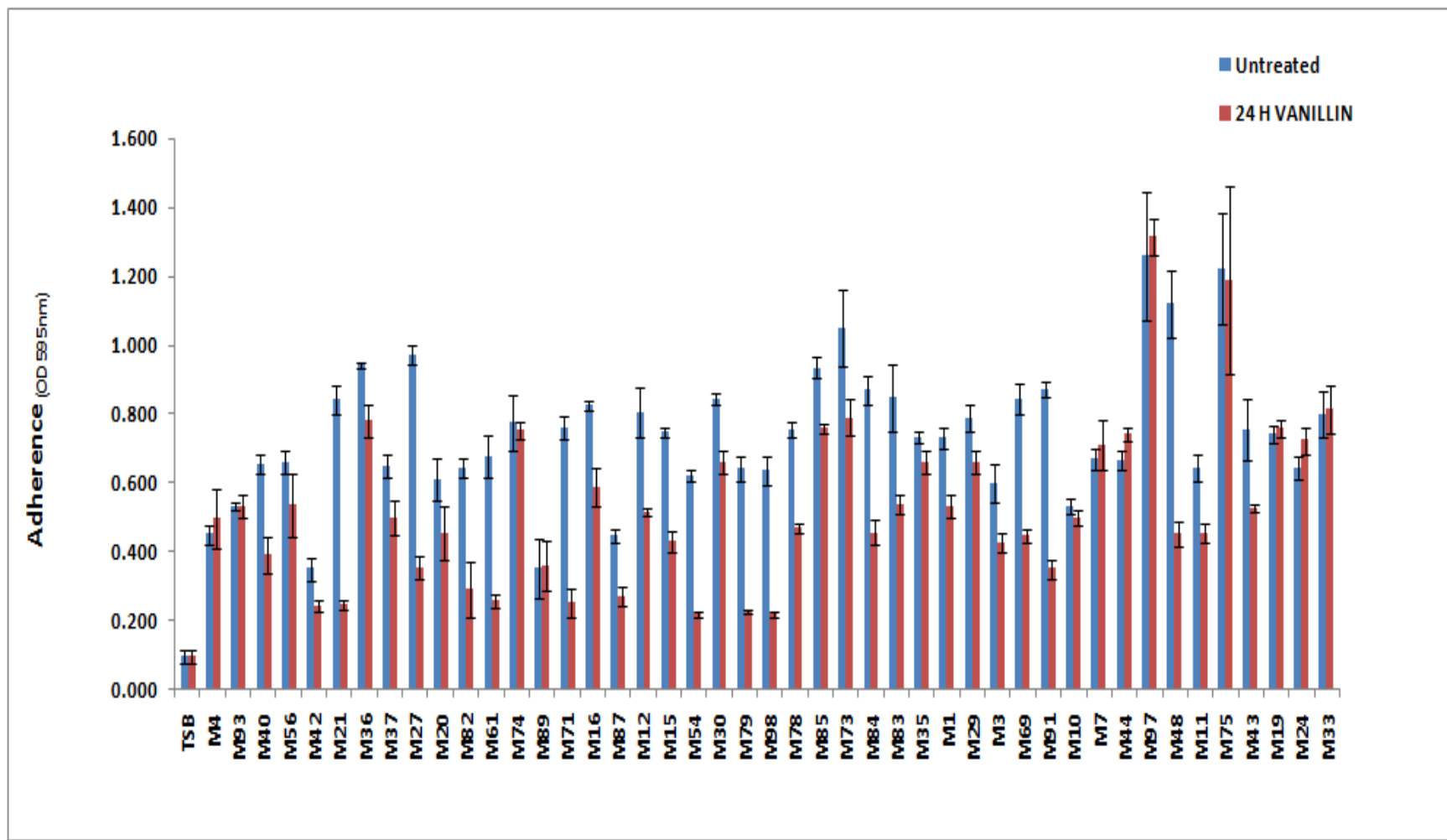


Figure 5.6. Effect of 5 µg/ml vanillin on initial attachment of *Aeromonas* spp. isolates using microtiter plate assays.

Following cinnamaldehyde treatment of pre-formed biofilms, 7% (3/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 93% (42/45) of isolates (Fig. 5.7). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed increased detachment in the pre-formed biofilm assay. Treatment of pre-formed *Aeromonas* spp. biofilms resulted in statistically significant altered adherence ($p = 0.001$).

Following furanone treatment of pre-formed biofilms, 4% (2/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 96% (43/45) of isolates (Fig. 5.8). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also increased detachment in the pre-formed biofilm assay. Treatment of pre-formed *Aeromonas* spp. biofilms resulted in statistically significant altered adherence ($p = 0.001$).

Following SAHC treatment of pre-formed biofilms, 9% (4/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 91% (41/45) of isolates (Fig. 5.9). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed increased detachment in the pre-formed biofilm assay. Treatment of pre-formed *Aeromonas* spp. biofilms resulted in statistically significant altered adherence ($p = 0.001$).

Following vanillin treatment of pre-formed biofilms, 2% (1/45) of isolates demonstrated increased adhesion, while increased detachment was observed for 96% (43/45) of isolates (Fig. 5.10). The type strains, *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T also displayed increased detachment in the pre-formed biofilm assay. Treatment of pre-formed *Aeromonas* spp. biofilms resulted in statistically significant altered adherence ($p = 0.001$). All four treatments of pre-formed biofilms resulted in statistically significant alterations in adhesion ($p < 0.05$).

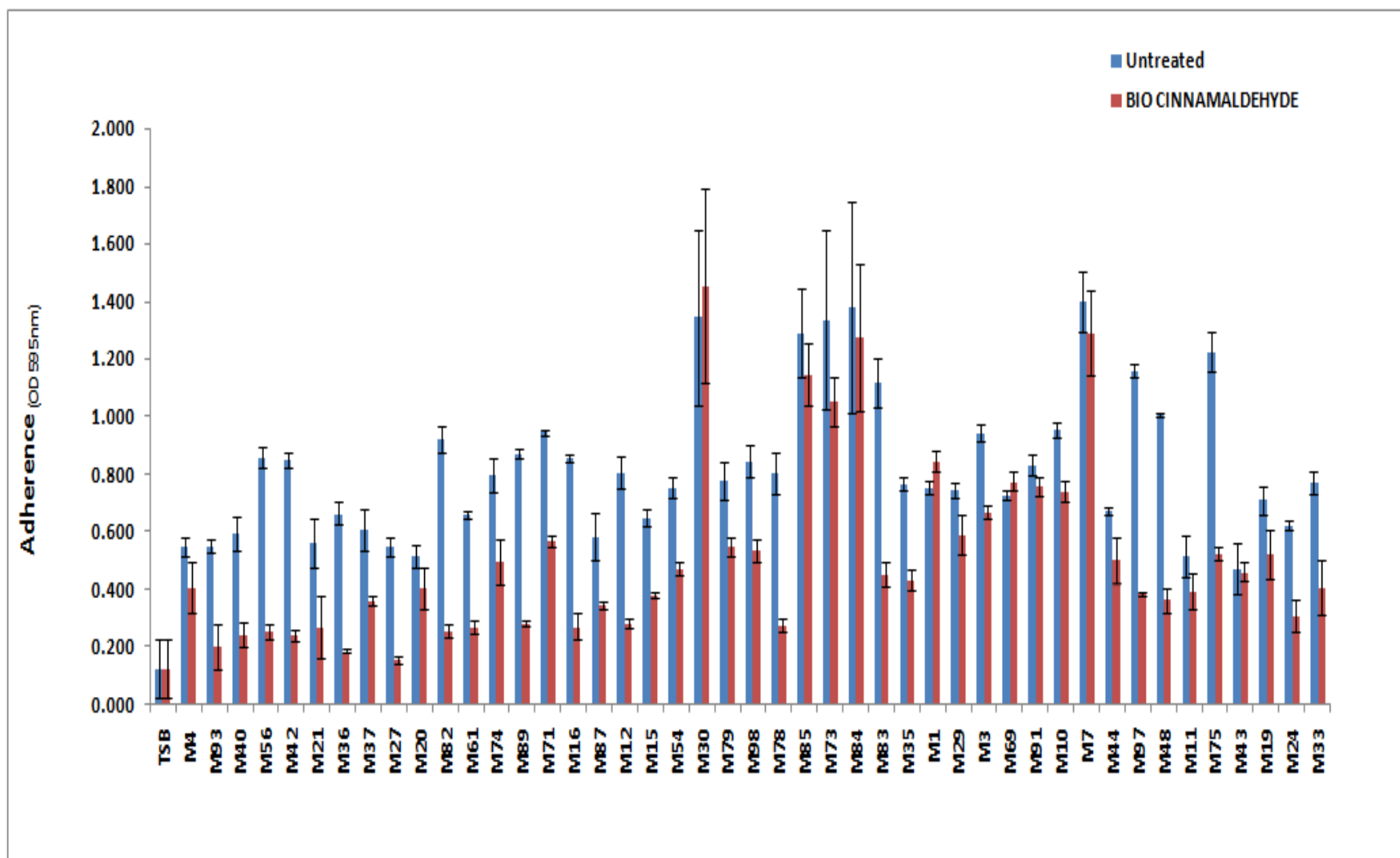


Figure 5.7. Effect of 5 µg/ml cinnamaldehyde on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

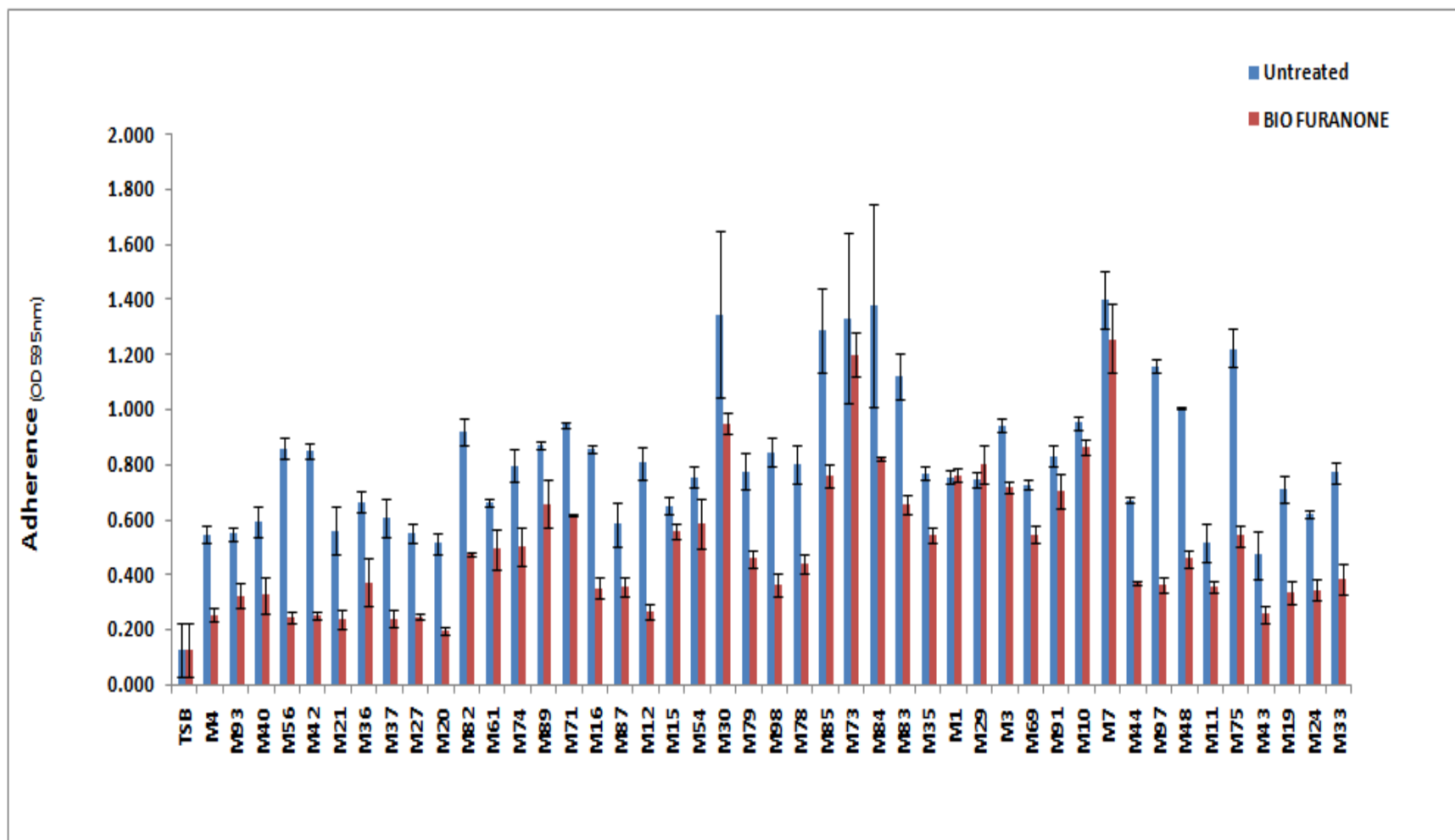


Figure 5.8. Effect of 5 µg/ml 2(5H)-furanone on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

