EVALUATING THE POTENTIAL OF ZOSTERIC ACID AND CAPSAICIN FOR USE AS NATURAL PRODUCT ANTIFOULANTS

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EVALUATING THE POTENTIAL OF ZOSTERIC ACID AND CAPSAICIN FOR USE AS NATURAL PRODUCT ANTIFOULANTS

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Thesis

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ABSTRACT

The undesired deposition of cells on a surface and the subsequent formation of a cell layer (biofilm) are called biofouling. Biofouling on the hulls of vessels adversely affects both fuel consumption and maximum attainable speeds. Traditional antifouling paints containing toxic organotin species or cupric oxide are highly effective in controlling biofouling, but pose severe environmental concerns. Alternative non-toxic antifouling coatings need to be sought. The main objective of this research was to study the antifouling performance of two natural product antifoulants (NPAs), capsaicin and zosteric acid. Two freshwater bacteria, isolated Lake Erie (LE) bacteria and *Pseudomonas putida* (Pp), as well as two marine bacteria *Vibrio parahaemolyticus* (Vp) and *Vibrio natriegens* (Vn) were applied as the target antifouling bacteria species.

Microtox assay indicated that the 5min-EC_{50} of capsaicin and zosteric acid were 11.75±1.02 mg/L and 442 ±100 mg/L, respectively. The static toxic assessments showed the EC_{50} of capsaicin and zosteric acid to be 10-23 mg/L and 240-380 mg/L, respectively in freshwater systems. With marine bacteria, capsaicin and zosteric acid had an EC_{50} range of 15-17 mg/L and 8-18 mg/L, respectively. Compared to those heavy metal based toxic antifoulants such as tributyltin or Irgarol 1051, capsaicin and zosteric acid have a relative low toxicity to organisms.

Attachment studies further assessed effectiveness of these two NPAs. A significant inhibition of bacteria attachment was achieved when aqueous NPA

concentration was increased. For instance, after 14 days the LE system depicted 93.5% and 98.5% less biofilm coverage when 20 ppm and 40 ppm capsaicin respectively was present. Biofilm coverage was reduced by 92.5% and 98.2%, respectively as zosteric acid increased from 50 ppm to 500 ppm. Similar results were observed when zosteric acid was entrapped at 1 wt % in Sylgard 184 silicone coatings. The changes of aqueous properties of pH, conductivity, dissolved oxygen, aqueous microbial population and biofilm formation on the coated slides were also evaluated. Aqueous changes and microbial attachment results led to the hypothesis that the primary antifouling mechanism of these two NPAs was to block the bacteria's active sites of attachment, instead of simply posing a lethal level to kill them.

Antifouling effectiveness of zosteric acid was also evaluated when it was entrapped into a different silicone coating. Although zosteric acid leached easier from RTV 11 than Sylgard 184, the effectiveness on antifouling decreased.

For freshwater system, capsaicin's effective concentration was 20 ppm. With zosteric acid, the effective concentration was 50 ppm in solution or when 1 wt % entrapped in Sylgard 184. Based on EC_{50} levels, this makes zosteric acid more effective than capsaicin as an antifoulant. For marine bacteria, Vp and Vn biofilm formation on the surface of solution was much easier than on the slides. Therefore, it was hard to accurately evaluate the antifouling effectiveness of NPAs. However, the bacterial colonization decreased as the concentration of NPAs increased.

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NOMENCLATURE

AF	Antifouling
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CFUs	Colony-forming Units
DBT	Dibutyltin
DO	Dissolved Oxygen
EC	Effective Concentration
EPS	Exopolysaccharides
FR	Fouling Release
GUs	Growth Units
IC	Inhibitory Concentration
IMO	International Maritime Organization
LD	Lethal Dosage
LE	Lake Erie
MBT	Monobutyltin
MPN	Most Probable Number
NPAs	Natural Product Antifoulants
PDMS	Polydimethyl Siloxane

P p	*Psudomonas putida*
SPC	Self-polishing Copolymer
TBT	Tributyltin
Vn	*Vibrio natriegens*
V p	*Vibrio parahaemolyticus*
ZA	Zosteric Acid

CHAPTER I

INTRODUCTION

1.1 Background on fouling

Biofouling is the undesirable growth of organisms on artificial and natural structures immersed in either seawater or freshwater. In seawater, the most visible and well-known forms of such fouling are the barnacles, limpets and seaweeds that attach to ships' hulls, seaside piers and coast defenses. Sticky, microbial biofilms attach first, and may even make it easier for the larger species to gain a foothold (White, 1999). In order to develop efficient and environmentally friendly controls for marine biofouling, more information about the dynamic processes that are involved needs to be obtained.

Biofouling can be considered to have four main stages, some of which can overlap or occur in parallel (Davis, 1995). In general, fouling starts from the moment an object is immersed in water. Whether metal, timber, stone or plastic, its surface rapidly accumulates dissolved organic matter and molecules such as polysaccharides, proteins and protein fragments. This conditioning process is regarded as the first stage of fouling. This stage begins within seconds, stabilizes within hours, and sets the scene for later events (Characklis et al., 1990). Bacteria and single-cell diatoms then sense the surface and settle on it, forming a microbial biofilm (Davis, 1995). This second stage of fouling involves the secretion of sticky muco-polysaccharides, sometimes in vast quantities, and

the production of other chemicals with important effects, for example, causing biocorrosion of the surface (Davis, 1995). The presence of adhesive exudates and the roughness of irregular microbial colonies help to trap more particles and organisms. The trapped organisms are likely to include algal spores, fungi and protozoa, some of which may be attracted by sensory stimuli (Davis, 1995). The transition from a microbial biofilm to a more complex community that typically includes multicellular primary producers, grazers and decomposers is regarded as the third stage of fouling. The fourth and final stage involves settlement and growth of larger marine invertebrates (such as barnacles, limpets, sea mats and sea squirts), together with the growth of seaweeds (Davis, 1995; Callow, 2002). "*Marine Fouling and its Prevention*" reported that nearly 2000 species had been identified on fouled structure (Callow, 2002). As such, fouling can be considered highly dynamic process (White, 1999). Water flow, mechanical damage and various other environmental factors can influence biofilm formation and community development (Kiil et al., 2002).

Fouling can cause serious problems in the shipping industry, since it increases the surface roughness of the hull and hence its frictional resistance of movement through water (Omae, 2003). It has been estimated that a vessel needs to expend 40% more fuel to maintain a normal speed after six months of fouling (Blunden, 1985). The additional cost caused by fouling is estimated about $1 billion per annum for US Navy alone (Callow, 2002). Also, the cost of cleaning and repainting, taking into account the loss of earning while the ship is immobile in dry-dock, can be significant. Furthermore, the

maneuverability of the ship maybe reduced when heavily fouled, the risk of accidents is increased (Kiil et al., 2002).

In maritime applications, many strategies have been tested to prevent fouling on ship hulls. These include copper or lead sheathing, heating /cooling of the ship hull, protein-based coatings, radioactive surfaces, ultraviolet radiation, ultrasound, magnetic fields, chlorine release, gel surfaces, and natural antifoulants (Kiil et al., 2002). The most effective method is to use antifouling paints. The self-polishing copolymer (SPC) paints containing tributyltin (TBT) introduced in 1974, were so called to indicate the 'polishing' effect as the polymer dissolves away during normal vessel operation, releasing TBT (Elbro, 2001). TBT killed the settling fouling organisms while simultaneously smoothing the surface. Being very lipid soluble, it was rapidly taken up by cells, where it inhibited energy transfer processes in respiration and photosynthesis. The SPC system was extremely successful, and had been applied all over the world for more than twenty years (Dahl and Blanck, 1996; Davis, 1999; Evens, 2000). Because of using TBT, the reduced fuel costs, as well as less frequent need to dry-dock and re-paint vessels, were estimated to be worth US$5.7 billion per annum to the shipping industry during the mid-1990s (Evens, 2000). However, TBT was shown to effect non-target organisms, including a number of shellfish, at levels much lower than ever envisioned. The most sensitive invertebrate species, the dog whelk, *Nucella lapillus*, exhibited imposex (imposition of male sexual characters on the female) at concentrations below 1 ng/L. Its disappearance from rocky shores in areas of high boating activity has been attributed to the presence of TBT from antifouling paints (Dahl and Blanck, 1996).

The International Maritime Organization (IMO) already imposed a worldwide ban on TBT-based paints on vessels larger than 82 feet (25 m) by Jan. 1st, 2003. A complete ban on the use of any paints containing TBT was set for Jan. 1st, 2008 (Konstantinou, 2004). Therefore, there is an urgent need to seek out suitable non-toxic or less toxic alternatives.

Typically, three main directions were explored to search alternative antifoulants (Konstantinou, 2003). One is "TBT-free" coatings containing copper or zinc combined with organic booster biocides (Voulvoulis et al., 2002). Another one is to enhance the fouling release capability of silicone coatings. They function by minimizing the adhesion strength of attached organisms, which are removed as the vessel moves through the water or by special cleaning procedures (Brady, 1997). Both Sigma Paints (Amsterdam, The Netherlands) and Akzo Nobel (Gateshead, UK) have commercialized foul release coatings based on silicone polymers (Elbro, 2001). The third approach is to seek non-toxic antifoulants from natural substances, which might repel or inhibit the adhesion of fouling organisms. It has been noted that many organisms in the sea remain free from fouling. Active natural compounds have been extracted from many types of organisms including bacteria, corals, sponges, seaweeds and sea grasses (Yebra et al., 2004).

1.2 Research Objectives

Two natural product antifoulants (NPAs) were investigated to determine their effectiveness on antifouling. Capsaicin is the natural extract from chili pepper, and zosteric acid is the natural extract from zosteric marina, or eelgrass. The approach of

incorporating natural product antifoulants into fouling release (FR) coatings is one of the innovative strategies in the antifouling industry. However, two major drawbacks to this approach are the limited knowledge on how the NPAs act against fouling, and how the incorporation of NPAs affects their release from the coating and the adhesion properties of the coatings.

The overall objective of this work was to evaluate the effectiveness of each NPA on antifouling. To achieve this goal, it was necessary to obtain the optimal concentration of capsaicin and zosteric acid as antifoulants. Experiments were also conducted to provide some insight on how they act against fouling and affect the aqueous properties when directly present in water and/or entrapped in a silicon coating. Several key tasks were performed to reach the objective:

- Perform toxicity assessments to determine if these two NPAs are too toxic to be antifoulants. Both qualitative (Microtox assay) and quantitative (static toxicity assessment) methods were used to obtain the EC_{50} of these two NPAs.
- Ascertain the optimal concentration of suspended capsaicin and zosteric acid on antifouling in freshwater systems. This approach used systems with the different NPAs at various concentrations with either enriched Lake Erie bacteria or *Pseudomonas putida*.
- Evaluate the antifouling effectiveness using the optimal concentration of bulk entrapped zosteric acid for freshwater containing enriched Lake Erie bacteria

- Determine the effectiveness of suspended capsaicin and zosteric acid on antifouling in marine water. *Vibrio parahaemolyticus* and *Vibrio natriegens* were used as the model marine cultures.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Microbes in a natural environment are most often found in biofilm rather than in pelagic fluids. Biofilm are localized concentrations of microorganisms attached to a substratum and consist of a population of a single species, or more often a multi-species community (Characklis, 1990).

Attachment in biofilm is advantageous for microorganisms. The benefits include availability of nutrients concentrated at surface; access to a flowing system which increases the availability of diffusible nutrients; and the presence of a glycocalyx matrix to restrict predation by phagocytosis or exposure to toxicant (Dagostino, 1991). In biotechnological or bioremediation process it is often the aim to promote biofilm formation, and maintain active, high density biomass (Characklis, 1990). In other situations, biofouling can seriously restrict effective heart transport, membrane processes and potentate macrofouling with loss of transportation efficiency (Dagostino, 1991). Although biofilms are advantageous for bacteria, they can be considered a "disadvantage" to other systems. Biofouling can be defined as the undesirable accumulation of microorganisms, plants, and animals on surfaces immersed in water. In the case of ships, the adverse effects caused by this biological settlement are well known:

• High frictional resistance, due to generated roughness, leading to an increase of weight and subsequent potential speed reduction and loss of balance (Ywbra, 2004). To compensate for this, higher fuel consumption is needed, causing increased emissions of harmful compounds (Rascio, 2000). It is estimated that on average, fuel consumption increases 6% for every 100 μm increase in the average hull roughness from fouling organisms (Townsin, 2003; Liu et al., 1997). This could result in an overall increased voyage's cost as much as 77% (Abbott et al., 2000).

• An increase of the frequency of dry-docking operations. This leads to lost time and wasted resources when remedial measures are applied. A large amount of toxic wastes can also be generated during this process (Abbott et al., 2000; Rouhi, 1996).

Among all the different solutions proposed throughout the history of navigation, tributyltin self-polishing copolymer (TBT-SPC) paints have been the most successful in combating biofouling on ships. The widespread use of these paints, estimated to cover 70% of the present world fleet (Gerigk et al., 1998; Champ, 2000), has led to important economic benefits. For instance, the US Navy has annually saved $100-150 million (Dagostino, 1991). Unfortunately, the TBT-SPC systems had adverse effect on the environment. For example, it has been shown that extremely low concentrations (20 ng/L) of TBT cause defective shell growth as well as imposex in the oyster. The number of gastropod species which has been affected by imposex was reported to be more than 140 (Swain, 1998; Champ, 2001). The degradation rate of organotin compounds is not high in the environment. Actually, Morita (1989) reported that the bioaccumulation in fish and shellfish was to about 5,000 times that of concentration in water (average 4.6

ng/L). That is a huge threat to the health of human beings. These facts forced the development of national regulations in countries all over the world (Champ, 2000):

- TBT-containing compounds have been prohibited on vessels less than 25 m in length.
- The release rate of TBT-containing compounds has been restricted; and
- The use of free TBT-holding compounds in paints has been eliminated.

Thus, the paint industry has been urged to develop TBT-free products able to replace the TBT-based ones. However the new products must yield the same economic benefits and cause less harmful effects on the environment.

2. 2. The process of biofouling

The organisms, which take part in biofouling are primarily the attached or sessile forms occurring naturally in the shallow water (Pickard et al., 1982). As mentioned in Chapter I, nearly 2000 species had been identified on fouled structures. Anderson et al. (2000) later increased the number to more than 4000 species. It is important to note that this is still a very small proportion of the known marine species. Almost all species in the water can participate in fouling. As mentioned previously, the fouling process has been considered to consist of four general stages: molecular fouling, microfouling (bacteria), developed microfouling (fungi) and macrofouling (Davis, 1995). The process is depicted in Figure 2.1.

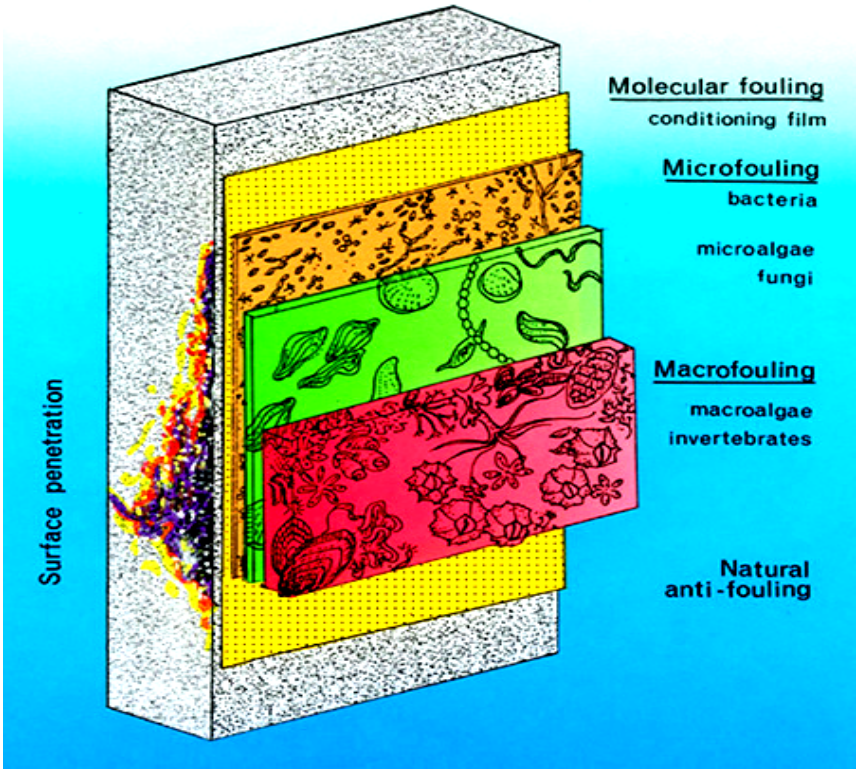

Figure 2.1 Four stages of biofouling (Davis, 1995)

In submerged clean surfaces, dissolved organic materials, such as polysaccharides, proteins and protein fragments will accumulate on the surface to form a condition layer within several minutes (Characklis et al., 1990). A theory developed in the 1940s tried to explain the adhesion of molecules to a surface in terms of physical force, referred as "double-layer theory" (Characklis et al., 1990). The mechanisms of this action can be accomplished by the electrostatic and Van der Waals forces between the surface and the molecules in the water. Studies have shown that the condition layer not only selectively increases adhesion of the other nutrients, but actually aids in the adhesion of bacteria. In addition, in environments where nutrients are plentiful, the nutrients act as a bacterial primer that increases the ability of the bacteria to attach to a surface.

Once the conditioning layer forms, it will assist the bacteria in water to attach on the surface. It is believed that in the initial stage, the cell is spinning and rotating as part of the mechanisms for sensing the environmental conditions and determine if the surface has potential for colonization. This is called a chemosensory mechanism. The initial attachment is the ability of bacteria to "swim" using flagella for propulsion, referred to as flagellar-mediated motility (Lawrence, 1987). Often non-swimming bacteria are considered to have reduced biofilm forming ability (O'Toole and Kolter, 1998a; Pratt and Kolter, 1998). When the bacteria attach to the surface, they start to produce slime (exopolysaccharides, EPS) forming a microcolony in a polysaccharide matrix. This acts to form a bridge between the bacteria and the conditioning film (Flint, 1997; Kumar, 1998). The EPS bridge is actually a combination of electrostatic, covalent and hydrogen bonding, dipole interactions and hydrophobic interactions. Initially the bonds between the bacteria and EPS may not be strong and can easily be removed by flowing water. However, with time, these bonds are strengthened making attachment irreversible. Figure 2.2 shows the bacteria attachment process. After the bacteria attach on the surface, growing daughter cells are released from the microcolony. The daughter cells attach to vacant sites on the substrate surface and grow; subsequently releasing their own daughter cells to form biofilm or microfouling (Kjelleberg et al., 1982).

In each stage, there are many factors that effect bacterial attachment. These include the pH and temperature of the contact surface, flow rate of the fluid passing over the surface, nutrient availability, length of time the bacteria is in contact with the surface, bacterial growth stage and surface hydrophobicity (Flint, 1997; Kumar, 1998).

Simultaneously bacteria use a variety of strategies to change and adapt to a surface. These include changes in their behavior to occupy the substrate and optimize its utilization to reproduce and survive changes in the environment.

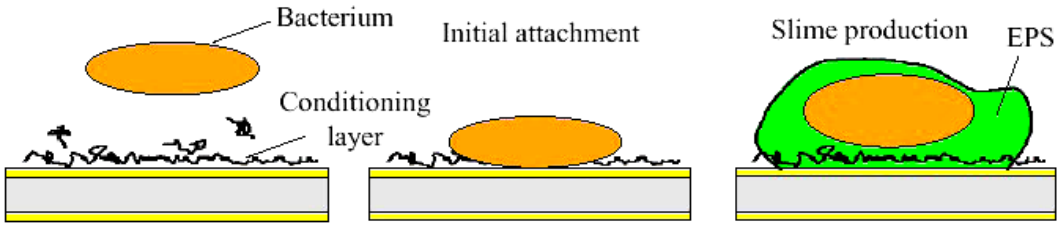

Figure 2.2 The process of bacteria attachment (Green, 2001)

Swimming spores of green alga (i.e., *Entermopha,* the major biofouling alga) attach rapidly once they have "detected" a suitable surface (microfouling) for settlement. The microfouling results in firm attachment to the substratum. Figure 2.3 is the Cartoon representation of the stages involved in *Enteromorpha* zoospore settlement and adhesion. During the surface sensing phase, the swimming spore undergoes a characteristic pre-settlement behavior. This involves a switch from random swimming to a 'searching' pattern of exploration close to the substratum (Callow, 2002). The spore appears to become temporarily adhered to the substratum as it spins like a 'top' on its apical dome, the flagella acting as propellers. The spore then commits itself to irreversible permanent adhesion involving the secretion of a powerful adhesive from pre-formed cytoplasmic vesicles (Callow, 2002). A cell wall is formed later.

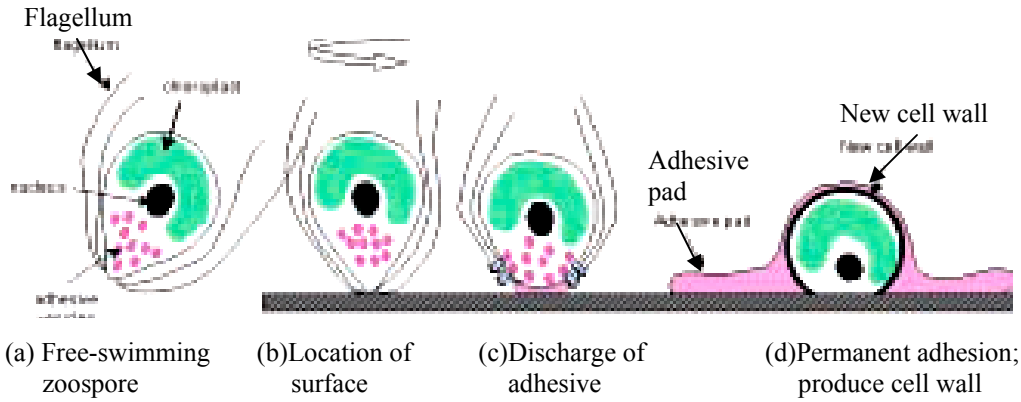

Figure 2.3 *Enteromorpha* zoospore settlement and adhesion (Callow, 2002)

Macrofouling usually describes the attachment and growth of barnacle larvae on the surface. Barnacle larvae swim around freely in the water column. In order to complete the transition to adult life, it must attach to a hard substrate (Callow, 2002). The larvae explore a surface by using a pair of attachment organs, which secrete an adhesive from unicellular glands. After selection of an appropriate site on which to settle, the barnacle larvae release proteinaceous cement onto the paired organs. Initially fluid, the permanent cement flows around and embeds the attachment organs, curing within one to three hours to form a discrete matrix (Callow, 2002). The firmly attached organism subsequently metamorphoses into the calcified adult barnacle. Barnacles and other hard foulers, such as oysters and tube worms, are the target antifouling organisms. They make the hull of ship rough. Once attached, they act as platforms for other fouling organisms (Rittschof, 2001). Figure 2.4 (Yebra et al., 2004) gives an image of fouled ship hulls. If the second stage can be prevented, then perhaps macrofouling will not occur.

Figure 2.4 Samples of heavily fouled hulls (Yebra et al., 2004)

2.3 The influence factors on biofouling

The local severity of biofouling depends upon a large number of parameters. Some of these are given by the water conditions and depend on the geographical location and the surface of the vessel. Consequently, these parameters cannot be modified to control the growth of the fouling organisms. Temperature is undoubtedly one of the most important parameters. It is widely known that fouling is generally greater in regions with high water temperatures (WHOI, 1952). This is clearly related to the fact that temperature appears to be the principal condition determining the breeding periods and growth rate of aquatic organisms. In regions where seasonal variations in temperature occur, the reproduction and the growth of many species are completely suppressed during the low-temperature period and only one generation can be produced in the course of the few warm months (Yebra, 2004). Conversely in tropical climates, where the seasonal changes in conditions are relatively small, fouling may continue without interruption throughout the year (Swain, 1998).

According to WHOI (1952), most of the common marine fouling forms are unable to withstand low salinities, which affect the growth rate and the maximum size attained and cause several malformations. However, slime, algae and bryozoa are commonly found in low-salinity waters, and some species do prefer such conditions (WHOI, 1952). The amount of solar radiation also plays an important role in the upper layers of the oceans and consequently for ship's fouling. Apart from influencing temperature and salinity, it directly affects the photosynthesis rate of the plants and thus controls the nutrition of the animals (WHOI, 1952). Because the salinity is very low for freshwater, limited research was found about its effect on the fouling in freshwater condition.

Dissolved gases may be important to the determination of corrosion rates and biological growth in water (Chandler, 1985). A basic assumption is that surface water is saturated with the atmospheric gases (mainly O_2, N_2, and CO_2). However, biological processes such as respiration and photosynthesis can alter their concentrations. In fact, algal activity can lead to super-saturation of the upper layers (Pickard et al., 1982). The oxygen concentration varies from 0 ~ 0.8 vol. %, although it is rarely outside the range of 0.1–0.6 vol. % (Pickard et al., 1982).

Polluted waters may be harmful either directly through toxic effects, or indirectly through depletion of oxygen or reduction of the solar radiation available for the photosynthesis (Yebra, 2004). Silt and other suspended matter may asphyxiate sessile organisms or produce substrates unsuitable for the attachment of many forms (WHOI, 1972). It may also interfere with the food assimilation of animals which use water

filtering. While, some contaminants may enrich the nutrient supply and thus enhance the fouling.

It is also well known that fouling is not the same in deep waters as in coastal areas (WHOI, 1952). Marine bacteria and organisms, in general, are much less plentiful in oceanic waters compared to coastal waters. Therefore depth is another parameter affecting the intensity of fouling. However it has no direct influence in the case of ships as they are always in contact with superficial waters.

Another major factor controlling initial colonization is the nature of the surface. All surfaces have a physicochemical property called surface energy. Surface energy is described by the way in which solvent molecules interact with a surface. Surfaces with low surface energies have little ability to interact with biological adhesives, thus, form poor adhesive bonds (Brady et al., 2000). These surfaces are relatively easy to clean. On the other hand, those surfaces with high surface energies lead to a dramatic increase in adhesion strength between the coated surface and the aquatic organisms. Thus heavy fouling occurs on the surface (Brady et al., 2000). Cowan (1991) reported that an ideal coating surface for low adhesion is one with a surface energy of about 25 mJ/m^2. The roughness of a surface is another important parameter affecting bacteria attachment and biofilm formation (Cowan, 1991). Generally, as the surface roughness increases, the fouling level increases. Compared to surface energy, surface roughness plays the predominate role in bacteria attachment and biofilm formation (Dagostino, 1991).

Finally, the interactions between the different organisms also modify the process of fouling. Bacteria in a host-associated biofilm may cause significant mortality to their

hosts, degrade the host tissue, and increase the drag on their hosts. Bacteria and other higher species may also compete for nutrients, inhibit gaseous exchange, block incident light, and even secrete secondary metabolites which may inhibit attachment (Steinberg et al., 1997).

2.4 Antifouling

Some of the disadvantages of biofouling have been recognized and combated for more than 2000 years. Fouling is combated by a variety of cleaning techniques and by killing the species. Killing is most effective either immediately before or after the microorganisms attach. By far, the most common antifouling techniques are based upon broad-spectrum biocides that kill settling organisms (Rittschof, 2001). Common biocides include strong oxidants and metals such as copper, zinc, and tin. There are two basic mechanisms of toxic metal action (Rittschof, 2001). One is death by metal ion overload. Free metal ions are essential cofactors and usually in short supply. As a result, organisms have efficient active transport mechanisms for obtaining metal ions, but mechanisms for shutting off their metal ion pumps have not evolved (Goyer et al., 1991). In the presence of metal-based antifouling technology, organisms overload themselves with metals. The metals disrupt their normal enzymatic and metabolic functions, and the organisms die (Goyer et al., 1991; Stadtman, 1991; Cheney et al., 1996). The other mechanism is death by uncoupling of oxidative phosphorylation and the electron transport. Organometal compounds such TBT are lipophylic. Lipophylic molecules partition into membranes and

disrupt essential membrane fuctions, such as the electron transport process required for generation of cellular energy through oxidative phosphorylation (Boyer, 1989).

2.5 Effect of antifouling on the Environment

Toxic metals have long-term impacts on freshwater and marine environments. This is because metals are biologically conserved and recycled. Two major biological processes result in buildup of toxic metals in the environment: 1) continuous conservation by organisms of free ions such as copper and zinc (Goyer, 1991), and 2) "reorganification" of metals like tin (Boyer, 1989). Both processes accumulate toxic compounds until nontarget species are impacted.

The antifouling coatings containing TBT were very effective. For instance, TBT effective leaching rate was only 0.6 $\mu g/cm^2 d$ to reduce 80% barnacles' attachment; 2 $\mu g/cm^2 d$ for *hydrozoyans* and 30 $\mu g/cm^2 d$ for *bryozoan* (Dahl et al., 1996). At these leach rates, once painted, TBT coatings could last 5 years. However, the negative impact of TBT on the aqueous environment was also significant. TBT is toxic to many aquatic organisms, including fish. Acute toxicity, to some fish, occurred at a few milligrams per liter, while chronic toxicity can be found at concentrations in the order of micrograms per liter. Evidence of the disruption of the endocrine system, e.g., the induction of imposex was seen at 0.5 ng/L in dogwhelks (Yebra et al., 2004).

Some benthic invertebrates are also very sensitive to TBT in sediments. Populations of benthic invertebrates such as polychaetes and amphipods have been

shown to be reduced as a result of exposure to TBT in sediments (Evens, 1995; Swain, 1998; Santos et al., 2003). The result is a worldwide ban on use of TBT antifouling coatings. This ban on TBT lead to an increase in vessels using alternative "TBT-free" coatings containing copper combined with organic booster biocides (IMO 1998; Julian, 1999).