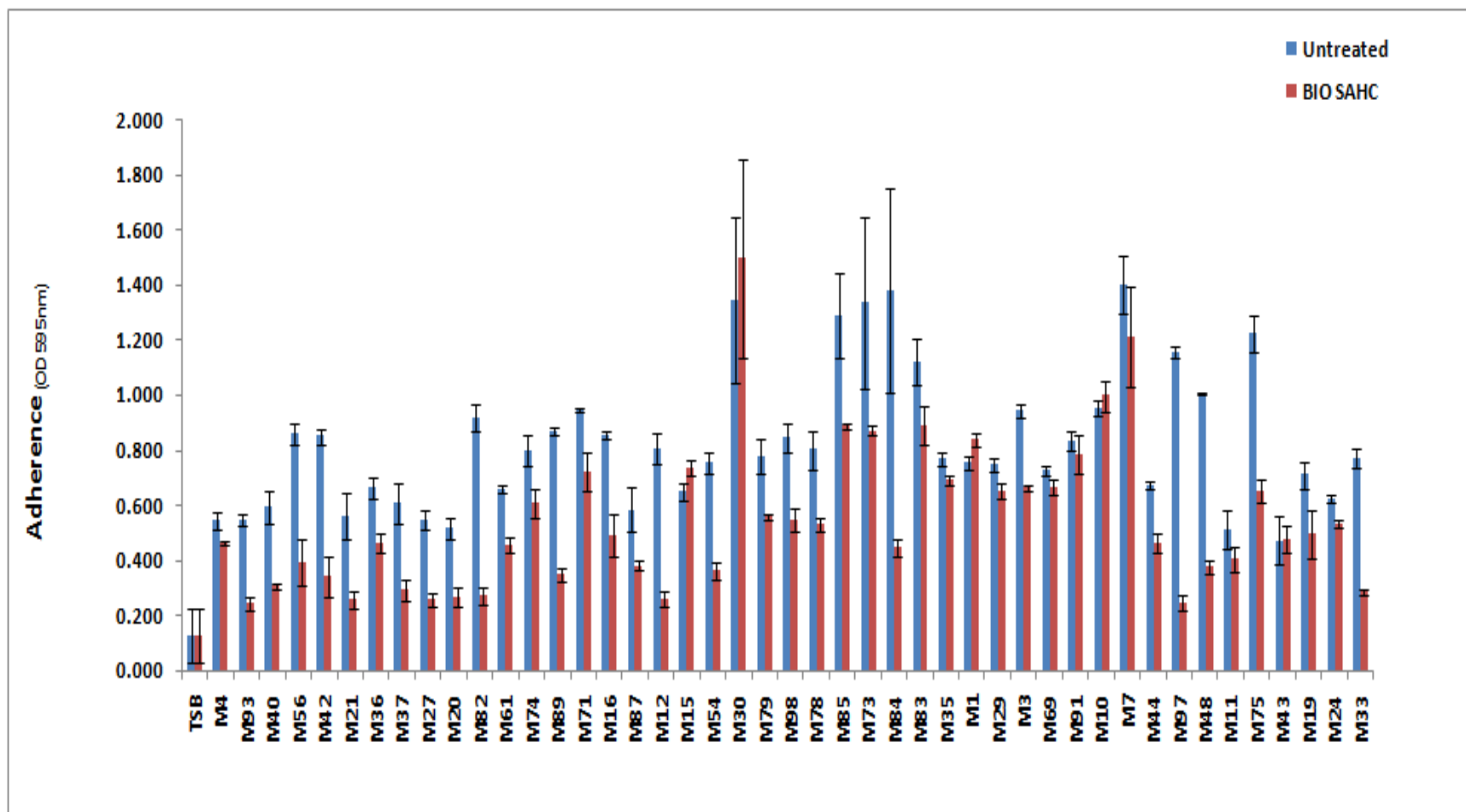


Figure 5.9. Effect of 5 μ g/ml S-adenosylhomocysteine (SAHC) on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

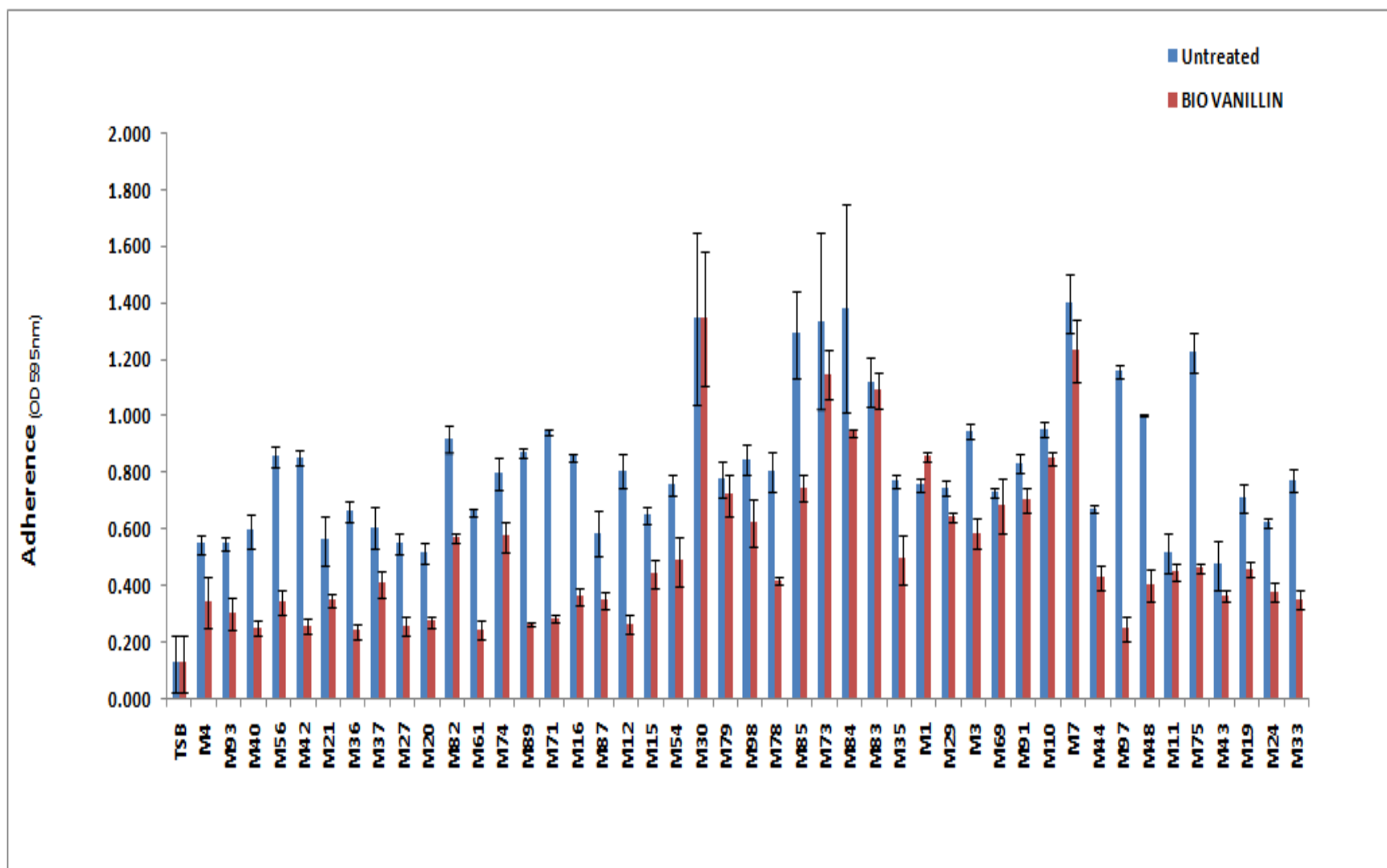


Figure 5.10. Effect of 5 µg/ml vanillin on pre-formed biofilms of *Aeromonas* spp. isolates using microtiter plate assays.

Based on biofilm persistence data (Table 5.2), for 4.5% (2/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by addition of cinnamaldehyde at the time of inoculation. For 2.2% (1/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by addition of 2(5H)-furanone at the time of inoculation (Table 5.2). For 4.5% (2/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by addition of SAHC at the time of inoculation (Table 5.2). Finally, for 11.1% (5/45) of the isolates, up to $\geq 75\%$ of the biofilm was destroyed by addition of vanillin at the time of inoculation (Table 5.2).

Based on biofilm persistence data (Table 5.2) for the effect of QSIs on pre-formed biofilms, for 22.2% (10/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by addition of cinnamaldehyde. For 11.1% (5/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by addition of 2(5H)-furanone (Table 5.2). For 8.8% (4/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by addition of SAHC (Table 5.2). Finally, for 15.6% (7/45) of the isolates, $\geq 75\%$ of the pre-formed biofilm was destroyed by addition of vanillin (Table 5.2).

Table 5.2. Biofilm persistence in the presence of cinnamaldehyde, 2(5H)-furanone, SAHC and vanillin exposure on initial attachment (24 H) and pre-formed biofilms (BIO) of *Aeromonas* spp. isolates

Isolates	% Biofilm persistence*							
	24 H Cinnamaldehyde	24 H Furanone	24 H SAHC	24 H Vanillin	BIO Cinnamaldehyde	BIO Furanone	BIO SAHC	BIO Vanillin
M1	91	59	68	69	115	101	114	116
M3	78	126	100	66	66	73	66	56
M4	113	99	102	113	67	31	81	51
M7	64	63	100	107	92	89	85	87
M10	92	138	99	93	75	89	105	87
M11	78	125	81	66	69	60	72	83
M12	50	64	66	59	23	20	20	20
M15	45	36	73	51	48	83	116	60
M16	36	48	44	68	20	31	50	32
M19	52	59	123	103	68	36	63	56
M20	38	86	76	69	72	18	36	38
M21	49	46	18	20	33	26	30	51
M24	121	135	175	115	37	44	82	50
M27	55	44	51	29	7	29	31	31
M29	81	52	75	81	75	109	85	84
M30	34	63	67	75	109	68	112	100
M33	63	66	66	102	44	40	25	35
M35	105	68	87	89	48	65	88	58
M36	46	40	35	81	11	46	63	21
M37	51	86	71	73	49	24	35	58
M40	47	77	80	52	25	43	39	27
M42	65	102	62	57	16	18	30	18
M43	84	99	91	65	97	38	102	69
M44	99	56	95	113	69	45	62	56
M48	52	91	52	35	27	38	28	31
M54	57	35	24	24	55	73	38	58
M56	43	74	75	78	18	17	37	29
M61	70	81	25	28	27	69	62	22
M69	61	99	58	47	108	70	90	92
M71	15	68	76	24	54	60	73	19
M73	55	3	98	73	77	89	62	85
M74	53	61	59	97	55	56	72	67
M75	49	82	78	97	36	38	48	31
M78	44	70	75	56	22	46	60	43
M79	56	56	39	24	65	51	66	92
M82	65	63	63	35	16	44	18	56
M83	66	82	69	59	33	53	77	97
M84	31	51	56	46	92	56	26	65
M85	76	33	42	79	88	54	65	53
M87	110	102	50	50	48	51	56	49
M89	123	149	66	103	21	71	30	18
M91	33	100	52	33	89	82	93	82
M93	69	113	114	100	18	47	28	41
M97	14	29	91	105	25	23	12	12
M98	34	58	78	23	57	33	58	69%
ATCC 15468T	30	66	56	89	74	44	23	87
ATCC 7966 ^T	34	76	99	77	82	37	15	96

*Biofilm persistence = (OD₅₉₅ X - OD₅₉₅ negative control) / (OD₅₉₅ positive control - OD₅₉₅ negative control) × 100%, where x corresponds to the tested antimicrobial agent (Tre-Hardy *et al.*, 2008).

Table 5.3 summarises the overall effects of DNase I and QSI treatments on initial attachment and biofilm detachment of *Aeromonas* spp. isolates.

Table 5.3. Summary table for effect of DNase I and QSIs on initial attachment and pre-formed biofilms of *Aeromonas* spp. isolates

Treatments	Initial attachment			Pre-formed biofilms		
	% Increase (number of isolates)	% Decrease (number of isolates)	% No effect	% Increase (number of isolates)	% Decrease (number of isolates)	% No effect
1 mg/ml DNase I	47 (21/45)	53 (24/45)	-	40 (18/40)	60 (27/45)	-
5 µg/ml Cinnamaldehyde	11 (5/45)	89 (40/45)	-	7 (3/45)	93 (42/45)	-
5 µg/ml 2(5H)-furanone	16 (7/45)	84 (38/45)	-	4 (2/45)	96 (43/45)	-
5 µg/ml SAHC	9 (4/45)	87 (39/45)	4 (2/45)	9 (4/45)	91 (41/45)	-
5 µg/ml Vanillin	22 (10/45)	78 (35/45)	-	2 (1/45)	96 (43/45)	2 (1/45)

5.4. Discussion

Diverse strategies are being applied to control bacterial biofilms due to their increased resistance to antimicrobial agents, including the use of matrix-degrading enzymes, quorum-sensing inhibitors, and quorum-quenching enzymes (Francolini and Donelli, 2010; Xiong and Liu, 2010). The present study investigated the effects of DNase I, a matrix-degrading enzyme, quorum sensing inhibitors [(5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone and S-adenosylhomocysteine], as well as the phytochemicals cinnamaldehyde and vanillin, on initial attachment and detachment of *Aeromonas* spp. isolates from abiotic surfaces.

DNase I was more effective in reducing mature bacterial biofilms, with greater reduction for mature biofilms (60% of isolates) than on initial attachment (reduction for 53% of isolates), although this was not significant for the population as a whole. Similar results were obtained for *A. caviae* ATCC 15468^T and *A. hydrophila* ATCC 7966^T, where lower levels of adherence were observed in the initial attachment assay and increased detachment was observed in pre-formed biofilm assay. These results were similar to the findings of Whitchurch *et al.* (2002) for *P. aeruginosa*, where eDNA was involved in biofilm adhesion and maturation, as well as biofilm stabilization. The effect of DNase I did not appear to isolate-specific. The decreased adhesion following DNase I exposure, may be explained by the action of DNase I removing the secreted DNA adhesins, thus preventing effective attachment. The increased adhesion observed for some isolates may be explained by the exolytic action of DNase I facilitating the adhesion process and promoting attachment by degrading eDNA. This has been observed for *C. crescentus* biofilm, which matured faster in the presence of DNase I than in its absence (Berne *et al.*, 2010).

A. tumefaciens A136 detects a broad range of AHLs (all 3-oxo, C6, C8, C10, C12, C14, C6-3-hydroxy, C8-3-hydroxy and C10-3-hydroxy) which is why in the present study, the *A. tumefaciens* A136 reporter responded to all isolates (100%) producing a signal (blue colour) in the presence of X-gal. *C. violaceum* CV026 detects short and medium AHLs (C6, C6-3-oxo C8, C8-3-oxo), and produced the purple violacein pigment. The *C. violaceum* CV026 reporter responded to 33% of isolates by the production of violacein. Swift *et al.* (1997) observed that *A. hydrophila* and *A. salmonicida* produce diffusible AHL. *Aeromonas* spp. isolates isolated from patients with malaria were shown to produce C4-HSL and C6-HSL as their major two types of AHLs. *A. hydrophila* was shown to produce both C4-HSL and C6-HSL, while *A. sobria* only produced C4-HSL (Chan *et al.*, 2010). *A. hydrophila* was shown to produce C4-HSL, as the

major AHL, and *A. caviae* was shown to produce 3-oxo-C6-HSL (Medina-Martínez *et al.*, 2006). *Aeromonas* spp. isolates isolated from municipal activated sludge produced C4-HSL and C6-HSL (Morgan-Sagastume *et al.*, 2005). This implies that *Aeromonas* spp. isolates in the present study may also produce diverse AHLs, specifically C4-HSL and C6-HSL as major AHLs.

Another strategy to inhibit bacterial biofilm formation is QS inhibition because microorganisms communicate using QS signals and these signals play important roles in initiating biofilm formation and in the detachment of biofilms. QSIs were more effective in reducing bacterial biofilm (mature biofilm) compared to preventing initial attachment. In the present study, although increased biofilm dispersal was observed with all QSIs, vanillin and 2(5H)-furanone were more effective (reduction for 96% of isolates) compared to SAHC and cinnamaldehyde and this might be due to their varied modes of action.

Vanillin and furanones inhibit both short and long chain AHLs and *A. hydrophila* growth and biofilm formation was reduced up to 17% by 1 mg/ml 2(5H)-furanone and up to 46.3% by 0.25 mg/ml vanillin (Ponnusamy *et al.*, 2009; Viana *et al.*, 2009). Brominated furanones have been widely used due to their antibacterial ability, anti-QS activity and anti-biofilm effect. These molecules competitively prevent binding of the QS molecules to the receptor or decrease the receptor concentration thus inhibiting or inactivating the signal transduction of QS (Dong *et al.*, 2007). This effectiveness of quorum sensing inhibition using brominated furanones has been observed with *P. aeruginosa* was observed, as well as reduced virulence factors production, biofilm formation, respectively (Hentzer *et al.*, 2002). 2(5H)-furanone also caused a significant reduction (32%) in QS-mediated biofilm formation of *A. hydrophila* (Ponnusamy *et al.*, 2010), this was, however, lower than the $\geq 75\%$ reduction of the biofilm obtained in the study (Table 5.2).

Vanillin was more effective in reducing pre-formed biofilm (mature biofilm). Following vanillin treatment at the time of attachment, decreased adhesion was observed for 78% (35/45) of isolates in the initial attachment assay while detachment was observed for 96% (43/45) of isolates. Ponnusamy *et al.* (2009) also described the potential use of vanillin as a QSI for *A. hydrophila* biofilm formation, where it had an effect on the adherence and detachment of *A. hydrophila*.

Cinnamaldehyde was also more effective on mature biofilms than on initial attachment. Cinnamaldehyde has been observed to inhibit biofilm formation of *E. coli* and *P. aeruginosa*, as well as swimming motility of *E. coli* (Niu and Gilbert, 2004). Following cinnamaldehyde treatment, an 11% reduction in biofilm mass was observed for *Burkholderia multivorans* (Brackmann *et al.*, 2009), which was lower than the $\geq 75\%$ reduction (Table 5.2) obtained in present study for *Aeromonas* spp. isolates.

S-adenosylhomocysteine (SAHC), an intermediate formed during the synthesis of both AHLs and AI-2, can be directly used as QSI because it mimics the action of SAM-utilizing enzyme that donates the nitrogen atom from SAM to become AHLs (Musk and Hergenrother, 2006). Following SAHC treatment, 87% (39/45) of isolates (Fig. 5.5) displayed decreased adhesion at the time of inoculation, while 91% (41/45) of isolates (Fig. 5.9) displayed increased detachment for pre-formed biofilms. Based on the present data, in the initial attachment assay (Table 5.3), cinnamaldehyde was most effective, followed by SAHC, 2(5H)-furanone then lastly vanillin. For the pre-formed biofilm assay, 2(5H)-furanone and vanillin were the most effective, followed by cinnamaldehyde then SAHC (Table 5.3).

The *A. tumefaciens* A136 and *Chromobacterium violaceum* CV026 biosensor strains were effective and useful in phenotypic detection of AHLs, although further analysis such as

liquid-liquid and solid-phase extraction methods (Wang *et al.*, 2010) could be used to identify and characterize AHLs from different *Aeromonas* spp. isolates. This could provide relevant information on the exact AHL molecules produced by the study isolates. Because QSI compounds attenuate virulence of pathogenic bacteria without affecting growth, they have been termed anti-pathogenic drugs, as opposed to being antibacterial drugs. QSIs and the DNase I enzyme are thus alternative treatment options that may be used to treat diseases and outbreaks associated with aeromonad species because these treatments interfere with crucial stages of biofilm formation, i.e., QS and degradation of the adhesin eDNA. The toxicity and carcinogenic effects as well as poor stability in aqueous solutions have greatly limited the utilization of halogenated furanones as antimicrobials (Hentzer and Givskov, 2003). Vanillin could be a potential alternative to the toxic chemicals, and has potential practical application in aquaculture systems (Ponnusamy *et al.*, 2009). The source and identity of the eDNA has to be established in order to clarify its specific role in the ability of *Aeromonas* spp. to form biofilms.

CHAPTER SIX

CONCLUSIONS

The presence of *Aeromonas* spp. isolates in mixed spp. biofilms has been reported previously from the clinical and industrial settings (Asha *et al.*, 2004; Azad *et al.*, 1999; Bomo *et al.*, 2004; Dogruoz *et al.*, 2009; Nayak *et al.*, 2004). *Aeromonas* spp. isolates from seawater and diverse cultured fish displayed different levels of biofilm formation on polystyrene, which was affected by alteration of nutrient availability, but not by temperature. Although biofilms have been studied for *A. hydrophila* and *A. caviae*, studying biofilm formation by a variety of members of this genus provides more relevant information on the behaviour of diverse species from diverse sources. The ability of *Aeromonas* spp. to autoaggregate, rather than its hydrophilic nature, appears to be a significant characteristic for aeromonad biofilm formation. The role of motility and other surface-associated appendages (A-layer) and extra-cellular enzymes (protease and gelatinase) in adherence and biofilm formation did not appear statistically significant and requires further investigation.

Majority of *Aeromonas* isolates showed a high level resistance to β -lactams (penicillins), trimethoprim and sulphamethoxazole, and were susceptible to augmentin, piperacillin-tazobactam, aztreonam, 2nd and 3rd generation cephalosporins, carbapenems, macrolides, tetracyclines, fluoroquinolones and aminoglycosides, and appeared to have susceptibility profiles similar to environmental, fish and clinical isolates. For most isolates, the MICs were at the highest concentration of antimicrobial agents tested (32 $\mu\text{g/ml}$), except for ceftazidime for which most isolates were highly resistant with (MICs > 32 $\mu\text{g/ml}$). Of the five antimicrobial agents, fluoroquinolones, which are the drugs of choice to treat bacterial fish diseases and clinical

human *Aeromonas* infections, were highly effective in the present study. This conclusion was reached following analysis of the disk diffusion data and MIC levels for analysis of this drug.

Evaluating the antimicrobial concentration required to eradicate biofilms by *Aeromonas* spp. isolates could facilitate effective treatment of these biofilms using antimicrobial agents, as cells in a biofilm appear to be more resistant to antimicrobial agents and/or biofilm inhibition molecules. In the present study, all antimicrobial agents used had an effect on pre-formed biofilms by *Aeromonas* spp. isolates (MBICs); however, majority of isolates displayed significant detachment at the highest concentration (4096 µg/ml) of these antimicrobial agents and this was somehow expected as this was the highest concentration. The sub-MIC, MIC and supra-MIC exposures of all antimicrobial agents had an effect on both initial attachment and pre-formed biofilms of *Aeromonas* spp. isolates.

Since the *Aeromonas* spp. isolates in the present study eliminated diverse classes of antimicrobial agents, this suggests the presence of broad-substrate range efflux pumps and/or multiple efflux pumps. Since microorganisms have multiple efflux pump systems, EPIs with a broad spectrum of activity need to be used so they can be effective against diverse, multiple efflux pump systems present in different microbial pathogens. Further experiments are required to explore the type of efflux pumps, substrate profiles, and regulation mechanisms of these pumps in these local *Aeromonas* spp. isolates. All EPIs (CCCP, NMP and PAβN) displayed significant reduction on biofilm formation by *Aeromonas* spp. isolates in the initial attachment and mature biofilm assays, although a much greater effect was observed on pre-formed biofilm [detachment] (Table 4.4). In the present study, CCCP was most effective, followed by NMP then lastly PAβN, for both the initial attachment and pre-formed biofilm assays (Table 4.4). Therefore, the EPIs do inhibit different efflux pump systems used by *Aeromonas* spp. isolates

and thus could prevent elimination of antimicrobial agents within these cells, and thus multidrug resistance. These EPIs also can be used as potential inhibitors of biofilm formation by *Aeromonas* spp. isolates, as they caused reduction of adherence and detachment of majority of the isolates. Although more work still needs to be done with these inhibitors and their mechanism, efflux pump inhibition represents one of the control strategies to limit aeromonad biofilms and could not only prevent disease outbreaks but also increase effectiveness of existing therapeutic agents.

The *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains were effective and useful in phenotypic detection of AHLs, although further analysis such as liquid-liquid and solid-phase extraction methods (Wang *et al.*, 2010) could be used to identify and characterize AHLs from different *Aeromonas* spp. isolates. This could provide relevant information on the exact AHL molecules produced by the study isolates. Based on the present data, in the initial attachment assay (Table 5.3), cinnamaldehyde was most effective, followed by SAHC, 2(5H)-furanone then lastly vanillin. For the pre-formed biofilm assay, 2(5H)-furanone and vanillin were the most effective, followed by cinnamaldehyde then SAHC (Table 5.3). QSIs and the DNase I enzyme are thus alternative treatment options that may be used to treat diseases and outbreaks associated with aeromonad species because these treatments interfere with crucial stages of biofilm formation, i.e., QS and degradation of the adhesin eDNA. The toxicity and carcinogenic effects as well as poor stability in aqueous solutions have greatly limited the utilization of halogenated furanones as antimicrobials (Hentzer and Givskov, 2003). Vanillin has been revolutionarily developed, it adds to the suitability for the commercial use. Also, vanillin could be a potential alternative to the toxic chemicals, and it has more advantage in its applications to

aquaculture systems (Ponnusamy *et al.*, 2008). The source and identity of the eDNA has to be established in order to clarify its specific role in the ability of *Aeromonas* spp. to form biofilms.

Future work will entail understanding fully, the factors involved in and affecting biofilm formation by *Aeromonas* spp. isolates and their correlation with biofilm formation by these isolates. Although higher concentrations of antimicrobial agents had an effect on the detachment of *Aeromonas* pre-formed biofilms, evaluating whether these would be practical to use in aquaculture systems and the exact antimicrobial concentration required to eradicate biofilms by *Aeromonas* spp. isolates could facilitate effective treatment. Further experiments are required to explore the type of efflux pumps, substrate profile, and regulation mechanisms of these pumps in these South African *Aeromonas* spp. isolates. Additionally, to determine the importance of multi-drug efflux pumps for biofilm resistance to antimicrobial agents and a better understanding of the mechanisms by which EPIs work will allow for more effective control of biofilm-associated diseases. Identifying and characterizing AHLs from different *Aeromonas* spp. isolates could provide relevant information on the exact AHL molecules produced by the study isolates, and the diversity between different species and geographical origins. The source and identity of the eDNA has to be established in order to clarify its specific role in the ability of *Aeromonas* spp. to form biofilms.