Biocide containing coatings are already used and applied as antifoulants. About 18 compounds are currently used as antifouling biocide worldwide (Yonehara, 2000; Thomas, 2001). Some of them, such as chlorothalonil, dichlofluanid, diuron, Irgarol 1051, sea-nine 211, zinc pyrithione and zineb are approved for use by Health and Safety Executive (HSE) as alternative "tin-free" antifouling products in the UK (HMSO, 1998; Voulvoulis et al., 2002). Since these alternatives to TBT are also toxic, their contamination in the aquatic environment has been a topic of increasing attention. For instance, the mode of action of the most popular booster biocide, Irgrol 1051, is based on the inhibition of photosynthetic electron transport in chloroplasts that can occur at concentrations < 1 mg/L (Noyelles et al., 1982). However, the lowest EC_{50} reported for algae and crustaceans are 450 ng/L and 0.4 mg/L, respectively (Scarlett et al., 1997). Dahl and Blanck (1996) also showed chronic effects on periphyton communities at concentrations 60 ~ 250 ng/L. The work by Okamura et al. (2000) described the phytotoxicity of Irgarol 1051 to various seaweeds at concentration range of 0.6 ~ 5.9 μg/L. Some of the concentrations reported are at levels which may be sufficient to pose a risk to aquatic life (Thomas et al., 2001) and to damage microalgal communities (Dahl and Blanck, 1996), macroalgae (Scarlett et al., 1997), corals (Owen et al., 2002),

seagrasses (Scarlett et al., 1999) and therefore indirectly herbivorous mammals, such as dugongs. In Sutton harbor, a concentration of 127 ng/L of Irgarol 1051 significantly inhibited the growth of *E. intestinalis* (Scarlett et al., 1999b). Therefore, the HSE have led to restrictions in the use of booster biocides (Thomas et al., 2002). As a result, in the UK, only paints containing dichlofluanid, zinc pyrithione or zineb as the active biocide can be applied on vessels < 25 m in length (Voulvoulis et al., 2002a). Irgarol 1051, chlorothalonil and Sea-nine 211 containing formulations can only be used on vessels >25 m (Voulvoulis et al., 2002a). Diuron is no longer approved for use as an active ingredient in antifouling paints on any size of vessel (Voulvoulis et al., 2002a). Therefore, more reliable and effective non-toxic techniques should be developed in the antifouling field.

2.6 Non-toxic antifouling technologies

2.6.1 Silicone foul-release coating

To date, no alternative has been able to reach a sufficient degree of development to replace biocide-based A/F coatings. Thus, improved products derived from the current ones and new binder systems and booster biocides will dominate the A/F market during the coming years.

Foul-release coatings are an attempt to prevent the adhesion of fouling organisms by providing a low-friction, ultra-smooth surface on which organisms has great difficulties in settling (Elbro, 2001). Foul-release coatings were produced almost simultaneously with self-polishing copolymers. However, the latter proved to be much

more effective as well as cheaper. Thus the development of foul-release systems did not take off until the 1990s, after the first bans of TBT-based products (Elbro, 2001).

Many studies have been performed to elucidate the properties that a coating should possess to resist adhesion (Brady, 1996; 1999; Candries, 2001). The main ones are summarized by Yebra (2004):

- A flexible, linear backbone which introduces no undesirable interactions.
- A sufficient number of surface-active groups which are free to move to the surface and impart a surface energy in the desired range.
- Low elastic modulus.
- A surface which is smooth at the molecular level to avoid infiltration of a biological adhesive leading to mechanical interlocking.
- Molecules which combine all of the above factors and are physically and chemically stable for prolonged periods in the marine environment.

These properties are mainly possessed by two families of materials: fluoropolymers and silicones. Fluoropolymers form non-porous, very low surface-free energy surfaces with good non-stick characteristics (Brady, 2001). With assembling oriented perfluoroalkyl groups on the surface, very low values of surface-free energy are achieved (Brady, 1999; 2001). Nevertheless, a drawback of these materials is the limited mobility due to the stiffness added by the F atoms, which hinder the rotation about a backbone bond (Hare, 1997). Therefore, the fouling which does accumulate on the surface is not easily released.

Silicones, which are applied in thick layers (Brady, 2001), markedly improved the non-stick efficiency of fluoropolymers. Poly (dimethylsiloxane)-based fouling-release coatings are the most used today due to their low surface energy, low microroughness, and low elastic modulus and low glass transition temperature (Lindner, 1992). These surfaces present "moving targets" to the functional groups of aqueous adhesives due to their conformationally mobile surfaces. The mechanical locking of biological glues are minimized and fouling-release are enhanced (Yebra, 2004).

Polysiloxanes substituted by fluorine might seem to be attractive candidates for surfaces with low bioadhesion. This could lead to polymers with the main advantages of each type, such as low surface-free energy and the elastic properties of silicones (Brady, 2001; Thunemann, 2001). To improve the performance of the coatings, most commercial poly(dimethylsiloxane)-based coatings also contain fluid additives, and it is suggested that these migrate to the coating surface where they create weak surface layers that further promote fouling-release (Truby et al., 2000; Burnell et al., 1998). For instance, incorporation of oil into silicon foul release coatings decreased both barnacle and pseudo-barnacle adhesion (Burnell er al., 1998). One drawback of this technology was that once the reservoir of oil has been exhausted, the coating becomes brittle, cracks and fouls. The service life limit of the present coatings is approximately two years (Brady, 2001).

The performance of the foul-released coatings has been tested on real ships operating under different conditions. The conclusion of these studies is that most coatings could prevent a fouling coverage greater than 20% of the total surface for three years (Swain, 1998; Ryle, 1999; Anderson, 1998). However, this technology is still expensive.

In addition, the coatings exhibit poor adhesion to the substrate, are easily damaged (cutting, tearing and puncturing) and have poor mechanical properties (Swain, 1998; Ryle, 1999; Anderson, 1998).

Some recent studies focused on the investigation of the surface properties of marine organisms with respect to biofouling control. As an example, Swain (1998) reported that the non-fouling condition of porpoise and killer whale has been attributed to the outermost surface being composed of a glycoproteinaceous material with low surface energy. Baum et al., (2002) studied the skin of the pilot whale, which showed a hydrated jelly nanorough surface characterized by a pattern of nanoridge-enclosed pores, of a pore size below the average value for the skin of most of the marine biofouling organisms. Currently, the technique is not successful, but left room for improvements in the same direction.

2.6.2 Natural Product Antifouls (NPAs)

Another alternative to toxic antifoulant is natural product antifoulant (NPA). Initial searchers for natural antifoulants involved biologists and natural products chemists. Source organisms, such as tropical sponges and octocorals, were chosen because they don't foul when alive and are rich sources of novel secondary metabolites (Bakus et al., 1974; Davis et al., 1989). Since the early 1990s, there has been a dramatic increase in research looking for potential natural products antifoulants. This has resulted in identification of many new antifoulant compounds from a variety of marine invertebrates. The research community has reported the identity of over 100 additional

natural products with antifouling activity, including bromo Tyrosine, Oroidin, steroid, lentil Lectin and zosteric acid (Clare, 1996). The compounds can be divided into two broad classes: (1) compounds extracted from organisms that have antifouling activity which may never reach the surface of the organisms, and (2) compounds found in the water-bathing organisms that have antifouling activity and are likely to serve a role in deterring growth of epibiota (Devis, 1989). Most potent natural product compounds are often too structurally complex to be commercially synthesized. Alternative compounds must have a potency that makes them practical and be amenable to cost effective synthesis (Rittschof, 2001).

The mechanisms of action of natural product antifoulant are very complex, because it depends on the structures of each compound. Currently, extensive studies on the mechanism of NPAs still stay at a hypothesis level. There are two known hypotheses that have been proposed to explain the biofouling inhibition of NPAs against bacteria attachment. The first one assumes that the NPAs contain special chemicals that interfere with the communication signals, such as homoserine lactones, between the bacteria to prevent the biofilm formation (Davies, 1996). In the second hypothesis, it is assumed that the NPA molecules are released from the coating. These free-floating NPA molecules bind with the specific binding sites on the surface of the target organism, thus blocking the specific interaction organism with the surface (Sundberg, 1997).

2.6.3 Capsaicin and zosteric acid used as NPAs

Capsaicin is the “hot” ingredient in chili peppers and Tabasco sauce. Its chemical name is 8-methyl-N-vanillyl-6-nonenamide. Capsaicin has 10-15 million of Scovlle Heat Units, while the hottest peppers, habaneros, only have a Scoville heat unit of 0.2-0.3 million (Watts, 1995). As a result, capsaicin has been registered by the EPA as a bird, animal and insects repellent. Specifically, it is used to repel birds, voles, deer, rabbits, squirrels, insects and attacking dogs (ITRC, 2000). In 1991, it was reclassified by the EPA as a biochemical pesticide.

Capsaicin was first used as antifouling compound by a Japanese company, Saiden Chemical Industry. A capsaicin soaked fishing net was placed in sea water for six months. Compared to those ones with no capsaicin, less alga and mollusks were found on the net (Koho, 1980). Watts (1995) reported that they used capsaicin, which was mixed with a suitable corrosion resistant epoxy resin and a hardening catalyst, to apply as an antifouling coatings. They also suggested that capsaicin can be mixed with a silicon dioxide and then solubilized into a free-flowing homogeneous liquid. After that, capsaicin-based sustained-release biorepellent coating was anglicized by Bullat’s group (Bullat et al., 1999) who studied the coating structures and capsaicin diffusion rate. Jaggari and Newby (2003) pointed out that its non-toxic, non-biocidal, non-leaching properties can make capsaicin a viable alternative to organotin compound. In order to optimize the antifouling performance of capsaicin, they used silicone as the carrier by both bulk entrapped and surface immobilization. While bacteria liberally inhabited the control coating, their presence on the capsaicin-incorporated coating was found to be

minimal. However, these former studies didn't discover the antifouling mechanisms of capsaicin. Furthermore the effective antifouling concentration of this natural product antifoulant was not achieved.

Zosteric acid, p-(sulphooxy) cinnamic acid, is the natural extract from *zostera marina* or eelgrass. Zosteric acid has been applied as natural product antifoulant in recent years (Zimmermann, 1995; Zinn, 2000; Berlot, 2002). All the studies indicated that the zosteric acid can prevent biofouling from some bacteria, algae, barnacles and tubeworms at non-toxic concentrations. The antifouling property of zosteric acid results from binding to attachment site on cell surface, thereby preventing cell adhesion. Zosteic acid also was first synthesized in Zimmermann's lab, followed by incorporation into silicon foul release coating. After painted on the panels and exposed to the marine environment at Moss Landing, California, for 60 days, no hard fouling was present on the coatings in which zosteric acid had been incorporated. Coatings without the natural antifoulant were fouled with both calcareous organisms and slimes. Furthermore, in laboratory assays, the attachment dropped from 90% to 30% compared to the control surface as the concentration of zosteric acid increased from 1 $\mu g/cm^2$ to 200 $\mu g/cm^2$ (Zimmerman et al; 1995).

CHPTER III

EXPERIMENTAL METHODS

3.1 Reagent and antifoulant sources

3.1.1 Reagent sources

All coating experiments were performed with two FR coatings, Sylgard 184 or RTV 11. The Sylgard 184 is an elastomeric polydimethyl siloxane (PDMS) kit manufactured by Dow Corning. RTV 11, a calcium carbonate-filled PDMS manufactured by GE Silicones, is a two components silicone elastomer kit. One contained calcium carbonate, hydroxi-terminated PDMS and ethyl silicate 40; the other contained a dibutyltin dilurate catalyst. Naturally extracted capsaicin, or 8-methyl-N-vanilly-6-nonenamide, was purchased from Aldrich Chemical Co. The active component of extract contains 65% of 8-methyl-N-vvanillyl-6-nonenamide and 35% of 8-methyl-N-vanillylnonanamide (i.e.,dihydrocapsaicin). Zosteric acid, or p-(sulphooxy) cinnamic acid, was synthesized by following the known procedures (Zimmerman et al., 1995) by Dr. Newby's group in the Chemical Engineering Department (Song, 2004). All of the chemicals used for bacteria maintenance were purchased from Fisher Scientific in biotechnology grade. Agar was purchased from Sigma Chemical.

3.1.2 Antifoulant stock solution

Zosteric acid has very high water solubility. Therefore, once synthesized it was dissolved directly in water to yield stock solutions of 3 mg/L, 10 mg/L, 50 mg/L, 100 mg/L, 250 mg/L and 500 mg/L for subsequent toxicity and attachment experiments. Capsaicin has limited water solubility (the saturated concentration is only 60 mg/L). Two methods were used to obtain the required capsaicin solution. One approach pre-dissolved 16 mg capsaicin into 0.8 ml ethanol (Turgut, et al., 2004). Then water was added slowly in 0.2 ml increments until the final volume of 3.3 ml was achieved. The solution was then gently heated to drive off the ethanol. The resulting 6,400 mg/L stock solution was then diluted with distilled water to obtain 3, 5, 10, 20, 30 and 40 mg/L concentrations used in the future experiments.

The second method dissolved capsaicin into Lake Erie water directly. The required amount of capsaicin was added to proper volume of water. The solution was heated to 40 °C for 24 hours with constant mixing. Heating the solution to only 40 °C accelerated the rate at which capsaicin dissolved without re-precipitation when it cooled to room temperature. Using this approach a maximum concentration obtained was 60 mg/L.

3.2 Water source

3.2.1 Fresh water source and characterization

Ten gallons of water was obtained from Lake Erie (LE, Cleveland, OH). The sample was obtained approximately 100 ft off the shoreline of Edgewater Park at Edgewater Street. Water was collected both from the surface and about one meter below the water level. Subsequent samples were obtained from the same location as needed. The water was characterized immediately upon returning to the laboratory. The pH, conductivity and DO were determined by pH meter, conductivity meter and DO meter. A portion of the water was stored in a refrigerator (4°C) in a tightly sealed container until the experiments were initiated. Approximately 200 ml of the collected water was used to isolate and enrich the indigenous bacteria (see section 3.4) for use in attachment studies.

3.2.2 Marine water source

It was difficult for us to collect real marine water sample. Therefore, synthetic marine water (Difco 2216) was used for the attachment study and toxicity assessment. The synthetic water was composed of (g/l): 5.00 bacto peptone, 1.00 bacto yeast extract, 3.24 Na_2SO_4, 1.8 $CaCl_2$, 0.55 KCl, 0.16 Na_2CO_3, 22.0 mg H_3BO_3, 8.00 mg K_2HPO_4 and 1 liter distilled water. In addition, 19.45 g and 30 g NaCl was applied for *Vibrio natriegens* and *Viibrio parahaemolyticus,* respectively. The solution was sterilized in autoclave at 123 °C for one hour prior to initiating the experiments.

3.3 Bacteria sources, isolation and maintenance

3.3.1 Freshwater bacteria

Pseudomonas putida (Pp) is gram-negative rods with polar flagella. It is a rapidly growing bacterium frequently isolated from most temperate soils and waters. *P. putida* was selected as the model freshwater organism. Pure strain was purchased from American Type Culture Collection (ATCC 12633) in freeze-dried form. The species were revived and maintained in 250 ml Erlenmeyer flasks containing sterile nutrient solution comprised of (g/l): 1.33 KH_2PO_4, 2.67 K_2HPO_4, 1.0 NH_4Cl, 2.0 Na_2SO_4, 2.0 KNO_3, 0.05 $FeSO_4.7H_2O$, 0.2 $MgSO_4.7H_2O$, 1 glucose and 1ml trace metals. The trace mental solution was comprised of (g/l): 3.7 $CaCl_2.2H_2O$, 2.5 H_3BO_3, 0.87 $MnCl_2$, 6.5 $FeCl_3$, 0.44 $ZnCl_2$, 0.29 Na_2MoO_4, 0.01 $CoCl_2$, 0.001 $CuCl_2$ and 1 L distilled water. Each week 10% by volume of the batch culture were transferred to fresh nutrient solution to maintain the bacteria. The bacteria were maintained in a Lab-line Orbit Environmental shaker operated at 30.5±1.5 °C and 150 rpm.

The Lake Erie bacteria (LE) were isolated and enriched from the water collected in section 2.2.1. An aliquot of the Lake Erie water was subjected to the most probable number (MPN) plate method to assess the initial microbial activity (section 3.7.1). The colonies were counted three days after growth on nutrient agar. Then, an inoculating loop was used to inoculate the colonies (bacteria) into sterilized flasks each containing 150 ml sterile nutrient solutions. As with *P. putida*, weekly transfers of the batch culture were

used to maintain the L.E bacteria for future experiments.

Both cultures were also grown on agar slants for long-term storage. The agar solution consisted of (g/l): 0.053 KH_2PO_4, 0.1068 K_2HPO_4, 2 NH_4Cl, 2 Na_2SO_4, 0.2 $MgSO_4.7H_2O$, 15 Agar, 3 Tryptic Soy Broth and 1 liter distilled water. Hot agar solution was transferred into a sterilized tube and against the wall to make an agar slant. Once solidified, isolated bacteria colonies (LE or Pp) were transferred onto the slant surface by using an inoculating loop. The triplicate slants for each bacteria species were stored in a dark container at room temperature. Every six months the colonies growing on the slant surface were transferred to fresh agar slant.

3.3.2 Marine bacteria

Two typical marine bacteria *Vibrio natriegens* (Vn) and *Vibrio parahaemolyticus* (Vp) were selected as the model marine organisms (Lawrence, 1998; Levett 1988). The Vn (ATCC 14048) and Vp (ATCC 17802) pure strains were purchased from American Type Culture Collection (Rockville, MD) in freeze-dried form. The species were revived and maintained in 250 ml Erlenmeyer flasks containing 150 ml sterile bacto marine broth nutrient solution mentioned previously in section 3.2.2. Similar to the freshwater bacteria, weekly 10% by volume transfers were used to enrich and maintain the marine bacteria for future experiments. The cultures were grown in a Lab-line Orbit Environmental shaker operated at 30.5±1.5 °C and 150 rpm.

Agar slants were used for long-term storage of the marine bacteria culture. The nutrient composition used for the marine bacteria agar solution was (g/L): 0.053 KH_2PO4, 0.1068 K_2HPO_4, 2 NH_4Cl, 2 Na_2SO_4, 1 KNO_3, 0.2 $MgSO_4.7H_2O$, 15 Agar, 5 Bacto peptone, 1 Bacto yeast extract and 1 L of distilled water, as well as 19.45 g and 30 g NaCl for Vn and Vp, respectively.

3.4 Toxicity of antifoulants

Both a qualitative and quantitative method was used to measure the toxicity of capsaicin and zosteric acid. The first method was Microtox assay. It was based on reconstituted freeze-dried *Photobacterium phosphoreum* where inhibition of natural luminescence of photobacteria as toxicity endpoint. The Microtox Toxicity analyzer determined the EC_{50}, the effective concentrations of chemical which reduce the light output of bacteria by 50% after being in contact with bacteria over a certain time period. The Microtox Toxicity analyzer (Model 500) was operated via manufacture's guidelines (Microbics Corporation, CA). Although the Microtox assay does provide a quantitative evaluation of toxicity, it is still "qualitative" in that an indicator organism is used instead of the real microbe(s).

The other approach was a tube quantitative assessment method to obtain the static EC50, the concentration of chemical which kill the bacteria by 50% after being in contact with bacteria over a certain time period. Six milliliters of each enriched bacteria

strains (LE and Pp for freshwater or Vn and Vp for marine) were added to 125 ml amber flasks (to prevent photo-oxidation reactions) containing 54 ml of the corresponding nutrient solution with different concentrations of capsaicin. For the freshwater system, the concentrations were 5, 10 and 20 mg/L of capsaicin. For marine bacteria system, the concentrations were 2, 5 and 10 mg/L for *V. natriegens*, and 2, 10, 20 and 30 mg/L for *V. parahaemolyticus*. The concentrations used were based on the results from the Microtox analysis. Control flasks (i.e., no capsaicin) were also conducted to evaluate normal microbial activity. Each bacteria-capsaicin concentration combination was conducted in triplicate for reproducibility and reliability purposes. After one week of growth in shaker (30.5±1.5 °C and 150 rpm), the activity was determined by MPN method (section 3.7.1). The number of colony forming units (cfu) per liter for each condition was then compared with the control experiment.

The same approach was used for zosteric acid. Zosteric acid was used at 125 mg/L, 250 mg/L and 500 mg/L to determine the EC_{50} for both freshwater bacteria. Concentrations for the marine bacteria assessment were 5 and 10 mg/L for Vn, and 10, 20 and 100 mg/L for Vp.

3.5 Coating preparation

3.5.1 Evaluation of NPA in suspension

A 10:1 by mass of base elastomen: curing agent of Sylgard 184 was first mixed using a glass rod and then transferred to the glass substrate. Each coating was prepared by spreading a small amount of silicone mixture onto a 1.25 cm × 7.5 cm glass slide at an average thickness of 200 μm (measured in cross-section under an optical microscope) over an area of 1.25 cm × 1.25 cm. The coatings were allowed to cure for 48 hours at ambient conditions. The slides were sterilized in an autoclave at 123 °C for 60 min immediately prior to bacterial attachment studies. The same procedure was followed to make RTV 11 coatings but using 0.5% wt of catalyst and 99.5% wt of base elastomer.

3.5.2 Zosteric acid entrapped in silicone

Pure silicone was prepared according to the manufacture's procedure. The zosteric acid bulk entrapped silicone was prepared by blending a solution having 10% zosteric acid, 45% pyridine and 45% water in the base elastomer first. After drying off the solvent (pyridine and water), the curing agent was added. The same method was used to spread the silicone mixture on the slide surface to obtain a uniform 200 um coating over an area of 1.25 cm ×1.25 cm (Barrios, 2004). The coatings were cured under ambient conditions for 48 hours and sterilized at 123 °C for 1 hour before attachment studies.

3.6 Bacteria attachment study

3.6.1 NPAs dissolved in solution

In order to ascertain if the NPAs interfere with bacteria growth by blocking their attachment to the surface or simply kill them, the initial antifouling performance of the NPAs was first determined by dissolving them directly into the solution instead of incorporating into the coatings. For freshwater bacteria, the silicone-coated slides were immersed in 60 ml amber bottles (to prevent photo-oxidation reactions) containing 27 ml sterilized Lake Erie water containing 3 ml of enriched bacteria (either LE or P. p) and capsaicin to obtain final concentration of 0 mg/L, 20 mg/L or 40 mg/L. The slides were placed in the bottle at a 40 degree angle with all coatings face down to ensure that the attachment was not simply the result of settlement of species and/or organic matter. Two control experiments were performed at the same time. One was silicone-coated slide, the other was an uncoated or plain glass slide. Each control slide was placed in amber bottles containing 30 ml enriched Lake Erie water. The purpose of control experiments was to compare the aqueous properties. Both the physiochemical and biological characteristics of the water of the three parallel experiments (controls, Lake Erie bacteria and *P. putida*) were tracked with time to assess the impact of capsaicin on the surrounding ecosystem. Every other week, bottles were opened for thirty minutes to refresh the air. Triplicate bottles were sacrificed at days 0, 1, 3, 7, 10, 14, 21 and 28 to assess the water properties and biofilm formation. The pH, DO (in mg/L), temperature, conductivity (in mg/L) and

aqueous MPN of the sacrificed bottles were determined as described in section 3.2.1. Variations in the surface morphology of silicone coating due to biofilm formation and growth were examined within one hour after the samples were removed. An Olympus microscope with an attached video system was used to capture the images of interest. The JKR and contact angle (for bulk modulus and surface energy) evaluation were employed to ascertain the characteristics of the coating (Barrios, 2004).

Attachment studies with marine bacteria followed a slightly modified procedure. Twenty-seven milliliter of sterilized synthetic marine water was used in 60 ml amber bottles. Based on the toxicity assessment studies, the concentrations of capsaicin were 5 and 3 mg/L, 30 and 10 mg/L for Vn and Vp, respectively. Triplicate amber bottles were sacrificed at weeks 0, 1, 2, 3, 4, 6 and 8. As with the freshwater system, air was refreshed every other week.

The entire procedure was then repeated with zosteric acid as the NPA. As with capsaicin, the concentrations of zosteric acid employed were based on the toxicity assessment. For LE and Pp, the concentrations were 50, 100 and 500 mg/L, and 50 and 500 mg/L, respectively. The concentrations of zosteric acid in marine bacteria attachment study were quite different from that applied in freshwater. Vn evaluated zosteric acid at 5, 10 and 20 mg/L. Vp used 20 and 40 mg/L.

Additional attachment studies were conducted for L. E bacteria using zosteric acid concentration of 5 and 10 mg/L. The concentrations were selected based on a concurrent

leach study (Barrios, 2004). These were conducted to augment the bulk-entrapped study described below.

3.6.2 Zosteric acid was entrapped in silicone

Evaluation of the antifouling activities with entrapped zosteric acid in silicone was performed only with Lake Erie bacteria. Four entrapped zosteric acid coated-slides of 0%, 0.3%, 0.5% and 1% by weight were tested. Each coating was immersed in triplicate amber bottles (60 ml of volume) containing 30 ml enriched Lake Erie bacteria. Care was taken to place the coating facing the bottom of the bottle. Bottles were achieved every week. The biofilm formation was investigated by using an Olympus microscope.

3.7 Key analysis methods

3.7.1 Enumeration of aqueous bacteria via MPN

Organisms in aqueous solution were enumerated by the most probable number (MPN) determination. The MPN should be judged as an estimate of growth units (GUs) or colony-forming units (CFUs) instead of individual bacteria. The MPN procedure is a dilution method that was combined with a plate count method to estimate the microbial activity in the aqueous solution. One half milliliter of the initial bacteria (freshwater or marine) solution was transferred into a sterilized 10 ml test tube containing 4.5 ml sterile diluent (5.5 g NaCl for freshwater bacteria, 19.45 g for Vn and 30 g for Vp dissolved in 1

L distilled water). After mixed uniformly, the concentration of bacteria was diluted to one tenth (10^{-1}). Then, 0.5 ml of the mixture was transferred to 4.5 ml diluent. The procedure was repeated until the initial bacteria solution was diluted to 10^{-6} for freshwater and 10^{-9} for marine water. Triplicate dilution vials were conducted. Two drops (～0.1 ml) of each diluted solution were pipetted to sterilized petric dish containing 13 ml cured agar solution (section 3.3). The solution was gently spread over the agar surface and stored at ambient condition for bacteria growth. The colonies formed on the surface of the agar were counted after 3 days. The concentration of the bacteria N (cfu/L) was calculated by using the formula below (Maturin, 1998):

$$N = \frac{\sum C \times 10^3}{[(1 * n1) + (0.1 * n2)]d \times 0.1}$$

Where:

ΣC is the sum of the colonies counted

n1 is the number of dishes counted at the first dilution

n2 is the number of dishes counted at the second dilution

d is the dilution from which the first counts were obtained

3.7.2 Biofilm image analysis and the coverage determination

After the slides were taken from the bottles, they were rinsed gently by using distilled water to get rid of the settlement of organisms on the silicone surface. Then the attached biofilm image was evaluated with an optical microscope (IX 70, Olympus). The transmitted light mode was used for Sylgard® 184 transparent coatings, and the reflected

light mode was used for RTV 11 non-transparent coatings. The surface morphology was determined using different degrees of magnification to identify the differences in shapes between types and quantity of bacteria attached to the silicone surface. The pictures were saved in a computer which is connected with the microscope.

The biofilm coverage on the surface of silicon was roughly determined by using a "pixel count method". The biofilm formation pictures were edited by using the Microsoft Photo Editor. Randomly, a sub-sampling area of 120×120 pixels for each picture was selected. Then, the edited picture was printed out by using a RM125 laser printer. The print-out picture size was adjusted suitable to the printer. Another sub-sampling area of 60×60 pixels was selected in the printed biofilm image picture. Dark pixels were counted within this area. Because the silicone and glass slide are transparent, the picture background was white if no bacteria attached on the silicon surface. The surface coverage of the darker area to the total area was assumed to be the surface coverage of the bacteria.

3.7.3 Statistical analysis

In order to determine if the presence of NPAs causes significant effect on the pH, DO, conductivity and MPN in the solution, a statistical analysis was conducted. Tukey's comparisons were performed among levels of bacteria exposed to different concentrations of NPAs. The confidence level was assumed 95% for all the statistical

analysis procedure. The p-value of the ANOVA table and the intervals of subtracted means between each condition indicated if a significant difference existed. MINITAB 14 software did all statistical analysis. The detailed results are shown in appendix.

CHEPTER IV

TOXICITY ASSESSMENT OF CAPSAICIN AND ZOSTERIC ACID

4.1 Introduction

Both qualitative and quantitative methods were used to evaluate the toxicity of capsaicin and zosteric acid. A standard Microtox assay (5-minute test) was performed to achieve the qualitative EC_{50} of capsaicin and zosteric acid. Two of the reactive ingredients of zosteric acid, p-coumaric acid and chlorosulfonic, were also evaluated. The results were then compared to the EC_{50} of three other potential non-toxic antifoulants, tannic acid, sodium benzoate and benzoic acid.

A static toxicity test was used to obtain the true quantitative EC_{50} of capsaicin and zosteric acid. Static evaluations were conducted for the NPAs in conjunction with *Pseudomonas putida,* enriched Lake Erie bacteria, *Vibrio parahaemolyticus* and *Vibrio natriegens*.

4.2 Microtox toxicity

Table 4.1 presents the toxicity result of Microtox test. The most toxic compound was chlorosulfonic acid with an EC_{50} of 5.21±2.17 mg/L. The EC_{50} value for p-coumaric acid was 32.15±2.67 mg/L. Therefore both reactants were much more toxic than that of their product, zosteric acid (EC_{50} of 442±100 mg/L). The EC_{50} of capsaicin was

11.75±1.02 mg/L. Obviously, capsaicin is more toxic than zosteric acid. Limited research was done to obtain the EC_{50} of capsaicin and zosteric acid as pesticides or antifoulants. Only the IC_{50} (Inhibitory Concentration) of capsaicin was reported at 1.67 mg/L (Molina-Torres, 1999). Since the IC was low, it is reasonable that the capsaicin has a low EC_{50} value. No available literature data for the EC_{50} of zosteric acid has been found. The Microtox assay value of capsaicin and zosteric acid not only provided a reference range of static toxicity assessment, but also gave a resonable concentration range of these two compounds for use either when suspended or entrapped for the later attachment study.

Table 4.1 Microtox toxicity results for compounds evaluated (± is 95% confidence range)

Compound	EC_{50} (mg/L)
Capsacin	11.75±1.02
Zosteric acid	442 ±100
p-Coumaric acid	32.15±7.67
Chlorosulfonic acid	5.21±2.17
Benzoic acid	6.83±2.92
Tannic acid	117.5±8.8
Sodium benzoate	559.1±74.3

Benzoic acid, sodium benzoate and tannic acid are three common compounds in food industry. Benzoic acid is widely used in the food industry as a preservative in acid food (Sieber et al., 1994). Tannic acid has numerous food and pharmacological applications as an anti-oxidant (Khan et al, 1999). Sodium benzoate is usually applied in acidic foods and products to control bacteria, mold, yeasts, and other microbes (Turantas et al., 1999). Their ability of inhibiting the proliferation of adhesive bacteria drew the

attention of researchers. Benzoic acid (Weisman et al, 1992; Railkin et al., 1993), tannic acid (Saha et al., 1996; Lau, 1998) and sodium benzoate (Vetere et al., 1999; Stupak et al., 2003) were already investigated as non-toxic antifoulants. In order to obtain the antifouling ability of these three compounds, the toxicities were also evaluated (Table 4.1).