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Appendix

Table A1. Biochemical and physiological characterization of 99 *Aeromonas* spp. isolates obtained from different fish hosts or origin

Code	Source	Species	A-layer	Gelatin hydrolysis	Motility (mm)		Hydrophobicity (%)	Hydrophobicity classification*	Autoaggregation (%)	Autoaggregation classification [#]
					Swimming	Swarming				
M1	Catfish	<i>A. culicicola</i>	-	+	40.0	10.0	6.96	HPL	72	SA
M2	Catfish	<i>A. hydrophila</i>	+	+	29.3	8.3	6.45	HPL	39	MA
M3	Catfish	<i>A. allosaccharophila</i>	+	+	60.7	9.0	23.39	MHPB	63	SA
M4	Catfish	<i>A. jandaei</i>	+	-	21.7	7.0	12.88	HPL	52	SA
M5	Catfish	<i>A. hydrophila</i>	+	+	36.7	12.3	16.80	HPL	41	MA
M6	Catfish	<i>A. hydrophila</i>	-	+	26.7	15.3	14.02	HPL	48	MA
M7	Catfish	<i>A. culicicola</i>	+	+	21.0	12.3	23.98	MHPB	65	SA
M8	Tilapia	<i>A. allosaccharophila</i>	-	+	14.7	8.3	0.27	HPL	57	SA
M9	Catfish	<i>Plesiomonasshigelloides</i>	+	+	26.7	5.7	1.34	HPL	69	SA
M10	Catfish	<i>A. culicicola/jandaei</i>	+	-	39.3	16.0	10.60	HPL	54	SA
M11	Catfish	<i>Aeromonas</i> spp.	+	+	60.0	13.3	10.72	HPL	74	SA
M12	Catfish	<i>A. bestiarum</i>	+	+	36.7	15.3	56.26	HPB	72	SA
M13	Catfish	<i>A. hydrophila</i>	-	+	60.0	16.3	20.76	MHPB	76	SA
M14	Tilapia	<i>A. hydrophila</i>	+	+	38.3	14.7	3.33	HPL	80	SA
M15	Tilapia	<i>Aeromonas</i> spp.	+	+	37.7	9.0	0.97	HPL	75	SA
M16	Tilapia	<i>A. jandaei</i>	+	+	30.7	9.0	2.58	HPL	75	SA
M17	Tilapia	<i>A. hydrophila</i>	+	+	31.0	11.7	4.34	HPL	50	SA
M18	Tilapia	<i>A. caviae</i>	+	+	37.3	15.0	33.99	MHPB	81	SA
M19	Tilapia	<i>A. jandaei</i>	+	+	40.0	14.7	10.90	HPL	45	MA
M20	Tilapia	<i>Aeromonas</i> spp.	+	+	34.7	15.3	15.04	HPL	66	SA
M21	Tilapia	<i>A. allosaccharophila</i>	+	+	36.0	9.0	30.18	MHPB	73	SA
M22	Seawater	<i>A. culicicola</i>	+	+	50.3	12.0	17.96	HPL	41	MA
M23	Seawater	<i>A. culicicola</i>	+	+	40.0	31.7	6.70	HPL	47	MA

M24	Seawater	<i>A. jandaei</i>	+	+	41.7	16.0	33.36	MHPB	58	SA
M25	Seawater	<i>A. culicicola</i>	+	+	42.0	23.0	12.04	HPL	73	SA
M26	Seawater	<i>Aeromonas</i> spp. 45	-	+	39.7	17.7	14.85	HPL	62	SA
M27	Seawater	<i>Aeromonas</i> spp. 45	+	+	43.0	35.3	4.60	HPL	55	SA
M28	Seawater	<i>A. jandaei</i>	+	+	41.0	10.3	18.38	HPL	66	SA
M29	Seawater	<i>A. culicicola</i>	+	+	51.0	15.3	29.24	MHPB	84	SA
M30	Seawater	<i>A. jandaei</i>	+	+	51.0	51.0	16.06	HPL	37	MA
M31	Seawater	<i>A. culicicola</i>	+	+	41.0	22.3	6.39	HPL	61	SA
M32	Seawater	<i>A. culicicola</i>	-	+	47.3	21.3	2.00	HPL	63	SA
M33	Seawater	<i>Aeromonas</i> spp. 310	+	+	43.0	26.7	24.58	MHPB	82	SA
M34	Seawater	<i>Aeromonas</i> spp. 45	-	+	51.0	15.3	12.97	HPL	50	SA
M35	Seawater	<i>A. culicicola</i>	+	+	41.0	46.7	34.31	MHPB	39	MA
M36	Seawater	<i>A. jandaei</i>	+	+	41.7	12.0	15.98	HPL	60	SA
M37	Seawater	<i>Aeromonas</i> spp. 45	+	+	45.0	21.7	3.21	HPL	65	SA
M38	Seawater	<i>A. culicicola</i>	+	+	41.7	20.0	0.97	HPL	56	SA
M39	Seawater	<i>A. culicicola</i>	+	+	40.0	12.3	9.73	HPL	62	SA
M40	Seawater	<i>A. culicicola</i>	+	+	41.0	19.0	20.78	MHPB	60	SA
M41	Seawater	<i>Aeromonas</i> spp.	-	+	39.7	22.7	0.57	HPL	26	MA
M42	Tilapia	<i>A. culicicola</i>	-	+	35.7	14.7	3.40	HPL	75	SA
M43	Tilapia	<i>Aeromonas</i> spp. 310	+	+	33.7	14.3	37.07	MHPB	67	SA
M44	Tilapia	<i>A. ichtiosmia</i>	+	+	33.7	13.0	13.39	HPL	53	SA
M45	Tilapia	<i>Plesiomonasshigelloides</i>	+	+	32.0	11.0	8.48	HPL	74	SA
M46	Tilapia	<i>Plesiomonasshigelloides</i>	+	+	34.7	8.0	21.32	MHPB	54	SA
M47	Tilapia	<i>Plesiomonasshigelloides</i>	+	+	15.0	8.0	19.27	HPL	55	SA
M48	Tilapia	<i>A. ichtiosmia</i>	+	+	17.7	8.3	1.84	HPL	74	SA
M49	Tilapia	<i>A. sobria</i>	+	+	30.3	17.3	18.19	HPL	51	MA
M50	Catfish	<i>A. hydrophila</i>	-	+	14.3	9.7	6.19	HPL	54	SA
M51	Catfish	<i>A. hydrophila</i>	+	+	59.3	8.7	20.31	MHPB	38	MA
M52	Tilapia	<i>A. hydrophila</i>	+	+	15.3	7.3	5.82	HPL	64	SA
M53	Catfish	<i>A. hydrophila</i>	+	-	53.0	67.3	10.22	HPL	60	SA

M54	Tilapia	<i>A. ichthiosmia</i>	+	+	39.7	9.7	2.85	HPL	78	SA
M55	Tilapia	<i>A. veronii/A. culicicola</i>	+	+	41.7	7.7	4.42	HPL	70	SA
M56	Tilapia	<i>A. culicicola</i>	+	+	40.3	8.3	61.02	HPB	34	MA
M57	Tilapia	<i>A. veronii</i>	+	+	39.3	6.0	11.41	HPL	65	SA
M58	Tilapia	<i>A. culicicola</i>	+	+	40.3	8.3	23.29	MHPB	64	SA
M59	Tilapia	<i>A. caviae</i>	+	+	32.0	11.0	3.48	HPL	42	MA
M60	Tilapia	<i>A. hydrophila</i>	+	+	34.7	14.0	9.36	HPL	74	SA
M61	Tilapia	<i>A. culicicola</i>	+	+	44.3	15.7	3.45	HPL	69	SA
M62	Tilapia	<i>A. hydrophila</i>	+	+	20.0	23.0	7.64	HPL	76	SA
M63	Tilapia	<i>A. veronii</i>	+	+	38.0	11.7	8.13	HPL	58	SA
M64	Tilapia	<i>A. hydrophila</i>	+	-	59.7	14.3	2.07	HPL	60	SA
M65	Tilapia	<i>A. hydrophila</i>	-	+	41.7	10.7	5.42	HPL	75	SA
M66	Tilapia	<i>Plesiomonasshigelloides</i>	-	+	40.0	15.7	45.80	MHPB	56	SA
M67	Tilapia	<i>Plesiomonasshigelloides</i>	+	+	40.0	47.7	29.08	MHPB	67	SA
M68	Goldfish	<i>A. caviae</i>	+	-	47.0	12.3	4.54	HPL	54	SA
M69	Goldfish	<i>A. bestiarum</i>	+	-	34.7	12.0	2.99	HPL	66	SA
M70	Goldfish	<i>A. bestiarum</i>	+	+	60.7	16.0	10.09	HPL	55	SA
M71	Goldfish	<i>A. bestiarum</i>	+	-	28.0	28.3	26.15	MHPB	56	SA
M72	Goldfish	<i>A. bestiarum</i>	+	+	35.0	12.0	18.29	HPL	74	SA
M73	Goldfish	<i>A. bestiarum</i>	+	+	59.7	13.7	13.95	HPL	70	SA
M74	Goldfish	<i>A. allosaccharophila</i>	+	-	54.0	13.0	52.64	HPB	57	SA
M75	Goldfish	<i>Aeromonas. spp. 45</i>	+	+	23.0	11.0	11.58	HPL	57	SA
M76	Goldfish	<i>A. salmonicida</i>	+	-	52.3	9.0	12.25	HPL	61	SA
M77	Goldfish	<i>A. salmonicida</i>	+	+	54.3	9.0	10.31	HPL	44	MA
M78	Goldfish	<i>A. bestiarum</i>	+	+	59.3	21.7	7.46	HPL	52	MA
M79	Goldfish	<i>A. bestiarum</i>	-	+	29.0	15.3	37.58	MHPB	62	SA
M80	Goldfish	<i>A. bestiarum</i>	+	+	40.0	20.0	19.21	HPL	78	SA
M81	Goldfish	<i>A. bestiarum</i>	+	+	54.0	12.3	29.56	MHPB	63	SA
M82	Goldfish	<i>A. culicicola</i>	+	+	52.7	13.7	15.57	HPL	82	SA
M83	Goldfish	<i>A. bestiarum</i>	+	+	40.0	15.3	69.09	HPB	64	SA

M84	Goldfish	<i>A. bestiarum</i>	-	+	49.7	14.0	11.41	HPL	54	SA
M85	Goldfish	<i>A. allosaccharophila</i>	-	+	23.3	19.0	10.54	HPL	56	SA
M86	Goldfish	<i>A. hydrophila</i>	+	+	37.3	10.3	18.26	HPL	59	SA
M87	Goldfish	<i>A. bestiarum</i>	+	+	60.0	9.0	45.31	MHPB	72	SA
M88	Goldfish	<i>A. bestiarum</i>	-	+	52.7	13.3	1.79	HPL	63	SA
M89	Goldfish	<i>A. allosaccharophila</i>	-	-	37.0	14.7	3.19	HPL	61	SA
M90	Goldfish	<i>A. bestiarum</i>	-	-	19.0	12.3	16.57	HPL	46	MA
M91	Goldfish	<i>A. bestiarum</i>	+	-	14.0	12.0	34.72	MHPB	74	SA
M92	Goldfish	<i>A. allosaccharophila</i>	+	+	38.0	40.7	18.25	HPL	69	SA
M93	Goldfish	<i>A. allosaccharophila</i>	+	+	41.7	18.7	8.88	HPL	69	SA
M94	Goldfish	<i>A. hydrophila</i>	+	-	54.0	29.0	8.57	HPL	51	SA
M95	Goldfish	<i>A. hydrophila</i>	+	+	61.0	21.0	6.77	HPL	61	SA
M96	Goldfish	<i>A. bestiarum</i>	+	+	41.7	13.7	4.21	HPL	56	SA
M97	Goldfish	<i>A. bestiarum</i>	-	+	44.3	9.7	17.06	HPL	58	SA
M98	Goldfish	<i>Aeromonas</i> spp. 310	+	+	28.3	13.0	0.49	HPL	52	SA
M99	Goldfish	<i>A. bestiarum</i>	+	+	40.0	14.7	4.69	HPL	67	SA

*HPL = hydrophilic, MHPB = moderately hydrophobic, and HPB = hydrophobic

#SA = strong autoaggregation ability, MA = moderate autoaggregation ability, WA = weak autoaggregation ability (Basson *et al.*, 2008).

Table A2. Relative caseinolytic and DNase activities of 99 *Aeromonas* spp. isolates obtained from different fish hosts or origin

Code	Source	Species	Casein hydrolysis zone diameter (mm)	RCA*	DNase hydrolysis zone diameter (mm)	RDA*
M1	Catfish	<i>A. culicicola</i>	21.7	0.4	15.3	0.4
M2	Catfish	<i>A. hydrophila</i>	19.0	0.5	12.7	0.4
M3	Catfish	<i>A. allosaccharophila</i>	16.3	0.4	13.7	0.3
M4	Catfish	<i>A. jandaei</i>	22.0	0.5	14.3	0.3
M5	Catfish	<i>A. hydrophila</i>	21.0	0.2	16.3	0.4
M6	Catfish	<i>A. hydrophila</i>	16.7	0.3	15.7	0.3
M7	Catfish	<i>A. culicicola</i>	18.3	0.8	23.7	1.3
M8	Tilapia	<i>A. allosaccharophila</i>	18.7	0.6	9.7	0.0
M9	Catfish	<i>Plesiomonasshigelloides</i>	16.0	0.4	16.7	0.6
M10	Catfish	<i>A. culicicola/jandaei</i>	18.3	0.9	18.3	0.3
M11	Catfish	<i>Aeromonas</i> spp.	10.0	0.0	18.7	0.3
M12	Catfish	<i>A. bestiarum</i>	14.3	0.1	16.0	0.4
M13	Catfish	<i>A. hydrophila</i>	16.3	0.3	18.3	0.3
M14	Tilapia	<i>A. hydrophila</i>	15.7	0.1	20.7	0.4
M15	Tilapia	<i>Aeromonas</i> spp.	19.7	0.7	14.3	0.3
M16	Tilapia	<i>A. jandaei</i>	18.3	0.8	16.3	0.4
M17	Tilapia	<i>A. hydrophila</i>	18.7	0.9	15.7	0.5
M18	Tilapia	<i>A. caviae</i>	17.3	0.8	19.7	0.6
M19	Tilapia	<i>A. jandaei</i>	16.3	0.5	10.3	0.0
M20	Tilapia	<i>Aeromonas</i> spp.	18.0	0.9	18.3	0.5
M21	Tilapia	<i>A. allosaccharophila</i>	20.7	0.9	18.7	0.2
M22	Seawater	<i>A. culicicola</i>	17.7	0.7	17.3	0.4
M23	Seawater	<i>A. culicicola</i>	15.3	0.3	16.3	0.4
M24	Seawater	<i>A. jandaei</i>	16.3	0.4	18.0	0.2
M25	Seawater	<i>A. culicicola</i>	17.7	0.7	20.7	0.2
M26	Seawater	<i>Aeromonas</i> spp. 45	14.7	0.5	17.7	0.4