The Microtox toxicity of benzoic acid was 6.83±2.92 mg/L. It was significantly more toxic than that of tannic acid and sodium benzoate with EC_{50}'s of 117.5±8.8 mg/L and 559.1±74.3 mg/L, respectively. Previous research indicated that the EC_{50} of benzoic acid was 16.5 mg/L when the photobacteria was exposed for 30-minute test (Kaiser et al., 1987). Furthermore, the 14-day EC_{50} to *Anabaena inaequalis* was reported as 9 mg/L (Stratton and Corke, 1982). Thus the EC_{50} value of benzoic acid achieved here was reasonable. No previously reported Microtox toxicity values were found for tannic acid or sodium benzoate. However, the ecotoxicity values were obtained by former research work. For instance, the LD_{50} value of tannic acid to rats was 2300 mg/L, and the EC_{50} of sodium benzoate to water flea was 500 mg/L. Therefore, it was not surprising for tannic acid and sodium benzoate to have high EC_{50} value to specific bacteria.

4.3 Static toxicity assessments

4.3.1 NPAs exposure to freshwater bacteria

The EC_{50} values of Microtox assay (section 4.2) provided an identified concentration range for compounds of interest for the static toxicity assessment. Table 4.2 contains the quantitative capsaicin toxicity assessment with the enriched Lake Erie consortium and *P. putida.* As anticipated, capsaicin eliminated or inhibited the bacteria

Table 4.2 Static toxicity assessment of capsaicin for freshwater bacteria (± is standard deviation of triplicate samples)

Bacteria system	MPN (cfu/L)	% decrease
Lake Erie		
Control	7.22±1.51E11	---
+ 5 mg/L capsaicin	5.85±0.05E11	19.0%
+ 10 mg/L capsaicin	4.75±0.32E11	34.2%
+ 20 mg/L capsaicin	4.16±0.08E11	42.4%
P. putida		
Control	1.13±0.10E12	---
+ 5 mg/L capsaicin	5.77±0.86E11	48.9%
+ 10 mg/L capsaicin	4.57±0.32E11	59.6%
+ 20 mg/L capsaicin	3.38±0.02E11	70.1%

growth effectively. As the capsaicin dosage increased, the more pronounced the toxic effect (i.e., decrease in microbial activity) was. For instance, the decrease in Lake Erie bacteria activity was 19% at 5 mg/L capsaicin and increased to 42% when 20 mg/L capsaicin was used. For *P. putida*, the microbial activity decreased by 48.9% with 5 mg/L capsaicin, and decreased by 70.1% when the concentration of capsaicin increased to 20 mg/L. Figures 4.1 and Figure 4.2 present the exponential fit line between the concentrations of capsaicin and the microbial activity for Lake Erie system and *P. putida* system, respectively. The EC_{50} values of each system were estimated based on the trend line forecast using this approach, the EC_{50} values of capsaicin were 22.6 mg/L and 10 mg/L for Lake Erie and *P. putida*, respectively.

Capsaicin was more "toxic" to *P. putida* than the Lake Erie bacteria system. This was evident by the EC_{50} values, and *P. putida* exhibiting a decrease in activity of twice that of the Lake Erie culture for each capsaicin dosage tested. In addition, statistical

analysis (P-value=0.029<0.05) indicated that capsaicin is significantly more toxic to *P. putida* than to Lake Erie bacteria system.

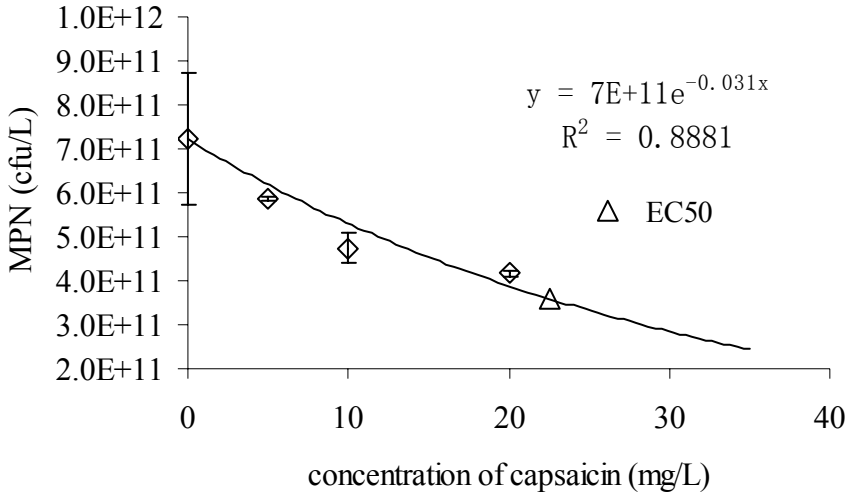

Figure 4.1 Static toxicity assessment of the MPN of Lake Erie bacteria changing with capsaicin concentration after exposed 7 days (Error bar represents the standard deviation, n=3). Results yield an EC_{50}= 22.6 mg/L.

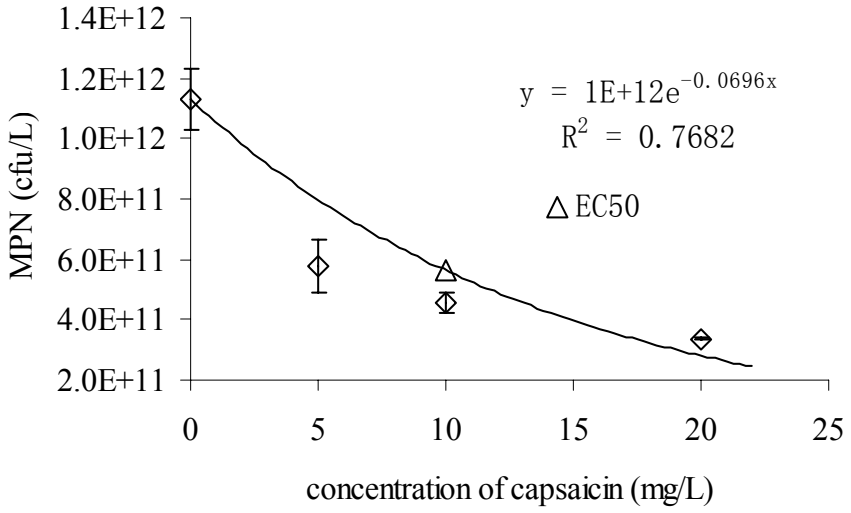

Figure 4.2 Static toxicity assessment of the MPN of *P. putida* changing with capsaicin concentration after exposed 7 days (Error bar represents the standard deviation, n=3). Results yield an EC_{50}=10 mg/L.

This was expected since Lake Erie bacteria culture contains several different kinds of bacteria. Plate counts qualitatively identified the presence of four significantly different species (with different colors of green, white, purple and yellow), some of which maybe more resistant and/or adaptable to the toxicity posed by capsaicin. *P. putida* being a single strain could not adapt as well.

The static toxicity results when zosteric acid was used as the NPA are given in Table 4.3. As with capsaicin, a trendline forecast method was applied to estimate the EC_{50} values for both Lake Erie (R^2=0.9797) and *P. putida* (R^2=0.8259) systems (Figures are located in Appendix A). The EC_{50} of zosteric acid was approximately 376 mg/L for the Lake Erie system. There was no statistical difference (P-value =0.577>0.05) between the EC_{50} value achieved with Microtox assay or statistic test (ANOVA table presented in Appendix C). The EC_{50} for *P. putida* was 242 mg/L, which was almost half of that obtained by Microtox assay. It was obvious, and statistically significant (P-value=0.001<0.05, ANOVA table in Appendix C), that *P. putida* was more sensitive than Lake Erie bacteria when exposed to zosteric acid. The reason was the same as that stated previously for capsaicin.

Table 4.3 Static toxicity assessment of Zosteric Acid (ZA) for freshwater bacteria (± is standard deviation of triplicate samples)

Bacteria	MPN (cfu/L)	% decrease
Lake Erie		
Control	9.40±1.4E11	---
+ 125 mg/L ZA	6.67±0.83E10	29.0%
+ 250 mg/L ZA	6.02±0.58E10	36.0%
+ 500 mg/L ZA	3.41±0.01E10	63.7%
P. putida		
Control	1.2E12±1.3E10	--
+ 125 mg/L ZA	6.48±0.17E11	46%
+ 250 mg/L ZA	5.03±0.2E11	58%
+ 500 mg/L ZA	3.38±0.12E11	72%

Because zosteric acid was synthesized in the laboratory, the "pureness" of the sample will directly affect the toxicity value. The product of zosteric acid was sacrificed at each synthesis step which was controlled by pH value. The static toxicity assessment was then performed with Lake Erie consortium for the product of each step. The results of EC_{50} were given in Table 4.4.

Table 4.4 Toxicity assessment for Zosteric acid to L.E bacteria at each synthesis step

		pH=11	pH=7	Final
EC_{50} (mg/L)	Before drying	517	482	402
	After drying	494	494	384
	Previous Value	376		
	Microtox Value	442		

From Table 4.4, zosteric acid after drying was slightly more toxic than that before drying. The only exception was for pH = 7. The reason is that the drying process drove away the water and methanol remaining in the sample. Once dry, the material was "purer" and could be more toxic to the bacteria. Even if methanol was in the sample, its effect on EC_{50} of ZA to *L.E* bacteria can be ignored. Neglecting methanol contribution was possible since its EC_{50} was 25,200 mg/L by 5-min Biotox test (Callow et al., 1999). The Biotox test, based on the luminescent bacterium *V. fischeri NRRLB-111,* is similar in principle to Microtox test that uses the marine bacterium *Photobacterium phosphoreum* (Callow et al., 1999). Statistical analysis (ANOVA table in Appendix C) indicates that zosteric acid became more toxic from control, to pH = 11, to the final step. The EC_{50} of ZA of the final step after drying is almost equal to the value we obtained previously. Therefore the purity between batches and their associated effectiveness was the same.

4.3.2 NPAs exposure to marine bacteria

A concurrent study with two pure cultures of marine bacteria, *Vibrio natriegens* and *Vibrio parahaemolyticus*, was performed to achieve the EC_{50} of capsaicin and zosteric acid. The results when capsaicin was used as the NPA are shown in Table 4.5. Table 4.6 gave the result for zosteric acid exposed to these two marine bacteria systems. The EC_{50} values of capsaicin and zosteric acid to marine bacteria were obtained by using the trendline forecast method (all the figures are located in Appendix A). The EC_{50} values of capsaicin to *V. natriegens* and *V. parahaemolyticus* systems were 17.0 mg/L and 15.6 mg/L, respectively. For zosteric acid, they were 7.4 mg/L and 18.0 mg/L, respectively. It is seen that the microbial activity of marine bacteria decreased as the concentration of

Table 4.5 Static toxicity assessment of capsaicin to marine bacteria

(± is standard deviation of triplicate samples)

Bacteria system	MPN (cfu/L)	% decrease
V. parahaemolyticus		
Control	2.44±0.38E16	---
+ 2 mg/L capsaicin	1.89±0.54E16	22
+ 10 mg/L capsaicin	1.86±0.05E16	23
+ 20 mg/L capsaicin	5.7±0.85E15	76
V. natriegens		
Control	1.57±0.10E12	---
+ 5 mg/L capsaicin	1.23±0.04E12	22
+ 10 mg/L capsaicin	1.00±0.32E12	36
+ 20 mg/L capsaicin	7.35±0.92E11	53

Table 4.6 Static toxicity assessment of Zosteric Acid (ZA) to marine bacteria
(± is standard deviation of triplicate samples)

Bacteria	MPN (cfu/L)	% decrease
V. parahaemolyticus		
Control	2.44±0.4E16	---
+ 10 mg/L ZA	1.41±0.63E16	42
+ 20 mg/L ZA	1.18±0.58E16	52
+ 100 mg/L ZA	8.5±0.91E15	65
V. natriegens		
Control	8.9±1.2E12	--
+ 5 mg/L ZA	6.14±0.32E12	31
+ 10 mg/L ZA	2.16±0.12E12	71

NPAs increased. For instance, the decrease of bacterial activity of *V. parahaemolyticus* was 23% at 10 mg/L of capsaicin, while it was 76% when the concentration was at 20 mg/L of capsaicin in the solution. The microbial activity of *V. natriegens* decreased by 31% at 5 mg/L of zosteric acid, and by 71% at 10 mg/L. It seems that *V. natriegens* was more sensitive (P-value is 0.031) than *V. parahaemolyticus* when they were exposed to zosteric acid for a same time. However, there was no statistically significant difference (P-value 0.654) between them when they were exposed to capsaicin.

4.4 Comparison

From the analysis of all the toxicity results, it is seen that the NPAs were more "toxic" to marine bacteria than freshwater bacteria (P-value=0.009). As the same result mentioned in section 4.3.1, the comparison ANONA table (shown in Appendix C) indicated that capsaicin and zosteric acid have no significant difference in toxicity to every kind of bacteria species (P-value=0.423). However, for a same compound or NPA, a significant difference between any two bacteria species was observed (P-value almost equal to zero). The most sensitive species to capsaicin and zosteric acid was *P. putita* and *V. natriegens,* respectively. The least sensitive one to both of the NPAs was the Lake Erie consortium. It was expected that zosteric acid would be less toxic than capsaicin since zosteric acid can be extracted from eelgrass, an aquatic plant. While, capsaicin is the hot component of chili-pepper.

From the toxicity assessment, capsaicin and zosteric acid are significantly better than that of currently used antifoulants. For example, the 48h-EC_{50} of TBT was 0.46 μg/L for *D. tertiolecta* and 10 day-LC_{50} of 2.31 μg/L for *M. koreana* (Cheung et al., 2003). The

10 day-LC_{50} of *Eohaustorius wasingtonianus* and *Eohaustorius estuaries* were 1.6 and 2.0 μg/L, respectively (Meador et al., 1993). Also, TBT has been shown to inhibit cell survival of marine unicellular algae at very low concentrations with the 72 h-EC_{50} ranging from 0.33 to1.03 μg/L (USEPA, 1985). For another antifoulant, Bromotyramine, an EC_{50} of 0.03-1 μg/mL was reported for *B. ampritite* (Schoenfeld et al., 2002). Because those compounds are highly toxic to many aquatic organisms, their exposure to non-target aquatic organisms such as mussels, clams and oysters at a few ng/L levels may cause structural changes, growth retardation, and death (Alzieu and Heral, 1984; Bryan and Gibbs, 1991). Maximum water TBT concentrations of 0.4 ng/L was suggested by Alzieu (1996) in order to ensure the protection of plankton in the marine environment. The biotoxicity of DBT (Dibutyltin) and MBT (Monobutyltin) was expected to be low since effective concentrations on various organisms obtained by several studies were some orders of magnitude higher than their elutriate concentrations obtained in the present study. For example, the EC_{50}/24-48 h of MBT and DBT on the water flea, Daphnia magna, was 1-10 mg/L (Vighi and Calamari, 1985; Acima, 1992), and 48 h-EC_{50} of dibutyltin on oyster larva was 0.1-0.2 mg/L (Thorn et al., 1991). All of these studies indicate that they are very toxic to the aquatic organisms in the marine environment. As such, their usage as antifoulants was inhibited. However, capsaicin and zosteric acid have relative lower toxic characteristics. In freshwater system, the EC_{50} of capsaicin and zosteric acid were 10-22 mg/L and 240-380 mg/L, respectively. With marine bacteria, capsaicin and zosteric acid had EC_{50} of 15-17 mg/L and 8-18 mg/L, respectively. Therefore they can be considered non-toxic natural products.

CHAPTER V

BACTERIAL ATTACHMENT USING CAPSAICIN AND ZOSTERIC ACID

5.1 Introduction

As mentioned in Chapter III, the antifouling effectiveness of capsaicin and zosteric acid were also assessed by attachment studies. The coated slides were immersed in water containing different bacteria and NPAs. The antifouling effectiveness was evaluated by analyzing of the properties changing in the aqueous solution and the biofilm formation on the coatings. First, the NPAs were suspended in the bacterial solution at different concentrations to perform the experiment. Then, zosteric acid (1 wt %) was entrapped in the silicone coatings to obtain the antifouling effectiveness. Two freshwater bacteria, an enriched culture isolated bacteria from Lake Erie and pure culture *P. putida*, and two marine bacteria *V. parahaemolyticus* and *V. natriegens* were used as the species of interest.

5.2 NPAs exposed to freshwater bacteria

5.2.1 NPAs suspended in the solution

In order to ascertain if the NPAs interfere with bacteria growth by blocking their attachment to the surface or simply kill them, the initial antifouling performance of the NPAs was first determined by dissolving them into water containing bacteria. Both the

physiochemical and biological characteristics of the water, as well as the biofilm formation on the coated slides were investigated. As mentioned in Chapter III, concentrations of NPAs selected were based on the toxicity assessments and to be able comparison the different effectiveness on fouling. Thus, 10, 20 and 40 mg/L of capsaicin, 50, 100 and 500 mg/L of zosteric acid were applied in the initial sets of experiment. Unfortunately, ethanol was accidentally left in solution with 10 mg/L capsaicin (forgot to drive off the ethanol after capsaicin dissolved in) and significantly affected the evaluation of the effectiveness of capsaicin on fouling. Therefore only the performance of 20 mg/L and 40 mg/L of capsaicin were considered. For case in comparison the 50 mg/L and 500 mg/L zosteric acid, the initial characteristics of control (without zosteric acid) condition were dramatically different from the conditions containing 100 mg/L zosteric acid, the effectiveness of 100 mg/L of zosteric acid will not be discussed together with 50 mg/L and 500 mg/L. The representative figures for the 100 mg/L zosteric acid system are located in Appendix B.

5.2.1.1 Change in temperature and pH

Temperature was considered as a potential factor that could impact other parameters. As shown in Figure 5.1, the most pronounced changes occurred with the first week because of the room temperature change. However, the temperature fluctuation throughout each experiment was insignificant (P-value>0.05), only deviating by ±1.6ºC on average. Its effect on the conductivity and pH change would be very small. Therefore, the effect of temperature on the changes in conductivity and pH can be neglected.

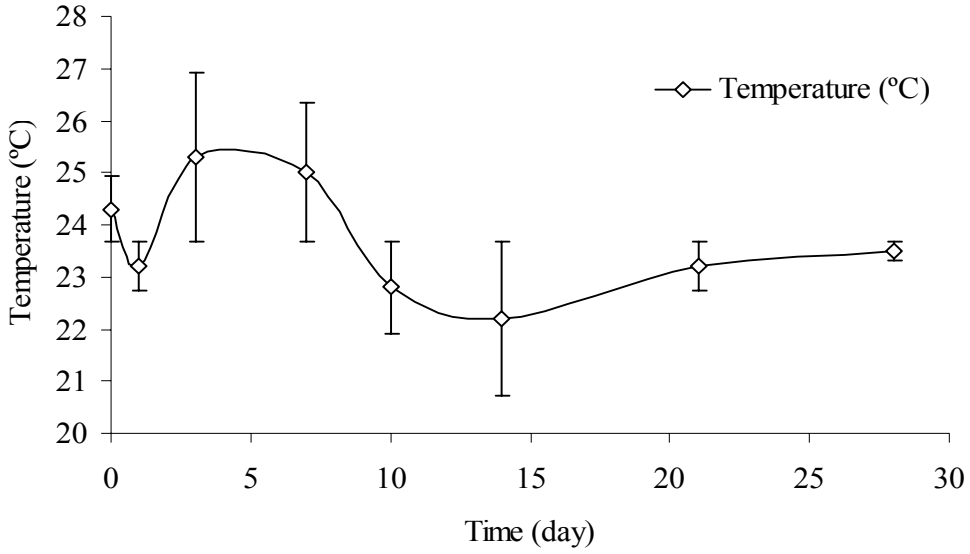

Figure 5.1 Representative of temperature change with time in aqueous solution for attachment studies, (error bars represent ± standard deviation, n=2)

Figure 5.2 is a representative graph of changes in pH due to the presence 20 and 40 mg/L capsaicin on *P. Putida* (Pp) and enriched Lake Erie (LE) bacteria. Initially two controls were investigated. There was no statistical difference (P-value=0.135, Appendix D) between silicone-coated slide (control 1) and plain slide (control 2), indicating that the silicone coating does not affect pH (refer to Figure B1 in Appendix B). Therefore, only control 1 will be used in subsequent evaluation of aqueous parameters. Stanley (2001) found that the pH of an unbuffered 1% wt/vol zosteric acid solution was 7.5, a weak base. However, because of the buffering characteristics of bacteria, the effect of NPAs on the initial pH value was minimal. For instance, capsaicin lowered the pH by ~0.1 while the presence of zosteric acid lowered the initial pH only by 0.1~0.2, depending on the concentration used.

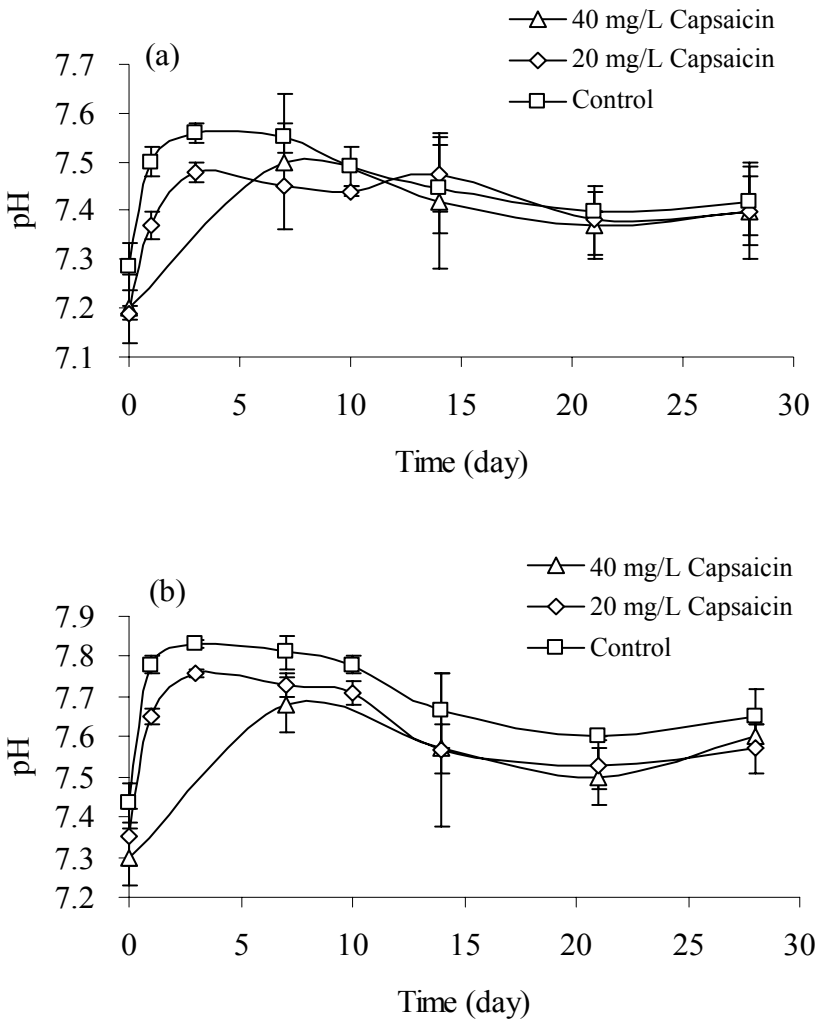

Figure 5.2 pH changing with time for *P. putida* (a) and *LE* (b) with different concentration of capsaicin, data missed on day 1 and day 3 for 40 mg/l. control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

As shown in Figure 5.2, the pH increased from day 1 to day 3 for all three capsaicin concentrations in each bacteria system. Bacteria can adjust the pH of a new environment to the optimal growth condition via biobuffering (release of exudates to change the pH). For the Pp system (Figure 5.2a), the optimal pH value with capsaicin was 7.4 (no statistical difference, P-value=0.492>0.05) for three concentrations

evaluated. For the LE system (Figure 5.2b), the presence of capsaicin initially exhibited a slightly lower pH. However, both the 20 mg/L and 40 mg/L treatment had stabilized at 7.6 by the end of the fourth week.

Zosteric acid exhibited the same trend as shown in Figure 5.3, with control samples having a higher pH. By the end of the fourth week, the control and 50 mg/L treatments were stable at about 7.4 and 7.6 for Pp and LE bacteria system, respectively. It indicated that the lower concentration of zosteric acid was insufficient to affect the final pH. With 500 mg/L zosteric acid, the Pp and LE bacteria pH was 7.1 and 7.3, respectively. It was seen that the higher concentration of zosteric acid have a significant (P-value equal to 0.004 and 0.007 for Pp and LE systems, respectively) effect on the final pH value in solution.

A separate assay that dissolved NPA directly into distilled water found that the synthesized zosteric acid would increase the pH by 0.57 and 1.03 from 4.89 (pH of pure DI water was found to be 4.89), as concentration increased to 50 mg/L and 500 mg/L, respectively (see Appendix E). Therefore, the presence of zosteric acid should increase the final pH. Surprisingly, it had an obvious decrease for both of Pp and LE systems with 500 mg/L zosteric acid being similar to the corresponding control condition. This implies that the biobuffering property of bacteria played a key role in the aqueous pH change; a high concentration of zosteric acid would inhibit the capacity of biobuffering characteristics. In other words, the effect of NPA's biobuffering inhibition on pH change is more than that of its inherent property on pH.

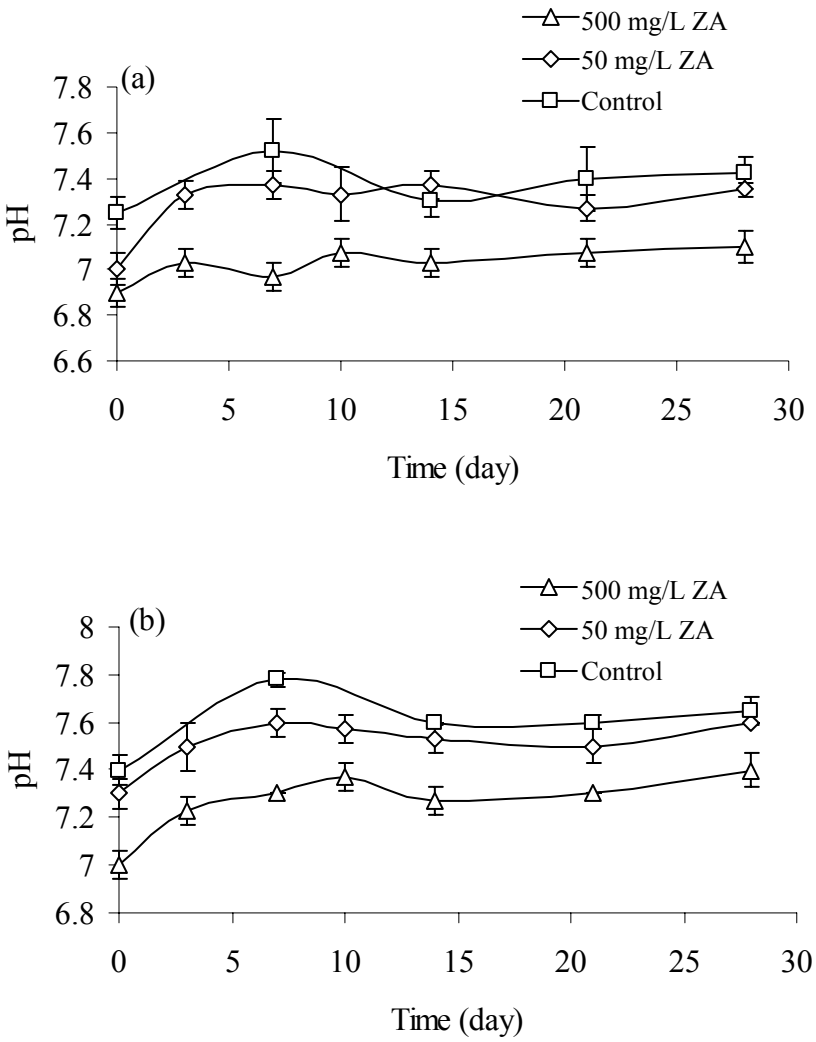

Figure 5.3 pH changing with time for *P. putida* (a) and *LE* (b) with different concentration of zosteric acid, data missed on day 3 for 500 mg/L. control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

5.2.1.2 Change in conductivity

The initial characterization ascertained that the conductivity of Lake Erie water was 320 mg/L. Transferring 3 mL of nutrient solution (with 7200 mg/L conductivity) to sterilized Lake Erie water would result in an initial conductivity prior to adding any

antifoulant of 740-770 mg/L (refer to Appendix E for data). As shown in Figure 5.4, this was the initial value for the controls. The controls also depicted conductivity values lower than the systems with capsaicin. For instance, 40 mg/L of capsaicin increased conductivity readings by 35 mg/L (as ascertained by a concurrent experiment with distilled water). Similarly, 500 mg/L zosteric acid increased conductivity readings by ~200 mg/L as shown in Figure 5.5 (Appendix E for data).

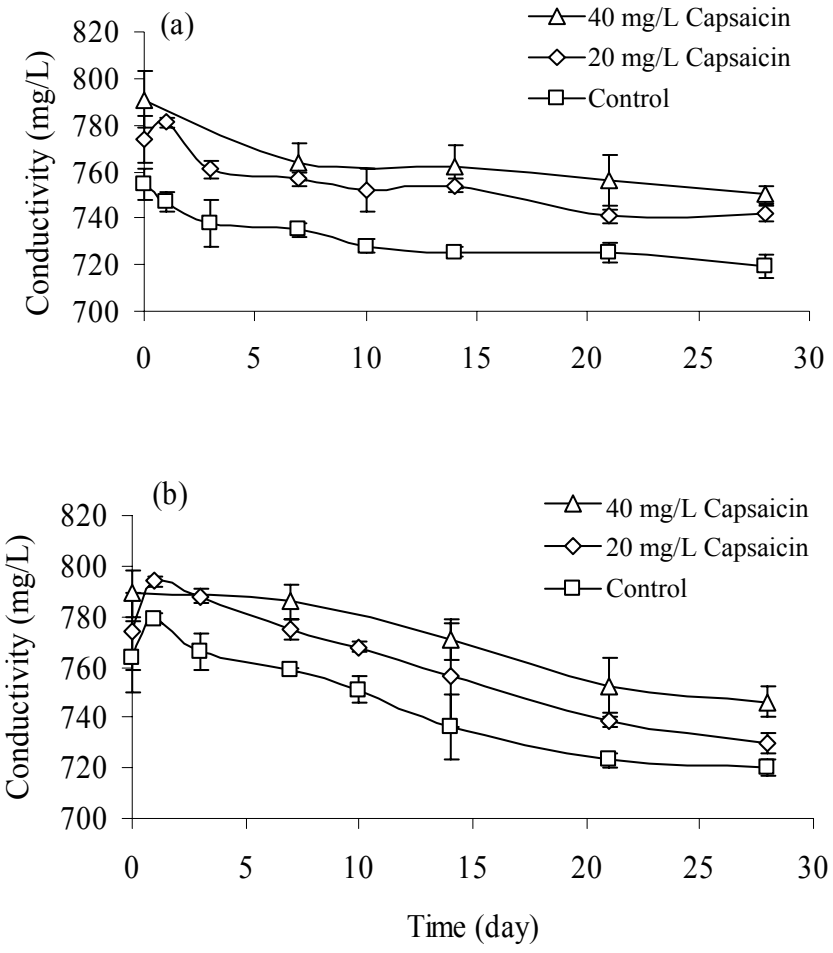

Figure 5.4 Conductivity changing with time for *P. putida* (a) and *LE* (b)with different concentration of capsaicin, data missed on day 1 and day 3 for 40 mg/L. control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

The conductivity trend was similar for each bacteria-capsaicin combination investigated. As with the *P. putida* system, the conductivity of LE system (Figure 5.4b) fluctuated during first two days. This was directly related to microbial activity that consumed inorganic nutrients during cellular maintenance, thereby decreasing conductivity. The more active the population, the more pronounced the decrease in conductivity. For instance, Kjekkeberg et al (1982) found that different bacteria growth on the surfaces exhibited growth rates up to ten times higher than that in the aqueous phase. In addition, this increase in growth rate was believed to be related to the adsorption of more nutrients and consumption by the bacteria on the surface of the substrate. According to this study, the more biofilm formation on the slide, the more relative decrease of nutrient including organic and inorganic (performed by conductivity) in solution should be achieved. For instance, by the end of four weeks, the conductivity decrease was 4.77%, 4.26% and 4.3% for the Pp control, 20 mg/L and 40 mg/L capsaicin, respectively with system. For LE system, this decrease was 5.76%, 5.43% and 5.32%, respectively, for the control, 20 mg/L and 40 mg/L conditions. After four weeks, for Pp system, there was a significant difference (P-value=0.003, Appendix D) of conductivity between the solution with capsaicin (both 20 mg/L and 40 mg/L) and without capsaicin. The same result was achieved (P-value=0.003, Appendix D) with the LE system. It implied that the presence of capsaicin and subsequent bacterial attachment to the coating surface was the primary factor on the difference of conductivity in solution.