M27	Seawater	<i>Aeromonas</i> spp. 45	15.7	0.5	15.3	0.5
M28	Seawater	<i>A. jandaei</i>	15.3	0.1	16.3	0.4
M29	Seawater	<i>A. culicicola</i>	16.7	0.1	17.7	0.5
M30	Seawater	<i>A. jandaei</i>	18.3	0.6	14.7	0.5
M31	Seawater	<i>A. culicicola</i>	17.7	0.2	15.7	0.6
M32	Seawater	<i>A. culicicola</i>	18.3	0.3	15.3	0.2
M33	Seawater	<i>Aeromonas</i> spp. 310	16.7	0.5	16.7	0.3
M34	Seawater	<i>Aeromonas</i> spp. 45	16.3	0.4	18.3	0.3
M35	Seawater	<i>A. culicicola</i>	19.3	0.9	17.7	0.5
M36	Seawater	<i>A. jandaei</i>	14.0	0.1	15.7	0.5
M37	Seawater	<i>Aeromonas</i> spp. 45	17.7	0.7	15.0	0.5
M38	Seawater	<i>A. culicicola</i>	18.3	0.5	15.3	0.6
M39	Seawater	<i>A. culicicola</i>	15.0	0.2	17.7	0.4
M40	Seawater	<i>A. culicicola</i>	15.7	0.2	16.3	0.4
M41	Seawater	<i>Aeromonas</i> spp.	16.7	0.2	16.3	0.4
M42	Tilapia	<i>A. culicicola</i>	16.7	0.4	18.0	0.6
M43	Tilapia	<i>Aeromonas</i> spp. 310	18.3	0.8	20.3	0.3
M44	Tilapia	<i>A. ichtiosmia</i>	16.7	0.7	16.3	0.4
M45	Tilapia	<i>Plesiomonasshigelloides</i>	18.7	0.9	19.3	0.5
M46	Tilapia	<i>Plesiomonasshigelloides</i>	20.0	0.6	16.7	0.5
M47	Tilapia	<i>Plesiomonasshigelloides</i>	21.7	0.8	9.7	0.0
M48	Tilapia	<i>A. ichtiosmia</i>	14.7	0.3	16.3	0.6
M49	Tilapia	<i>A. sobria</i>	11.0	0.0	20.7	0.4
M50	Catfish	<i>A. hydrophila</i>	17.0	0.1	15.7	0.3
M51	Catfish	<i>A. hydrophila</i>	18.3	0.6	18.3	0.6
M52	Tilapia	<i>A. hydrophila</i>	18.0	0.4	16.7	0.6
M53	Catfish	<i>A. hydrophila</i>	20.3	0.8	16.3	0.3
M54	Tilapia	<i>A. ichtiosmia</i>	16.7	0.7	19.3	0.4
M55	Tilapia	<i>A. veronii/A. culicicola</i>	17.3	0.7	14.0	0.4
M56	Tilapia	<i>A. culicicola</i>	18.7	0.3	17.7	0.4

M57	Tilapia	<i>A. veronii</i>	17.3	0.5	18.3	0.6
M58	Tilapia	<i>A. culicicola</i>	17.7	0.5	15.0	0.5
M59	Tilapia	<i>A. caviae</i>	19.0	0.8	15.7	0.3
M60	Tilapia	<i>A. hydrophila</i>	17.7	0.4	16.7	0.7
M61	Tilapia	<i>A. culicicola</i>	17.3	0.2	16.7	0.6
M62	Tilapia	<i>A. hydrophila</i>	15.7	0.6	18.3	0.8
M63	Tilapia	<i>A. veronii</i>	19.7	0.6	16.7	0.7
M64	Tilapia	<i>A. hydrophila</i>	21.3	0.8	18.7	0.6
M65	Tilapia	<i>A. hydrophila</i>	15.3	0.5	20.0	0.7
M66	Tilapia	<i>Plesiomonasshigelloides</i>	11.7	0.0	21.7	0.5
M67	Tilapia	<i>Plesiomonasshigelloides</i>	10.0	0.0	14.7	0.7
M68	Goldfish	<i>A. caviae</i>	10.3	0.0	15.0	0.5
M69	Goldfish	<i>A. bestiarum</i>	14.3	0.4	17.0	0.5
M70	Goldfish	<i>A. bestiarum</i>	15.3	0.6	18.3	0.5
M71	Goldfish	<i>A. bestiarum</i>	11.7	0.0	15.0	0.5
M72	Goldfish	<i>A. bestiarum</i>	15.3	0.3	15.0	0.5
M73	Goldfish	<i>A. bestiarum</i>	17.7	0.2	17.3	0.4
M74	Goldfish	<i>A. allosaccharophila</i>	15.3	0.8	14.7	0.6
M75	Goldfish	<i>Aeromonas. spp. 45</i>	15.7	0.6	15.7	0.4
M76	Goldfish	<i>A. salmonicida</i>	17.3	0.5	14.3	0.7
M77	Goldfish	<i>A. salmonicida</i>	18.7	0.6	15.3	0.5
M78	Goldfish	<i>A. bestiarum</i>	10.0	0.0	17.3	0.5
M79	Goldfish	<i>A. bestiarum</i>	18.3	0.8	15.3	0.5
M80	Goldfish	<i>A. bestiarum</i>	18.7	0.5	17.7	0.4
M81	Goldfish	<i>A. bestiarum</i>	9.0	0.0	15.3	0.6
M82	Goldfish	<i>A. culicicola</i>	16.3	0.5	15.7	0.3
M83	Goldfish	<i>A. bestiarum</i>	18.0	1.1	17.3	0.5
M84	Goldfish	<i>A. bestiarum</i>	20.7	1.0	18.7	0.8
M85	Goldfish	<i>A. allosaccharophila</i>	17.7	0.5	19.3	0.8
M86	Goldfish	<i>A. hydrophila</i>	15.3	0.5	18.0	0.9

M87	Goldfish	<i>A. bestiarum</i>	16.3	0.3	20.3	0.8
M88	Goldfish	<i>A. bestiarum</i>	17.7	0.8	16.7	0.6
M89	Goldfish	<i>A. allosaccharophila</i>	14.7	0.2	17.3	0.5
M90	Goldfish	<i>A. bestiarum</i>	15.7	0.4	18.7	0.5
M91	Goldfish	<i>A. bestiarum</i>	15.3	0.4	17.3	0.7
M92	Goldfish	<i>A. allosaccharophila</i>	11.0	0.0	17.7	0.4
M93	Goldfish	<i>A. allosaccharophila</i>	18.3	0.9	19.0	0.5
M94	Goldfish	<i>A. hydrophila</i>	17.7	0.6	17.7	0.7
M95	Goldfish	<i>A. hydrophila</i>	15.7	0.5	17.3	0.7
M96	Goldfish	<i>A. bestiarum</i>	15.3	0.4	15.7	0.8
M97	Goldfish	<i>A. bestiarum</i>	17.7	0.4	19.7	0.6
M98	Goldfish	<i>Aeromonas</i> spp. 310	16.0	0.5	21.3	0.8
M99	Goldfish	<i>A. bestiarum</i>	16.3	0.3	17.3	0.4

*RCA = relative caseinolytic activity, RDA=relative DNase activity, RCA/RDA=halo diameter (zone) – bacterial growth diameter/ bacterial growth diameter (Zacaria *et al.*, 2010).

Table A3. Biofilm formation of 99 *Aeromonas* spp. isolates following incubation at room temperature (RT, ≈21 °C), 30 °C, or 37 °C, under shaking or static conditions in nutrient-poor (EAOB) media, respectively

Code	Source	Species	RT EAOB	SD	RT EAOB	SD	30 EAOB	SD	30 EAOB	SD	37 EAOB	SD	37 EAOB	SD
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			SH*		ST*		SH*		ST*		SH*		ST*	
M1	Catfish	<i>A. culicicola</i>	0.187	±0.037	0.422	±0.047	0.180	±0.037	0.278	±0.009	0.239	±0.023	0.386	±0.045
M2	Catfish	<i>A. hydrophila</i>	1.759	±0.043	1.824	±0.146	0.282	±0.009	0.361	±0.037	0.301	±0.045	0.259	±0.011
M3	Catfish	<i>A. allosaccharophila</i>	0.465	±0.014	0.716	±0.052	0.682	±0.107	1.291	±0.183	0.388	±0.054	0.374	±0.121
M4	Catfish	<i>A. jandaei</i>	0.877	±0.058	1.188	±0.084	0.563	±0.014	0.259	±0.010	0.453	±0.081	0.500	±0.059
M5	Catfish	<i>A. hydrophila</i>	0.195	±0.016	0.280	±0.049	0.608	±0.059	0.564	±0.057	0.154	±0.019	0.171	±0.022
M6	Catfish	<i>A. hydrophila</i>	0.358	±0.039	0.213	±0.049	0.316	±0.031	0.174	±0.011	0.461	±0.017	0.169	±0.022
M7	Catfish	<i>A. culicicola</i>	0.200	±0.049	0.253	±0.031	0.196	±0.013	0.179	±0.009	0.597	±0.039	0.719	±0.050
M8	Tilapia	<i>A. allosaccharophila</i>	0.474	±0.084	0.640	±0.037	0.346	±0.045	0.565	±0.062	0.857	±0.048	0.799	±0.056
M9	Catfish	<i>Plesiomonasshigelloides</i>	0.142	±0.018	0.155	±0.024	0.154	±0.034	0.147	±0.016	0.162	±0.017	0.169	±0.049
M10	Catfish	<i>A. culicicola/jandaei</i>	2.242	±0.231	1.928	±0.168	1.492	±0.133	1.321	±0.179	0.658	±0.212	0.622	±0.047
M11	Catfish	<i>Aeromonas</i> spp.	0.328	±0.014	0.441	±0.028	0.622	±0.028	0.478	±0.108	0.465	±0.051	0.462	±0.095
M12	Catfish	<i>A. bestiarum</i>	0.249	±0.045	0.563	±0.087	0.168	±0.022	0.136	±0.024	0.120	±0.004	0.161	±0.006
M13	Catfish	<i>A. hydrophila</i>	0.227	±0.029	0.355	±0.037	0.194	±0.013	0.263	±0.028	0.232	±0.015	0.406	±0.093
M14	Tilapia	<i>A. hydrophila</i>	0.971	±0.046	0.736	±0.033	0.855	±0.053	1.017	±0.035	0.578	±0.017	0.958	±0.071
M15	Tilapia	<i>Aeromonas</i> spp.	0.292	±0.038	0.266	±0.023	0.193	±0.024	0.209	±0.045	0.183	±0.005	0.189	±0.017
M16	Tilapia	<i>A. jandaei</i>	1.638	±0.297	0.828	±0.025	1.380	±0.208	1.243	±0.037	0.703	±0.064	0.898	±0.041
M17	Tilapia	<i>A. hydrophila</i>	0.194	±0.004	0.168	±0.017	0.278	±0.061	0.168	±0.027	0.156	±0.022	0.150	±0.027
M18	Tilapia	<i>A. caviae</i>	0.147	±0.028	0.145	±0.006	0.187	±0.022	0.185	±0.021	0.150	±0.022	0.168	±0.025
M19	Tilapia	<i>A. jandaei</i>	0.349	±0.042	0.327	±0.062	0.155	±0.021	0.169	±0.033	0.989	±0.070	0.312	±0.028
M20	Tilapia	<i>Aeromonas</i> spp.	3.286	±0.182	2.437	±0.286	2.383	±0.284	1.234	±0.064	0.219	±0.069	0.318	±0.033
M21	Tilapia	<i>A. allosaccharophila</i>	1.690	±0.345	2.003	±0.074	0.457	±0.046	1.754	±0.338	0.154	±0.020	0.169	±0.040
M22	Seawater	<i>A. culicicola</i>	0.131	±0.003	0.135	±0.015	0.385	±0.113	0.138	±0.005	0.188	±0.026	0.148	±0.021
M23	Seawater	<i>A. culicicola</i>	0.633	±0.067	0.246	±0.023	0.348	±0.030	0.179	±0.090	0.250	±0.040	0.326	±0.034
M24	Seawater	<i>A. jandaei</i>	1.250	±0.112	1.181	±0.147	0.289	±0.033	0.260	±0.049	0.227	±0.013	0.164	±0.011
M25	Seawater	<i>A. culicicola</i>	0.182	±0.022	0.163	±0.013	0.164	±0.014	0.174	±0.018	0.270	±0.060	0.198	±0.006
M26	Seawater	<i>Aeromonas</i> spp. 45	1.673	±0.076	1.688	±0.106	1.264	±0.187	0.781	±0.044	0.285	±0.005	0.347	±0.058
M27	Seawater	<i>Aeromonas</i> spp. 45	1.220	±0.067	1.564	±0.061	0.442	±0.004	0.412	±0.096	0.238	±0.044	0.212	±0.011
M28	Seawater	<i>A. jandaei</i>	0.411	±0.032	0.171	±0.012	0.646	±0.079	0.227	±0.009	0.386	±0.045	0.317	±0.037