For the *P. putida*-zosteric acid combination (Figure 5.5a), conductivity remained relatively constant throughout the entire experiment. The specific levels were 760 mg/L and 930 mg/L for 50 and 500 mg/L zosteric acid, respectively. These levels were 25 mg/L and 190 mg/L higher than that of control solution. So, it is not surprising that the final values were significantly different (P-value=0.03). Similar results were obtained in the LE system, except that the initial difference was not as huge as with the Pp system. It was 20 mg/L and 180 mg/L, respectively for 50 mg/L and 500 mg/L zosteric acid solution compared to control. In addition, zosteric acid was still the key factor that made the conductivity different between the different conditions. As shown in Figure 5.5b, the Lake Erie system exhibited a gradual increase in conductivity levels within the first two weeks and stabilized during weeks 2-4. Because the components of bacterial species in LE system were more complex than that of Pp system, some kinds of bacteria in LE system could buffer the conductivity change or degraded zosteric acid into simple compounds to make a slight increase in conductivity.

5.2.1.3 Changes in dissolved oxygen (DO) levels

Figure 5.6 contains graphs of how DO levels changed with time when the aqueous microbes were exposed to NPA's in solution. For each system, the DO levels decreased as time increased, with the largest decrease corresponding to the highest NPA concentration. Several mechanisms could facilitate a decrease in DO. One is the temperature since saturated DO levels are a function of temperature. As mentioned

earlier, the change of temperature was not significant (±1.6°C) and would have had a maximum change in DO of ±0.27 mg/L (Calculation shown in Appendix E).

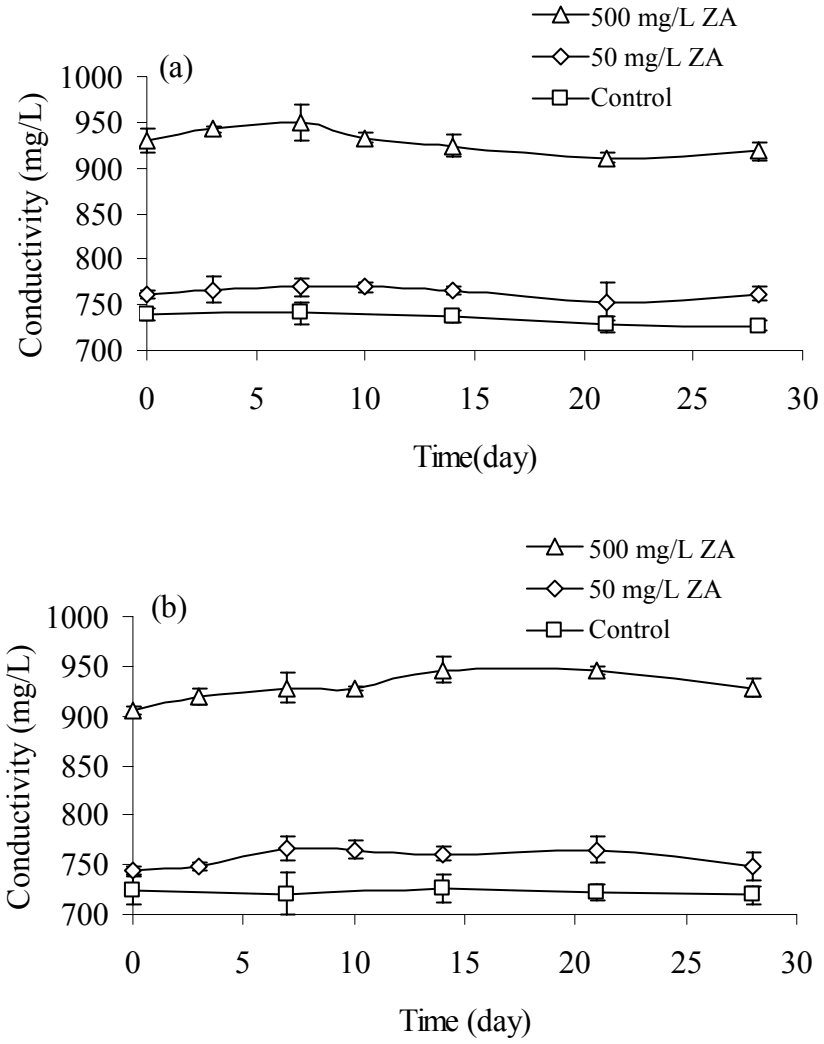

Figure 5.5 Conductivity changing with time for *P. putida* (a) and *LE* (b)with different concentration of zosteric acid, data missed on day 3 for 500 mg/L. control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

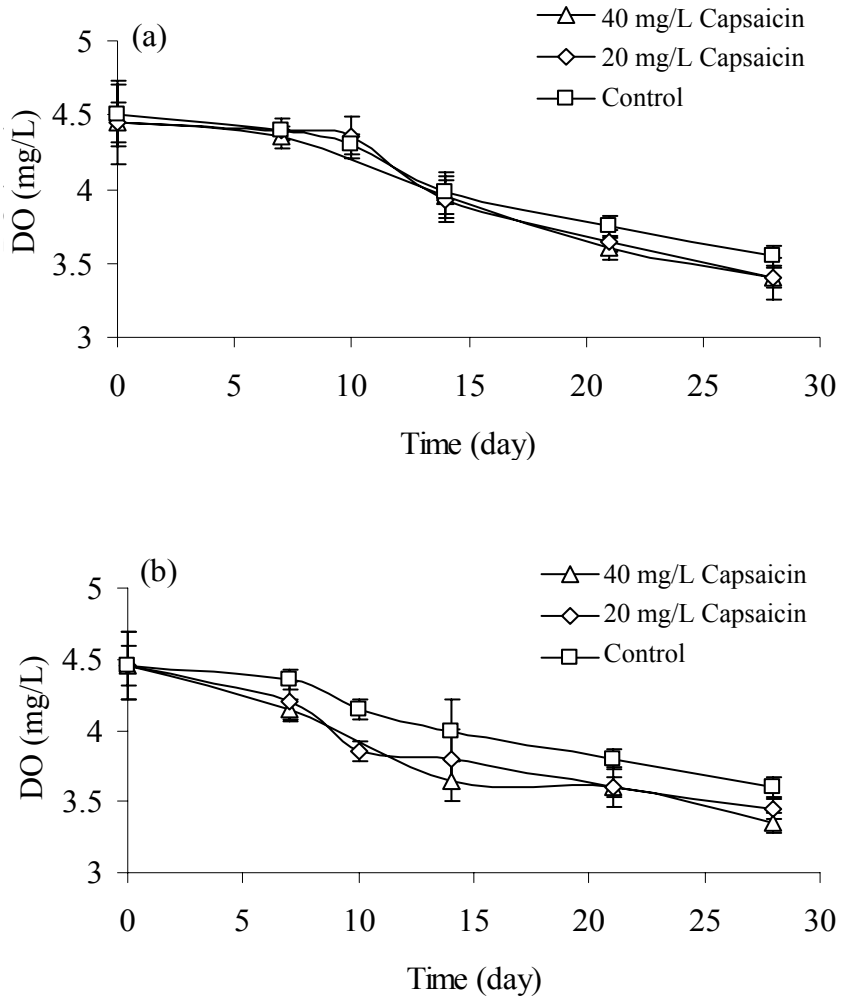

Figure 5.6 DO changing with time for *P. putida* (a) and *LE* (b) with different concentration of capsaicin, control represents no capsaicin in the solution (error bars represent ± standard deviation, n=3)

The other mechanism is the microbial activity. Both the aqueous bacteria culture (MPN result) and the biofilm formation on the slide are indications of microbial activity. DO is a critical need for bacteria respiration. If a new supply of oxygen were not provided, this would result in a decrease in DO. As mentioned in section 3.2, the bottles received

"oxygen refreshment" after 2 weeks. This approach introduced 10.4 mg O_2 into the headspace, yielding the necessary O_2 levels for respiration. Typically, consumption of every mol of oxygen can yield 0.514 mol biomass. So, the average yield biomass should be 0.049 mg in each bottle at the end of four weeks (detailed calculations were shown in appendix E).

It is important to note that the respiration of attached bacteria (in a biofilm) requires less DO than bacteria in suspension (Cookson, 1995). If the NPAs block the active surface sites on the bacteria, then less biofilm would be formed in comparison to the control. This would leave more bacteria in suspension (i.e., higher aqueous MPN) and cause a corresponding lower DO level. (As will be mentioned later (section 5.2.1.4), higher aqueous MPN with higher concentration of NPAs and a less corresponding biofilm formation on the slides were achieved.) Theoretically, a higher DO level in solution with less concentration of NPAs should be observed. After four weeks, there was an obvious difference of DO between control condition and conditions with capsaicin (Figure 5.6). But, the difference between the conditions with different concentration of NPA was insignificant (P-value=0.262 and 0.084 for Pp and LE system, respectively). For instance, DO was 3.55 mg/L, 3.4 mg/L and 3.4 mg/L in the solution of control, with 20 mg/L and 40 mg/L capsaicin, respectively for Pp system. For LE system, the DO level was 3.6 mg/L, 3.45 mg/L and 3.4 mg/L, respectively for control, 20 mg/L and 40 mg/L capsaicin. This implied that the higher NPAs resulted in the lower DO level and corresponded higher MPN in the solution.

Results with zosteric acid depicted the same trend as did capsaicin (Figure 5.7). However initial DO level for Pp system decreased by 0.1 (Figure 5.7a) and 0.05 for LE

system each having a large standard deviation (Figure 5.7b) when zosteric acid concentration was 500 mg/L.

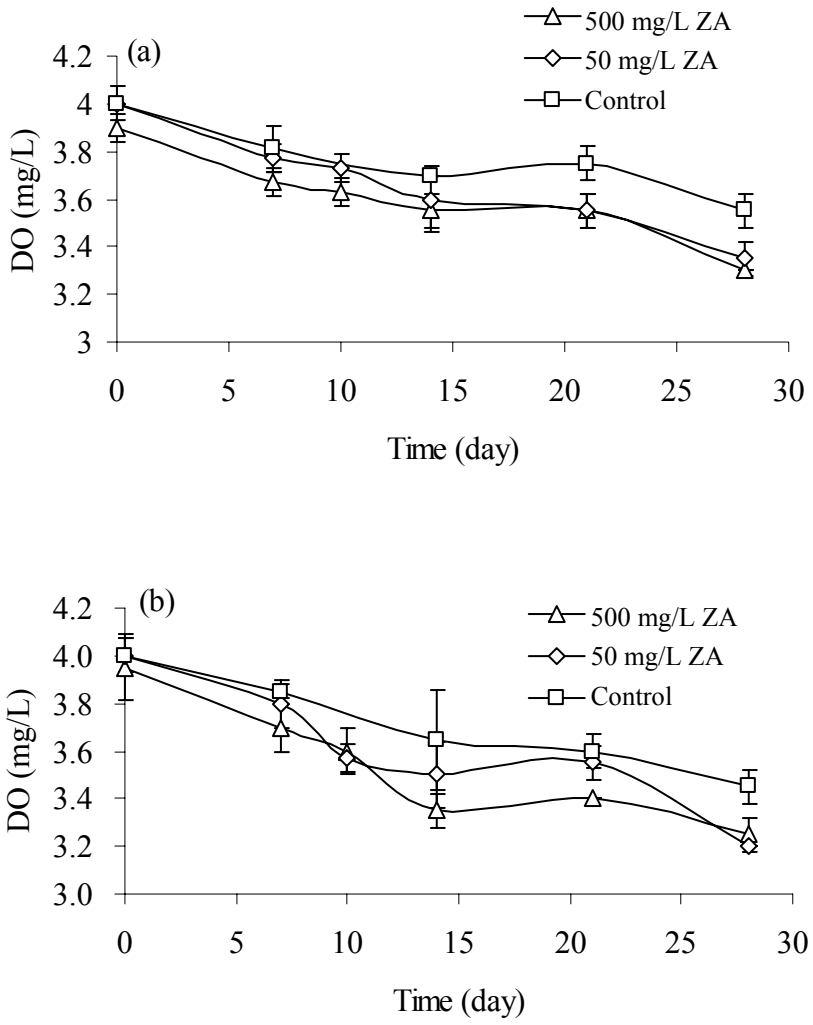

Figure 5.7 DO changing with time for *P. putida* (a) and *LE* (b) with different concentration of zosteric acid control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

This was expected since the saturated DO is a function of salt concentration that decreases as salt concentration increases. A separate assay identified that a lower concentration of NPAs dissolved in distilled water (20 mg/L and 40 mg/L of capsaicin, 50 mg/L of zosteric acid) would not affect the DO level (4.3 mg/L at 23.4°C). However, 500 mg/L zosteric acid would decrease the DO by 0.1 (Appendix E for tabulated results). So, the presence of 500 mg/L zosteric acid facilitated the decrease in the initial DO level.

5.2.1.4 Changes in aqueous microbial numbers

Figure 5.8 contains the results the aqueous microbial activity as determined by MPN for both cultures in the presence of 20 and 40 mg/L capsaicin. For each system, the aqueous microbial activity decreased with time. After one month of exposure to capsaicin, the MPN had decreased by 63.4% for the *P. putida* and 65% for the LE system. The decrease of MPN could be due to either the microbial activity decreased (i.e., bacteria died from either normal endogenous decay or exposure to toxic material) or the bacteria had attached onto the slides. As shown in Chapter IV, the EC_{50} of capsaicin to *P. putida* and LE bacteria was 5.5 mg/L and 22.6 mg/L, respectively. Thus a small portion of the MPN decrease could be associated with toxicity. As will be discussed in the following section, biofilm formation on the slides increased as time progressed. The consumption of DO (section 5.2.1.3) and presence of biofilm indicated that the decrease in MPN was primarily due to the biofilm formation.