M29	Seawater	<i>A. culicicola</i>	1.181	±0.115	1.163	±0.222	0.797	±0.094	0.761	±0.047	0.218	±0.012	0.272	±0.077
M30	Seawater	<i>A. jandaei</i>	0.142	±0.005	0.145	±0.016	0.512	±0.070	0.149	±0.010	0.603	±0.099	0.145	±0.032
M31	Seawater	<i>A. culicicola</i>	0.148	±0.024	0.171	±0.051	0.702	±0.129	0.298	±0.028	0.453	±0.005	0.225	±0.016
M32	Seawater	<i>A. culicicola</i>	0.234	±0.016	0.237	±0.030	0.224	±0.012	0.293	±0.015	0.168	±0.050	0.295	±0.060
M33	Seawater	<i>Aeromonas</i> spp. 310	1.031	±0.019	0.888	±0.052	0.525	±0.171	0.786	±0.082	0.430	±0.049	0.646	±0.079
M34	Seawater	<i>Aeromonas</i> spp. 45	0.132	±0.005	0.166	±0.052	0.342	±0.020	0.178	±0.019	0.149	±0.022	0.166	±0.008
M35	Seawater	<i>A. culicicola</i>	1.275	±0.089	1.636	±0.166	0.530	±0.072	0.770	±0.100	0.678	±0.062	0.550	±0.087
M36	Seawater	<i>A. jandaei</i>	0.454	±0.004	1.431	±0.141	0.265	±0.087	0.820	±0.081	0.155	±0.028	0.381	±0.017
M37	Seawater	<i>Aeromonas</i> spp. 45	1.257	±0.205	0.997	±0.031	0.177	±0.009	0.272	±0.034	0.370	±0.066	0.165	±0.009
M38	Seawater	<i>A. culicicola</i>	1.061	±0.056	1.333	±0.163	0.238	±0.039	0.937	±0.083	0.185	±0.016	0.191	±0.030
M39	Seawater	<i>A. culicicola</i>	1.719	±0.286	1.763	±0.042	1.323	±0.258	1.353	±0.226	0.147	±0.004	0.286	±0.045
M40	Seawater	<i>A. culicicola</i>	0.643	±0.027	0.345	±0.027	0.127	±0.003	0.169	±0.012	0.175	±0.025	0.237	±0.019
M41	Seawater	<i>Aeromonas</i> spp.	1.863	±0.106	1.559	±0.294	1.280	±0.096	1.216	±0.137	0.624	±0.074	0.512	±0.070
M42	Tilapia	<i>A. culicicola</i>	0.553	±0.069	0.163	±0.011	0.234	±0.040	0.163	±0.026	0.434	±0.005	0.162	±0.014
M43	Tilapia	<i>Aeromonas</i> spp. 310	0.133	±0.015	0.129	±0.010	0.141	±0.012	0.146	±0.008	0.284	±0.035	0.294	±0.075
M44	Tilapia	<i>A. ichthosmia</i>	1.325	±0.211	0.688	±0.074	1.427	±0.059	0.538	±0.031	0.388	±0.029	0.384	±0.063
M45	Tilapia	<i>Plesiomonasshigelloides</i>	1.237	±0.272	1.182	±0.110	0.487	±0.058	0.828	±0.112	0.463	±0.044	0.211	±0.011
M46	Tilapia	<i>Plesiomonasshigelloides</i>	0.153	±0.019	0.171	±0.018	0.187	±0.023	0.211	±0.012	0.429	±0.008	0.213	±0.003
M47	Tilapia	<i>Plesiomonasshigelloides</i>	0.129	±0.007	0.158	±0.025	0.224	±0.042	0.987	±0.064	0.772	±0.006	0.764	±0.035
M48	Tilapia	<i>A. ichthosmia</i>	0.179	±0.017	0.170	±0.011	0.199	±0.032	0.251	±0.038	0.461	±0.017	0.420	±0.054
M49	Tilapia	<i>A. sobria</i>	1.201	±0.072	0.941	±0.030	0.772	±0.112	0.769	±0.111	0.384	±0.094	0.270	±0.060
M50	Catfish	<i>A. hydrophila</i>	0.294	±0.034	0.429	±0.089	0.458	±0.058	0.169	±0.016	0.310	±0.066	0.302	±0.045
M51	Catfish	<i>A. hydrophila</i>	1.826	±0.233	1.841	±0.015	1.338	±0.137	1.500	±0.235	0.274	±0.050	0.376	±0.080
M52	Tilapia	<i>A. hydrophila</i>	1.114	±0.086	0.700	±0.039	1.333	±0.147	1.042	±0.129	0.794	±0.057	1.432	±0.393
M53	Catfish	<i>A. hydrophila</i>	1.413	±0.211	0.760	±0.085	1.331	±0.086	1.291	±0.037	0.554	±0.091	0.514	±0.189
M54	Tilapia	<i>A. ichthosmia</i>	1.005	±0.050	0.773	±0.085	0.697	±0.014	1.281	±0.026	0.429	±0.008	0.757	±0.033
M55	Tilapia	<i>A. veronii/A. culicicola</i>	0.189	±0.017	0.202	±0.089	0.193	±0.005	0.315	±0.054	0.152	±0.004	0.226	±0.029
M56	Tilapia	<i>A. culicicola</i>	0.507	±0.007	0.702	±0.102	0.613	±0.034	0.234	±0.019	0.592	±0.098	0.930	±0.048
M57	Tilapia	<i>A. veronii</i>	0.336	±0.043	0.214	±0.068	0.619	±0.070	0.593	±0.068	0.485	±0.050	0.457	±0.055
M58	Tilapia	<i>A. culicicola</i>	1.036	±0.046	1.439	±0.325	1.112	±0.107	1.314	±0.242	0.635	±0.080	0.696	±0.151

M59	Tilapia	<i>A. caviae</i>	0.165	±0.036	0.181	±0.057	0.154	±0.014	0.227	±0.035	0.153	0.013	0.397	±0.058
M60	Tilapia	<i>A. hydrophila</i>	1.239	±0.099	1.047	±0.186	0.862	±0.047	1.302	±0.036	0.288	±0.074	0.577	±0.058
M61	Tilapia	<i>A. culicicola</i>	0.206	±0.060	0.180	±0.055	0.420	±0.062	0.166	±0.015	0.177	±0.038	0.203	±0.030
M62	Tilapia	<i>A. hydrophila</i>	0.228	±0.027	0.215	±0.042	0.196	±0.030	0.189	±0.035	0.154	±0.021	0.237	±0.070
M63	Tilapia	<i>A. veronii</i>	0.274	±0.081	0.280	±0.026	0.194	±0.015	0.271	±0.053	0.231	±0.046	0.260	±0.078
M64	Tilapia	<i>A. hydrophila</i>	0.647	±0.022	0.554	±0.017	0.737	±0.090	0.238	±0.027	0.319	±0.096	0.529	±0.073
M65	Tilapia	<i>A. hydrophila</i>	1.170	±0.095	0.874	±0.020	0.977	±0.043	1.446	±0.209	0.239	±0.045	0.318	±0.108
M66	Tilapia	<i>Plesiomonasshigelloides</i>	0.963	±0.074	0.470	±0.064	0.795	±0.060	1.213	±0.066	0.548	±0.100	0.739	±0.060
M67	Tilapia	<i>Plesiomonasshigelloides</i>	0.802	±0.050	0.492	±0.029	0.849	±0.058	1.228	±0.012	0.569	±0.058	0.864	±0.051
M68	Goldfish	<i>A. caviae</i>	0.271	±0.071	0.242	±0.011	0.382	±0.071	0.271	±0.055	0.464	±0.086	0.485	±0.025
M69	Goldfish	<i>A. bestiarum</i>	0.747	±0.106	1.265	±0.081	1.456	±0.314	1.158	±0.085	0.360	±0.051	0.450	±0.168
M70	Goldfish	<i>A. bestiarum</i>	0.796	±0.072	0.783	±0.029	0.505	±0.044	1.264	±0.170	0.823	±0.199	0.807	±0.021
M71	Goldfish	<i>A. bestiarum</i>	0.623	±0.065	0.786	±0.067	0.530	±0.069	1.242	±0.115	0.350	±0.023	0.462	±0.107
M72	Goldfish	<i>A. bestiarum</i>	0.241	±0.010	0.207	±0.032	0.181	±0.021	0.232	±0.068	0.164	±0.012	0.420	±0.025
M73	Goldfish	<i>A. bestiarum</i>	0.555	±0.048	0.639	±0.028	0.806	±0.050	0.744	±0.050	0.318	±0.057	0.423	±0.068
M74	Goldfish	<i>A. allosaccharophila</i>	0.650	±0.038	0.630	±0.037	0.880	±0.080	0.709	±0.013	0.646	±0.015	0.804	±0.127
M75	Goldfish	<i>Aeromonas. spp. 45</i>	0.778	±0.068	0.401	±0.071	0.611	±0.012	0.496	±0.054	0.553	±0.114	0.887	±0.063
M76	Goldfish	<i>A. salmonicida</i>	1.438	±0.197	1.400	±0.063	1.231	±0.124	1.376	±0.160	0.629	±0.070	0.684	±0.032
M77	Goldfish	<i>A. salmonicida</i>	0.327	±0.039	0.295	±0.026	0.628	±0.062	0.289	±0.027	0.693	±0.108	0.619	±0.021
M78	Goldfish	<i>A. bestiarum</i>	0.473	±0.063	0.378	±0.007	1.467	±0.056	0.716	±0.156	0.776	±0.112	0.570	±0.060
M79	Goldfish	<i>A. bestiarum</i>	1.100	±0.065	1.125	±0.050	0.772	±0.034	0.834	±0.051	0.526	±0.067	0.435	±0.068
M80	Goldfish	<i>A. bestiarum</i>	1.136	±0.042	0.678	±0.015	0.408	±0.014	0.537	±0.023	0.192	±0.049	0.429	±0.057
M81	Goldfish	<i>A. bestiarum</i>	0.814	±0.155	0.548	±0.007	0.860	±0.091	1.219	±0.052	0.260	±0.044	0.414	±0.070
M82	Goldfish	<i>A. culicicola</i>	1.832	±0.082	1.649	±0.233	0.829	±0.057	1.405	±0.203	0.287	±0.076	0.393	±0.089
M83	Goldfish	<i>A. bestiarum</i>	1.550	±0.122	1.855	±0.179	0.583	±0.064	1.156	±0.127	0.186	±0.023	0.458	±0.070
M84	Goldfish	<i>A. bestiarum</i>	1.463	±0.157	1.151	±0.220	0.748	±0.019	1.272	±0.049	0.338	±0.087	0.781	±0.078
M85	Goldfish	<i>A. allosaccharophila</i>	0.837	±0.084	1.557	±0.335	1.014	±0.053	1.455	±0.282	0.279	±0.058	0.422	±0.050
M86	Goldfish	<i>A. hydrophila</i>	0.625	±0.111	0.781	±0.080	0.259	±0.025	0.373	±0.036	0.163	±0.017	0.234	±0.022
M87	Goldfish	<i>A. bestiarum</i>	0.377	±0.029	0.518	±0.036	0.369	±0.060	0.255	±0.053	0.183	±0.024	0.216	±0.010
M88	Goldfish	<i>A. bestiarum</i>	1.305	±0.200	0.861	±0.026	0.665	±0.058	1.129	±0.087	0.407	±0.091	0.342	±0.017

M89	Goldfish	<i>A. allosaccharophila</i>	0.551	±0.049	1.409	±0.264	0.592	±0.090	0.747	±0.079	0.167	±0.009	0.190	±0.009
M90	Goldfish	<i>A. bestiarum</i>	0.272	±0.048	0.343	±0.013	0.399	±0.078	0.429	±0.081	0.468	±0.010	0.514	±0.065
M91	Goldfish	<i>A. bestiarum</i>	0.537	±0.106	0.208	±0.003	0.271	±0.055	0.237	±0.002	0.472	±0.082	0.367	±0.032
M92	Goldfish	<i>A. allosaccharophila</i>	1.321	±0.193	0.781	±0.051	1.335	±0.030	0.979	±0.069	0.735	±0.021	0.648	±0.069
M93	Goldfish	<i>A. allosaccharophila</i>	1.426	±0.154	1.316	±0.142	0.501	±0.063	0.693	±0.099	0.284	±0.054	0.258	±0.031
M94	Goldfish	<i>A. hydrophila</i>	1.539	±0.198	1.240	±0.214	1.362	±0.204	1.292	±0.067	0.740	±0.106	0.636	±0.071
M95	Goldfish	<i>A. hydrophila</i>	0.923	±0.111	1.188	±0.012	1.108	±0.049	1.460	±0.228	0.648	±0.105	0.640	±0.098
M96	Goldfish	<i>A. bestiarum</i>	1.026	±0.059	1.724	±0.095	0.306	±0.057	0.572	±0.023	0.284	±0.044	0.256	±0.036
M97	Goldfish	<i>A. bestiarum</i>	0.180	±0.058	0.124	±0.016	0.386	±0.045	0.386	±0.103	0.257	±0.065	0.275	±0.120
M98	Goldfish	<i>Aeromonas</i> spp. 310	1.040	±0.056	1.209	±0.243	0.199	±0.061	0.315	±0.017	0.327	±0.110	0.254	±0.027
M99	Goldfish	<i>A. bestiarum</i>	1.282	±0.052	0.830	±0.042	0.198	±0.022	0.349	±0.051	0.213	±0.022	0.220	±0.016

*RT EAOB SH=room temperature (RT, ≈21 °C) under shaking conditions in nutrient-poor EAOB media, RT EAOB ST=room temperature (RT, ≈21 °C) under static conditions in nutrient-poor EAOB media, 30 EAOB SH=30 °C under shaking conditions in nutrient-poor EAOB media, 30 EAOB ST=30 °C under static conditions in nutrient-poor EAOB media, 37 EAOB SH=37 °C under shaking conditions in nutrient-poor EAOB media, 37 EAOB ST=37 °C under static conditions in nutrient-poor EAOB media, SD=standard deviation.

Table A4. Biofilm formation of 99 *Aeromonas* spp. isolates following incubation at room temperature (RT, ≈21 °C), 30 °C, or 37 °C, under shaking or static conditions in nutrient-rich (TSB) media, respectively

Code	Source	Species	RT TSB SH*	SD	RT TSB ST*	SD	30 TSB SH*	SD	30 TSB ST*	SD	37 TSB SH*	SD	37 TSB ST*	SD
M1	Catfish	<i>A. culicicola</i>	1.257	±0.111	1.435	±0.132	1.691	±0.158	1.059	±0.072	0.962	±0.049	1.665	±0.175
M2	Catfish	<i>A. hydrophila</i>	1.076	±0.087	1.203	±0.086	0.695	±0.040	1.277	±0.035	0.847	±0.055	1.357	±0.122
M3	Catfish	<i>A. allosaccharophila</i>	1.138	±0.056	0.722	±0.072	2.012	±0.053	2.192	±0.104	0.745	±0.090	0.756	±0.074
M4	Catfish	<i>A. jandaei</i>	1.448	±0.367	1.506	±0.142	2.164	±0.079	3.026	±0.230	0.565	±0.124	0.603	±0.120
M5	Catfish	<i>A. hydrophila</i>	1.579	±0.024	1.218	±0.155	1.144	±0.031	1.628	±0.359	1.275	±0.166	1.668	±0.453

M6	Catfish	<i>A. hydrophila</i>	1.318	±0.282	1.161	±0.100	1.825	±0.164	1.150	±0.196	1.793	±0.163	1.390	±0.151
M7	Catfish	<i>A. culicicola</i>	1.525	±0.119	0.448	±0.144	1.342	±0.288	2.506	±0.285	1.622	±0.373	1.492	±0.258
M8	Tilapia	<i>A. allosaccharophila</i>	1.722	±0.280	1.260	±0.319	1.298	±0.232	1.158	±0.156	0.711	±0.070	1.550	±0.224
M9	Catfish	<i>Plesiomonas shigelloides</i>	0.271	±0.048	0.292	±0.061	0.956	±0.086	1.686	±0.237	0.909	±0.093	0.382	±0.013
M10	Catfish	<i>A. culicicola/jandaei</i>	2.002	±0.133	1.150	±0.108	2.510	±0.077	1.821	±0.141	0.411	±0.093	0.349	±0.088
M11	Catfish	<i>Aeromonas</i> spp.	1.225	±0.098	1.038	±0.094	1.526	±0.347	1.584	±0.227	0.763	±0.202	0.577	±0.185
M12	Catfish	<i>A. bestiarum</i>	1.582	±0.318	2.648	±0.477	1.747	±0.153	1.150	±0.120	0.908	±0.077	0.627	±0.049
M13	Catfish	<i>A. hydrophila</i>	0.554	±0.163	0.480	±0.042	1.178	±0.053	1.417	±0.347	0.748	±0.125	0.826	±0.044
M14	Tilapia	<i>A. hydrophila</i>	1.639	±0.216	1.565	±0.312	1.767	±0.174	1.956	±0.144	1.459	±0.163	2.206	±0.102
M15	Tilapia	<i>Aeromonas</i> spp.	0.724	±0.056	0.423	±0.060	0.475	±0.052	0.467	±0.075	0.540	±0.075	0.597	±0.017
M16	Tilapia	<i>A. jandaei</i>	0.805	±0.060	2.030	±0.101	2.478	±0.204	2.670	±0.046	0.665	±0.044	0.534	±0.035
M17	Tilapia	<i>A. hydrophila</i>	0.735	±0.044	1.326	±0.215	1.364	±0.321	1.192	±0.084	1.209	±0.144	2.149	±0.143
M18	Tilapia	<i>A. caviae</i>	1.297	±0.046	0.854	±0.064	1.025	±0.149	1.238	±0.088	0.852	±0.059	0.764	±0.042
M19	Tilapia	<i>A. jandaei</i>	0.668	±0.055	0.796	±0.075	1.315	±0.194	0.533	±0.072	0.697	±0.075	0.425	±0.054
M20	Tilapia	<i>Aeromonas</i> spp.	1.290	±0.313	1.584	±0.024	1.068	±0.072	1.013	±0.111	0.695	±0.051	0.669	±0.008
M21	Tilapia	<i>A. allosaccharophila</i>	2.718	±0.069	1.431	±0.118	0.504	±0.011	0.662	±0.090	0.755	±0.042	1.702	±0.224
M22	Seawater	<i>A. culicicola</i>	0.539	±0.044	0.495	±0.075	0.462	±0.023	0.915	±0.107	0.797	±0.082	0.782	±0.132
M23	Seawater	<i>A. culicicola</i>	1.507	±0.251	1.154	±0.093	1.470	±0.124	1.603	±0.166	0.954	±0.052	1.651	±0.114
M24	Seawater	<i>A. jandaei</i>	1.825	±0.231	1.954	±0.027	4.000	±0.000	2.354	±0.263	1.404	±0.166	1.231	±0.170
M25	Seawater	<i>A. culicicola</i>	0.829	±0.064	0.993	±0.033	1.067	±0.075	1.347	±0.215	0.907	±0.074	1.233	±0.239
M26	Seawater	<i>Aeromonas</i> spp. 45	1.303	±0.284	1.216	±0.220	1.033	±0.013	0.882	±0.025	0.823	±0.046	0.918	±0.084
M27	Seawater	<i>Aeromonas</i> spp. 45	1.784	±0.084	1.856	±0.061	0.925	±0.035	1.281	±0.045	1.000	±0.006	2.230	±0.523
M28	Seawater	<i>A. jandaei</i>	0.866	±0.033	1.144	±0.031	1.480	±0.048	1.274	±0.106	0.759	±0.030	1.442	±0.184
M29	Seawater	<i>A. culicicola</i>	2.257	±0.313	1.553	±0.045	0.437	±0.037	1.358	±0.227	0.727	±0.059	0.822	±0.076
M30	Seawater	<i>A. jandaei</i>	1.043	±0.026	0.999	±0.084	0.377	±0.017	2.816	±0.061	1.085	±0.101	2.128	±0.179
M31	Seawater	<i>A. culicicola</i>	0.923	±0.084	0.625	±0.074	1.254	±0.015	1.285	±0.120	0.971	±0.067	1.674	±0.139
M32	Seawater	<i>A. culicicola</i>	1.080	±0.082	1.456	±0.285	0.940	±0.021	0.919	±0.078	0.565	±0.019	1.478	±0.269
M33	Seawater	<i>Aeromonas</i> spp. 310	1.630	±0.160	1.732	±0.363	1.987	±0.106	0.913	±0.076	1.659	±0.191	1.402	±0.438
M34	Seawater	<i>Aeromonas</i> spp. 45	0.594	±0.037	0.662	±0.095	0.802	±0.074	1.204	0.068	1.153	±0.016	2.674	±0.292
M35	Seawater	<i>A. culicicola</i>	1.725	±0.205	1.550	±0.406	1.533	±0.261	1.727	±0.179	1.676	±0.213	1.276	±0.104

M36	Seawater	<i>A. jandaei</i>	1.355	±0.107	1.581	±0.375	0.759	±0.040	1.200	±0.107	0.563	±0.079	1.782	±0.485
M37	Seawater	<i>Aeromonas</i> spp. 45	1.633	±0.030	1.154	±0.082	1.364	±0.032	1.663	±0.160	0.932	±0.084	1.656	±0.201
M38	Seawater	<i>A. culicicola</i>	0.880	±0.051	1.591	±0.117	1.428	±0.156	1.248	±0.283	1.463	±0.391	1.606	±0.259
M39	Seawater	<i>A. culicicola</i>	1.115	±0.048	1.710	±0.190	0.873	±0.003	0.937	±0.081	1.163	±0.182	1.404	±0.201
M40	Seawater	<i>A. culicicola</i>	0.738	±0.078	1.351	±0.181	0.896	±0.093	2.254	±0.149	0.785	±0.019	1.596	±0.304
M41	Seawater	<i>Aeromonas</i> spp.	1.574	±0.320	1.354	±0.426	1.516	±0.073	1.404	±0.391	0.766	±0.024	0.882	±0.025
M42	Tilapia	<i>A. culicicola</i>	1.405	±0.061	1.636	±0.333	1.300	±0.342	0.890	±0.098	1.597	±0.045	0.876	±0.088
M43	Tilapia	<i>Aeromonas</i> spp. 310	1.377	±0.121	1.237	±0.109	1.647	±0.362	1.707	±0.144	0.950	±0.045	2.291	±0.096
M44	Tilapia	<i>A. ichthiosmia</i>	2.211	±0.057	1.608	±0.068	1.790	±0.180	1.238	±0.131	0.433	±0.043	0.455	±0.142
M45	Tilapia	<i>Plesiomonasshigelloides</i>	1.481	±0.340	1.800	±0.092	1.105	±0.017	1.530	±0.042	1.132	±0.060	1.810	±0.135
M46	Tilapia	<i>Plesiomonasshigelloides</i>	0.554	±0.018	1.631	±0.279	1.271	±0.180	1.186	±0.048	0.725	±0.102	0.458	±0.097
M47	Tilapia	<i>Plesiomonasshigelloides</i>	1.595	±0.481	0.641	±0.067	1.393	±0.196	1.301	±0.118	1.130	±0.039	0.868	±0.097
M48	Tilapia	<i>A. ichthiosmia</i>	0.773	±0.073	0.984	±0.034	1.340	±0.177	1.394	±0.071	1.334	±0.239	1.367	±0.318
M49	Tilapia	<i>A. sobria</i>	1.535	±0.401	2.205	±0.172	1.231	±0.032	1.710	±0.280	1.533	±0.060	1.657	±0.242
M50	Catfish	<i>A. hydrophila</i>	1.703	±0.161	1.366	±0.348	1.470	±0.271	1.621	±0.484	0.736	±0.025	0.475	±0.082
M51	Catfish	<i>A. hydrophila</i>	1.622	±0.024	2.254	±0.277	1.074	±0.108	1.446	±0.101	0.381	±0.011	0.317	±0.128
M52	Tilapia	<i>A. hydrophila</i>	1.642	±0.202	1.210	±0.383	1.613	±0.167	1.532	±0.072	1.458	±0.234	0.786	±0.132
M53	Catfish	<i>A. hydrophila</i>	2.210	±0.051	1.609	±0.070	2.171	±0.096	1.867	±0.134	0.641	±0.031	1.688	±0.151
M54	Tilapia	<i>A. ichthiosmia</i>	1.308	±0.262	1.501	±0.394	1.514	±0.313	2.320	±0.043	0.712	±0.073	0.731	±0.180
M55	Tilapia	<i>A. veronii/A. culicicola</i>	1.418	±0.286	1.309	±0.042	1.628	±0.116	1.266	±0.061	1.101	±0.040	1.214	±0.033
M56	Tilapia	<i>A. culicicola</i>	1.571	±0.221	1.677	±0.399	1.633	±0.266	1.146	±0.036	1.487	±0.423	1.410	±0.126
M57	Tilapia	<i>A. veronii</i>	1.786	±0.066	1.434	±0.376	1.146	±0.237	0.822	±0.099	0.612	±0.015	0.729	±0.112
M58	Tilapia	<i>A. culicicola</i>	1.543	±0.256	1.559	±0.203	1.060	±0.096	1.571	±0.157	1.988	±0.251	0.868	±0.094
M59	Tilapia	<i>A. caviae</i>	1.270	±0.078	0.595	±0.030	1.354	±0.338	1.255	±0.241	0.696	±0.093	1.133	±0.133
M60	Tilapia	<i>A. hydrophila</i>	1.405	±0.236	1.414	±0.139	1.232	±0.130	1.575	±0.359	0.977	±0.121	1.361	±0.445
M61	Tilapia	<i>A. culicicola</i>	1.437	±0.218	0.822	±0.097	1.546	±0.020	1.722	±0.146	0.853	±0.036	1.455	±0.334
M62	Tilapia	<i>A. hydrophila</i>	0.325	±0.025	0.576	±0.064	0.324	±0.077	0.451	±0.068	0.180	±0.026	0.425	±0.023
M63	Tilapia	<i>A. veronii</i>	0.379	±0.061	0.546	±0.120	0.778	±0.025	1.656	±0.155	0.281	±0.048	0.769	±0.120
M64	Tilapia	<i>A. hydrophila</i>	0.812	±0.101	0.620	±0.058	1.194	±0.228	1.616	±0.195	1.195	±0.053	1.330	±0.137
M65	Tilapia	<i>A. hydrophila</i>	1.138	±0.045	1.208	±0.163	1.522	±0.267	1.665	±0.222	1.232	±0.144	1.799	±0.347

M66	Tilapia	<i>Plesiomonas shigelloides</i>	1.838	±0.209	2.084	±0.021	1.345	±0.183	2.188	±0.232	1.326	±0.201	1.816	±0.151
M67	Tilapia	<i>Plesiomonas shigelloides</i>	1.759	±0.105	1.413	±0.262	1.493	±0.134	1.533	±0.127	1.262	±0.115	1.110	±0.081
M68	Goldfish	<i>A. caviae</i>	0.724	±0.040	0.716	±0.110	1.638	±0.235	3.432	±0.557	0.543	±0.043	0.482	±0.059
M69	Goldfish	<i>A. bestiarum</i>	1.439	±0.208	1.181	±0.205	1.894	±0.127	2.461	±0.411	0.762	±0.017	3.145	±0.900
M70	Goldfish	<i>A. bestiarum</i>	1.380	±0.244	1.745	±0.308	2.318	±0.089	1.260	±0.236	1.442	±0.198	3.651	±0.494
M71	Goldfish	<i>A. bestiarum</i>	1.143	±0.235	0.785	±0.042	1.302	±0.177	0.665	±0.094	0.319	±0.068	0.428	±0.082
M72	Goldfish	<i>A. bestiarum</i>	0.413	±0.031	0.497	±0.071	0.560	±0.054	0.717	±0.107	0.232	±0.080	0.378	±0.060
M73	Goldfish	<i>A. bestiarum</i>	1.270	±0.212	1.488	±0.209	1.152	±0.130	1.548	±0.169	0.718	±0.059	0.737	±0.074
M74	Goldfish	<i>A. allosaccharophila</i>	1.404	±0.124	2.121	±0.282	3.267	±0.349	2.430	±0.195	0.435	±0.009	0.784	±0.096
M75	Goldfish	<i>Aeromonas. spp. 45</i>	2.119	±0.032	1.594	±0.264	1.735	±0.105	1.453	±0.273	1.162	±0.113	1.339	±0.213
M76	Goldfish	<i>A. salmonicida</i>	0.853	±0.078	0.861	±0.016	1.200	±0.109	1.442	±0.098	0.544	±0.014	0.447	±0.039
M77	Goldfish	<i>A. salmonicida</i>	0.728	±0.106	1.400	±0.177	1.142	±0.151	1.413	±0.291	0.537	±0.063	0.333	±0.047
M78	Goldfish	<i>A. bestiarum</i>	3.068	±0.494	1.997	±0.081	3.460	±0.326	1.815	±0.165	1.197	±0.000	3.471	±0.069
M79	Goldfish	<i>A. bestiarum</i>	0.777	±0.035	0.878	±0.044	1.552	±0.110	1.238	±0.172	1.145	±0.175	1.357	±0.232
M80	Goldfish	<i>A. bestiarum</i>	0.646	±0.096	0.889	±0.058	0.699	±0.119	1.147	±0.243	1.522	±0.159	1.410	±0.286
M81	Goldfish	<i>A. bestiarum</i>	1.490	±0.250	1.610	±0.056	1.822	±0.055	1.198	±0.088	0.632	±0.086	0.488	±0.258
M82	Goldfish	<i>A. culicicola</i>	1.411	±0.113	3.004	±0.378	1.551	±0.136	1.586	±0.036	0.629	±0.093	0.620	±0.078
M83	Goldfish	<i>A. bestiarum</i>	0.602	±0.066	0.505	±0.040	0.419	±0.053	0.651	±0.077	0.324	±0.130	0.277	±0.040
M84	Goldfish	<i>A. bestiarum</i>	1.477	±0.155	3.590	±0.358	2.102	±0.150	1.570	±0.484	0.757	±0.045	0.588	±0.064
M85	Goldfish	<i>A. allosaccharophila</i>	1.038	±0.120	1.085	±0.078	1.406	±0.244	1.579	±0.229	1.355	±0.086	0.493	±0.095
M86	Goldfish	<i>A. hydrophila</i>	0.702	±0.085	0.601	±0.084	0.780	±0.096	0.532	±0.078	0.698	±0.064	0.261	±0.099
M87	Goldfish	<i>A. bestiarum</i>	1.063	±0.057	1.804	±0.075	1.965	±0.312	1.414	±0.139	0.554	±0.036	0.508	±0.107
M88	Goldfish	<i>A. bestiarum</i>	0.433	±0.076	0.366	±0.021	1.506	±0.198	1.682	±0.186	0.504	±0.104	0.462	±0.090
M89	Goldfish	<i>A. allosaccharophila</i>	0.705	±0.098	0.437	±0.060	0.643	±0.083	0.502	±0.056	0.283	±0.047	0.308	±0.032
M90	Goldfish	<i>A. bestiarum</i>	0.850	±0.091	1.530	±0.112	1.074	±0.070	1.565	±0.204	0.485	±0.080	0.365	±0.102
M91	Goldfish	<i>A. bestiarum</i>	0.729	±0.114	0.723	±0.052	1.564	±0.382	1.580	±0.340	0.588	±0.065	0.465	±0.051
M92	Goldfish	<i>A. allosaccharophila</i>	1.609	±0.155	1.547	±0.231	1.630	±0.265	1.424	±0.345	0.474	±0.066	0.388	±0.069
M93	Goldfish	<i>A. allosaccharophila</i>	1.162	±0.113	1.451	±0.286	0.656	±0.042	0.354	±0.074	0.977	±0.082	1.897	±0.096
M94	Goldfish	<i>A. hydrophila</i>	1.248	±0.136	1.168	±0.094	2.580	±0.117	2.696	±0.440	0.428	±0.064	1.822	±0.836
M95	Goldfish	<i>A. hydrophila</i>	0.888	±0.094	1.334	±0.140	1.410	±0.199	1.930	±0.066	0.704	±0.049	0.522	±0.044

M96	Goldfish	<i>A. bestiarum</i>	1.755	±0.108	1.371	±0.189	0.739	±0.119	1.337	±0.273	1.640	±0.156	1.177	±0.200
M97	Goldfish	<i>A. bestiarum</i>	0.253	±0.038	0.563	±0.150	1.166	±0.147	1.988	±0.251	1.462	±0.123	1.873	±0.123
M98	Goldfish	<i>Aeromonas</i> spp. 310	1.507	±0.318	2.189	±0.325	1.130	±0.100	1.219	±0.168	1.060	±0.115	1.204	±0.093
M99	Goldfish	<i>A. bestiarum</i>	0.867	±0.032	0.962	±0.041	0.729	±0.020	0.867	±0.025	1.188	±0.169	1.321	±0.386

*RT TSB SH=room temperature (RT, ≈21 °C) under shaking conditions in nutrient-rich TSB media, RT TSB ST=room temperature (RT, ≈21 °C) under static conditions in nutrient-rich TSB media, 30 TSB SH=30 °C under shaking conditions in nutrient-rich TSB media, 30 TSB ST=30 °C under static conditions in nutrient-rich TSB media, 37 TSB SH=37 °C under shaking conditions in nutrient-rich TSB media, 37 TSB ST=37 °C under static conditions in nutrient-rich TSB media, SD=standard deviation.

Table A5. Resistance profiles of 99 *Aeromonas* spp. isolates obtained from different fish hosts or origin

Code	Source	Species	Resistance profiles
M1	Catfish	<i>A. culicicola</i>	NA,OX,T
M2	Catfish	<i>A. hydrophila</i>	AMP,AML,OX,W,RL
M3	Catfish	<i>A. allosaccharophila</i>	AMP,AML,OX
M4	Catfish	<i>A. jandaei</i>	AMP,AML,AMC,OX,W,RL
M5	Catfish	<i>A. hydrophila</i>	AMP,AML,OX,W,RL
M6	Catfish	<i>A. hydrophila</i>	AMP,AML,NA,OX,W
M7	Catfish	<i>A. culicicola</i>	AMP,AMLE,OX,T,W,RL
M8	Tilapia	<i>A. allosaccharophila</i>	AMP,AML,AMC,OX,T,W,RL
M9	Catfish	<i>Plesiomonasshigelloides</i>	OX
M10	Catfish	<i>A. culicicola/jandaei</i>	AMP,AML,CPD,OX,W,RL
M11	Catfish	<i>Aeromonas</i> spp.	AMP,AML,AMC,E,OX,W
M12	Catfish	<i>A. bestiarum</i>	AMP,AML,OX,W,RL
M13	Catfish	<i>A. hydrophila</i>	AMP,AML,OX,W,RL
M14	Tilapia	<i>A. hydrophila</i>	OX

M15	Tilapia	<i>Aeromonas</i> spp.	AMP,AML,AMC,FOX,OX
M16	Tilapia	<i>A. jandaei</i>	AMP,AML,CPD,CXM,FOX,OX,W,RL
M17	Tilapia	<i>A. hydrophila</i>	AMP,AML,OX,W,RL
M18	Tilapia	<i>A. caviae</i>	AMP,AML
M19	Tilapia	<i>A. jandaei</i>	AMP,AML,AMC,FOX,OX,W,RL
M20	Tilapia	<i>Aeromonas</i> spp.	AMP,AML,FOX,CPD,CXM,OX,W,RL,SXT
M21	Tilapia	<i>A. allosaccharophila</i>	AMP,AML,AMC,FOX,CPD,CXM,OX,W,S
M22	Seawater	<i>A. culicicola</i>	AMP,AML,OX,W,RL
M23	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M24	Seawater	<i>A. jandaei</i>	AMP,AML,OX,RL
M25	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M26	Seawater	<i>Aeromonas</i> spp. 45	AMP,AML,OX,RL
M27	Seawater	<i>Aeromonas</i> spp. 45	AMP,E,OX,W,RL
M28	Seawater	<i>A. jandaei</i>	AMP,AML,OX,RL
M29	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M30	Seawater	<i>A. jandaei</i>	AMP,AML,OX,RL
M31	Seawater	<i>A. culicicola</i>	AMP,AML,OX,W,RL
M32	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M33	Seawater	<i>Aeromonas</i> spp. 310	AMP,AML,ATM,NA,OX,W,RL
M34	Seawater	<i>Aeromonas</i> spp. 45	AMP,AML,OX,RL
M35	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M36	Seawater	<i>A. jandaei</i>	AMP,AML,E,OX,RL
M37	Seawater	<i>Aeromonas</i> spp. 45	AMP,AML,OX,T,W,RL
M38	Seawater	<i>A. culicicola</i>	ATM,OX,RL
M39	Seawater	<i>A. culicicola</i>	AMP,AML,OX,RL
M40	Seawater	<i>A. culicicola</i>	AMP,AML,ATM,OX,RL
M41	Seawater	<i>Aeromonas</i> spp.	AMP,AML,OX,RL
M42	Tilapia	<i>A. culicicola</i>	AMP,AML,NA,OX,RL
M43	Tilapia	<i>Aeromonas</i> spp. 310	AMP,AML,NA,OX,RL
M44	Tilapia	<i>A. ichthiosmia</i>	AMP,AML,OX,RL

M45	Tilapia	<i>Plesiomonasshigelloides</i>	AMP,AML,AMC,OX,T,W,RL
M46	Tilapia	<i>Plesiomonasshigelloides</i>	AMP,AML,E,NA,OX,W,S,RL,SXT
M47	Tilapia	<i>Plesiomonasshigelloides</i>	AMP,AML,OX,W,RL
M48	Tilapia	<i>A. ichtiosmia</i>	AMP,AML,OX,W,RL
M49	Tilapia	<i>A. sobria</i>	AMP,AML,AMC,OX,T
M50	Catfish	<i>A. hydrophila</i>	AMP,AML,CXM,E,NA,OX,W,RL
M51	Catfish	<i>A. hydrophila</i>	AMP,AML,AMC,OX,W,RL
M52	Tilapia	<i>A. hydrophila</i>	AMP,AML,AMC,CPD,CXM,E,OX,T,W,RL
M53	Catfish	<i>A. hydrophila</i>	AMP,AML,OX,T,W,RL
M54	Tilapia	<i>A. ichtiosmia</i>	AMP,AML,AMC,E,OX,T,W,RL
M55	Tilapia	<i>A. veronii/A. culicicola</i>	AMP,AML,OX,W,RL
M56	Tilapia	<i>A. culicicola</i>	AMP,AML,E,NA,OX,W,RL
M57	Tilapia	<i>A. veronii</i>	AMP,AML,AMC,CPD,CXM,C,E,OX,T,W,RL
M58	Tilapia	<i>A. culicicola</i>	AMP,AML,AMC,OX,W,RL
M59	Tilapia	<i>A. caviae</i>	AMP,AML,OX,T,W,RL
M60	Tilapia	<i>A. hydrophila</i>	AMP,AML,AMC,E,OX,T,W,RL
M61	Tilapia	<i>A. culicicola</i>	AMP,AML,OX
M62	Tilapia	<i>A. hydrophila</i>	AMP,AML,OX,RW,RL
M63	Tilapia	<i>A. veronii</i>	AMP,AML,AMC,OX,W,RL
M64	Tilapia	<i>A. hydrophila</i>	AMP,AML,CPD,FOX,OX,W,RL
M65	Tilapia	<i>A. hydrophila</i>	AMP,AML,OX,W,RL
M66	Tilapia	<i>Plesiomonasshigelloides</i>	AMP,AML,OX,RL
M67	Tilapia	<i>Plesiomonasshigelloides</i>	AMP,AML,OX,W,RL
M68	Goldfish	<i>A. caviae</i>	AMP,AML,AMC,CPD,OX,W,RL
M69	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CPD,OX,W,S,RL
M70	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CPD,OX,T,W,RL
M71	Goldfish	<i>A. bestiarum</i>	AMP,AML,CPD,CXM,E,OX,W
M72	Goldfish	<i>A. bestiarum</i>	AMP,AML,OX,W,RL
M73	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CXM,FOX,OX,W,RL
M74	Goldfish	<i>A. allosaccharophila</i>	AMP,AML,C,NA,OX,T,W,RL

M75	Goldfish	<i>Aeromonas</i> . spp. 45	AMP,AML,CPD,CXM,C,E,OX,W
M76	Goldfish	<i>A. salmonicida</i>	AMP,AML,AMC,CPD,OX,W,RL
M77	Goldfish	<i>A. salmonicida</i>	AMP,AML,AMC,CPD,OX,W,RL
M78	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CPD,CXM,OX,W,RL
M79	Goldfish	<i>A. bestiarum</i>	APM,AML,OX
M80	Goldfish	<i>A. bestiarum</i>	APM,AML,NA,OX,T,W
M81	Goldfish	<i>A. bestiarum</i>	AMP,AML,CXM,CTX,OX,W,RL
M82	Goldfish	<i>A. culicicola</i>	AMP,AML,AMC,ATM,CPD, CXM,E,FOX,OX,W,RL
M83	Goldfish	<i>A. bestiarum</i>	AMP,AML,OX,W,RL
M84	Goldfish	<i>A. bestiarum</i>	AMP,AML,OX,W,RL
M85	Goldfish	<i>A. allosaccharophila</i>	AMP,AML,AMC,OX,W,RL
M86	Goldfish	<i>A. hydrophila</i>	AMP,AML,AMC,OX,W,RL
M87	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,OX,W,RL
M88	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CPD,CXM,E,FOX,OX,W,RL
M89	Goldfish	<i>A. allosaccharophila</i>	AMP,AML,AMC,CPD,CXM,OX
M90	Goldfish	<i>A. bestiarum</i>	AMP,AML,AMC,CXM,OX,W,RL
M91	Goldfish	<i>A. bestiarum</i>	AMP,AML,OX,W,RL
M92	Goldfish	<i>A. allosaccharophila</i>	AMP,AML,CPD,OX,W,RL
M93	Goldfish	<i>A. allosaccharophila</i>	AMP,AML,C,NA,OX,W,RL
M94	Goldfish	<i>A. hydrophila</i>	AMP,AML,AMC,E,OX,T,W,RL
M95	Goldfish	<i>A. hydrophila</i>	AMP,AML,AMC,CXM,E,OX,T,W,RL
M96	Goldfish	<i>A. bestiarum</i>	E,OX,T
M97	Goldfish	<i>A. bestiarum</i>	E,OX,T
M98	Goldfish	<i>Aeromonas</i> spp. 310	AMP,AML,OX,W,RL
M99	Goldfish	<i>A. bestiarum</i>	AMP,AML,CPD,CXM,OX

AMP=ampicillin, AML=amoxicillin, AMC=augmentin, AZM=azithromycin, ATM=aztreonam, CAZ=ceftazidime, FOX=cefodoxitin, CPD=cefepodoxime, CXM=cefuroxime, CIP=ciprofloxacin, E=erythromycin, GN=gentamicin, NA=nalidixic acid, OX=oxacillin, TE=tetracycline, W=trimethoprim, S=streptomycin, RL=sulphamethoxazole, SXT=cotrimoxazole