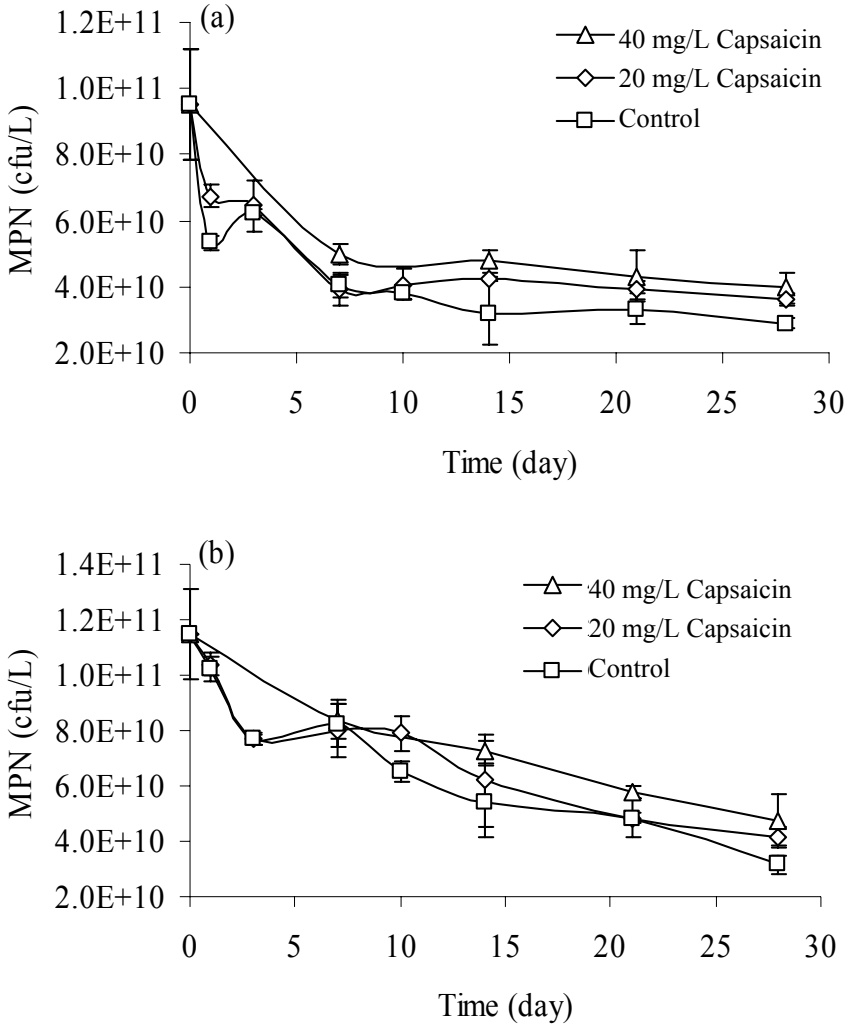

Figure 5.8 MPN in the solution changing with time for *P. putida* (a) and *LE* (b) with different concentration of capsaicin, data missed on day 1 and 3 for 40 mg/L. control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

It is very important to note that the higher the concentration of capsaicin present in the solution, the more MPN in the aqueous solution was observed. After 28 days, in Pp system, the MPN in the solution containing 40 mg/L capsaicin was 4×10^{10} cfu/L, 3.8% higher than that of 20 mg/L capsaicin and was 1.1×10^{10} cfu/L (11.2%) higher than that of control condition. Furthermore, the 20 mg/L capsaicin system had an MPN that was

7×10^9cfu/L (7.8%) higher than that of control (corroborated the finding in DO, section 5.2.1.3). Thus, the aqueous population with 40 mg/L capsaicin were statistically different (P-value=0.018) than the control. However, the MPN between 20 mg/L capsaicin and control was not significant (Tukey's comparison in Appendix D), indicating that lower concentration of capsaicin could not affect the aqueous population.

In LE system (Figure 5.8b), MPN was 1.6×10^{10} cfu/L (14%), 6×10^9 cfu/L (5.1%) and 1×10^{10} cfu/L (8.9%) higher between the conditions with 40 mg/L capsaicin and control, 40 mg/L capsaicin and 20 mg/L capsaicin, and 20 mg/L capsaicin and control, respectively (confirmed the finding in DO, section 5.2.1.3). Also, there was significant difference (P-value=0.018) between the condition with 40 mg/L capsaicin and control, but no significant difference (Appendix D) between 20 mg/L and control, 40 mg/L and 20 mg/L. It provided another evidence that lower concentration of capsaicin could not significantly change the bacteria concentration in solution. At the same time, biofilm formation on the slides decreased as concentration of capsaicin increased (Figure 5.10 in section 5.2.1.5). These two phenomena indicated that 1) the decrease of MPN in solution was primarily due to the bacteria attaching on the slides, and 2) the antifouling function of capsaicin may not be to simply kill the bacteria. If the mechanism was to simply kill the bacteria, the higher concentration of the antifoulant in solution, the less MPN should be observed.

As shown in Figure 5.9, zosteric acid amendments resulted in the aqueous microbial activity exhibiting similar trends as that with capsaicin. After four weeks, the MPN had averagely decreased by 67.3% for the *P. putida* system and 63.7% for the enriched LE bacteria.

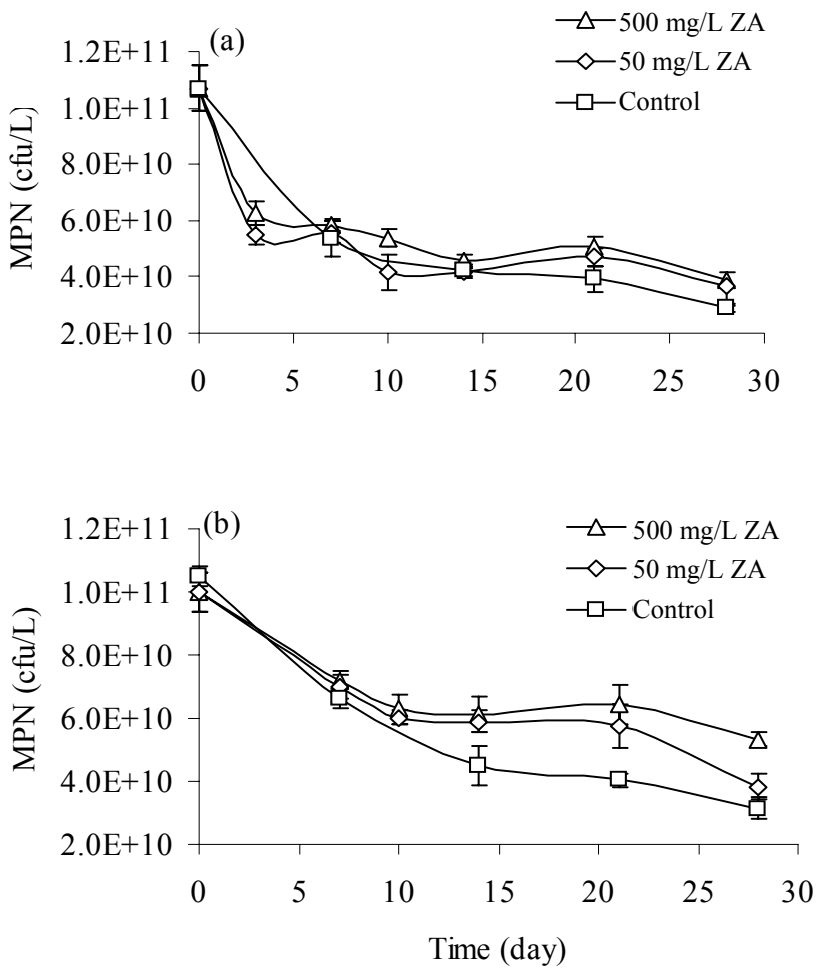

Figure 5.9 MPN in the solution changing with time for *P. putida* (a) and *LE* (b) with different concentration of zosteric acid, control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

The EC_{50} for *P. putida* and LE cultures were determined to be 170 mg/L and 380 mg/L, respectively (Section 4.2.2). As with capsaicin, the decrease in MPN in the presence of zosteric acid could be attributed to either toxicity or the formation of biofilm. Since zosteric acid was dispersed in solution, the formation of a biofilm would 'remove' the microbes from direct contact with the slightly toxic NPA. However, it was still observed that the higher concentration of zosteric acid present, the more MPN in the solution and a

corresponding less biofilm formation on the slide were achieved (Figure 5.11 in section 5.2.1.5). For instance, after 28 days in LE system (Figure 5.9b), the MPN in the solution containing 500 mg/L zosteric acid was 2.0×10^{10}cfu/L, 20% higher than that of 50 mg/L zosteric acid and was 2.2×10^{10}cfu/L (22%) higher than that of control condition. In addition, there was a 0.2×10^{10}cfu/L (2%) higher of 50 mg/L than that of control. The statistical analysis indicated that the MPN in 500 mg/L solution was significant higher (P-value=0.002) than that of 50 mg/L and control condition. The similar result was obtained in Pp-zosteric acid combination (P-value=0.048). Therefore, our biofilm formation and MPN results indicated that zosteric acid has the same antifouling mechanism as capsaicin—maybe not simply kill the bacteria, but block the attachment sites of bacteria to prevent them attaching onto the surface. If this is true, both compounds will be very attractive natural product antifoulants.

5.2.1.5 Effect of NPAs on biofilm formation

Figures 5.10(a)-(c) are representative morphology images of biofilm formation on the plain silicone coatings with and without the presence of capsaicin in the solution. As capsaicin concentration in solution increased, the amount of bacteria attachment (i.e., biofilm growth) to the coating surface decreased. Figure 5.10 (a) shows normal growth of Lake Erie bacteria on silicone (Sylgard® 184) coating after 14 days (The difference of biofilm formation on the slide was easier to be seen in day 14 than that of day 28) in absence of NPAs. When 20 mg/L of capsaicin was added, only a few colonies (6.5% compared to control) were found to have attached after 14 days (Figure 5.10b). With 40

mg/L capsaicin, even less colonies (only 1.5% compared to control) were found (Figure 5.10c), indicating that capsaicin inhibited bacteria colonies thereby preventing the formation of a biofilm. Comparison to control for biofilm formation was shown in Table 5.1. By the 28th day, the control surfaces were almost completely covered, while only a few colonies attached on the coatings that were immersed in solutions with capsaicin.

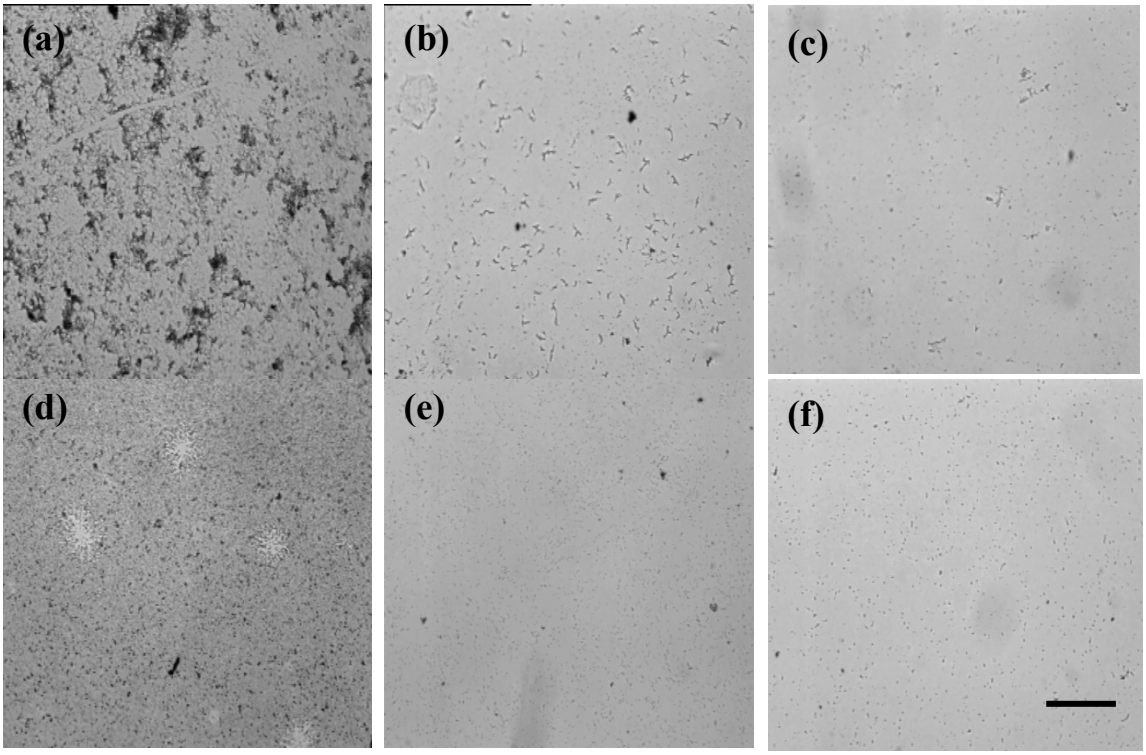

Figure 5.10 Coatings of Sylgard® 184 immerged in various bacteria solution after 14 days: (a)Lake Erie bacteria without capsaicin; (b) Lake Erie bacteria with 20 mg/L capsaicin; (c) Lake Erie bacteria with 40 mg/L capsaicin; (d) *P. putida* without capsaicin; (e*) P. putida* with 20 mg/L capsaicin; (f) *P. putida* with 40 mg/L capsaicin, bar =30 μm (Barrios took the pictures)

Table 5.1 Comparison to control for biofilm formation of suspended capsaicin in Lake Erie solution

Lake Erie	Pixels	% Relative
Control	1618	100.0
20 mg/L capsaicin	121	6.5
40 mg/L capsaicin	29	1.5

The pure strain, *P. putida,* was only "cocci" shape while the Lake Erie bacteria exhibited various shapes including rod, spirilla, and cocci. The attachment and growth of *P. putida* was also found to be different from that of Lake Erie bacteria. Figure 5.10(d) depicts the normal attachment and growth of *P. putida* on the silicone coating surface without NPAs. Figures 5.10(e) and (f) show that *P. putida* attachment to the coating surface decreased as concentration of capsaicin in solution increased. An almost clear coating (Figure 5.10(f)), indicating minimal attachment was observed when 40 mg/L of capsaicin was used.

Figure 5.11 contains images of biofilm formation for systems treated with 50 or 500 mg/l zosteric acid. The behavior of samples immersed solution containing zosteric acid exhibited similar trend treated with capsaicin: the bacteria population on the surface decreased as concentration of NPA increased. Even after only 14 days, the control samples for LE system had significant biofilm coverage (Figure 5.11 (a)). As shown in Figure 5.11(b), only a few colonies (7.5% compared to control) were observed on the surface when 50 mg/L zosteric acid was present. Similarly, the coating was almost clear (1.8% compared to control) with 500 mg/L zosteric acid (Figure 5.11c). Table 5.2 presents the comparison of biofilm formation. The *P. putida* systems exhibited less

biofilm than that of the same condition for LE system. The most reasonable explanation is that the LE system contains much more species of bacteria that that of *P. putida* system. Some kinds of bacteria could resist the effect of NPAs and still attached onto the surface of slides. However as shown in Figure 11 (d) (e) and (f), the attachment still decreased as the concentration of NPA increased.

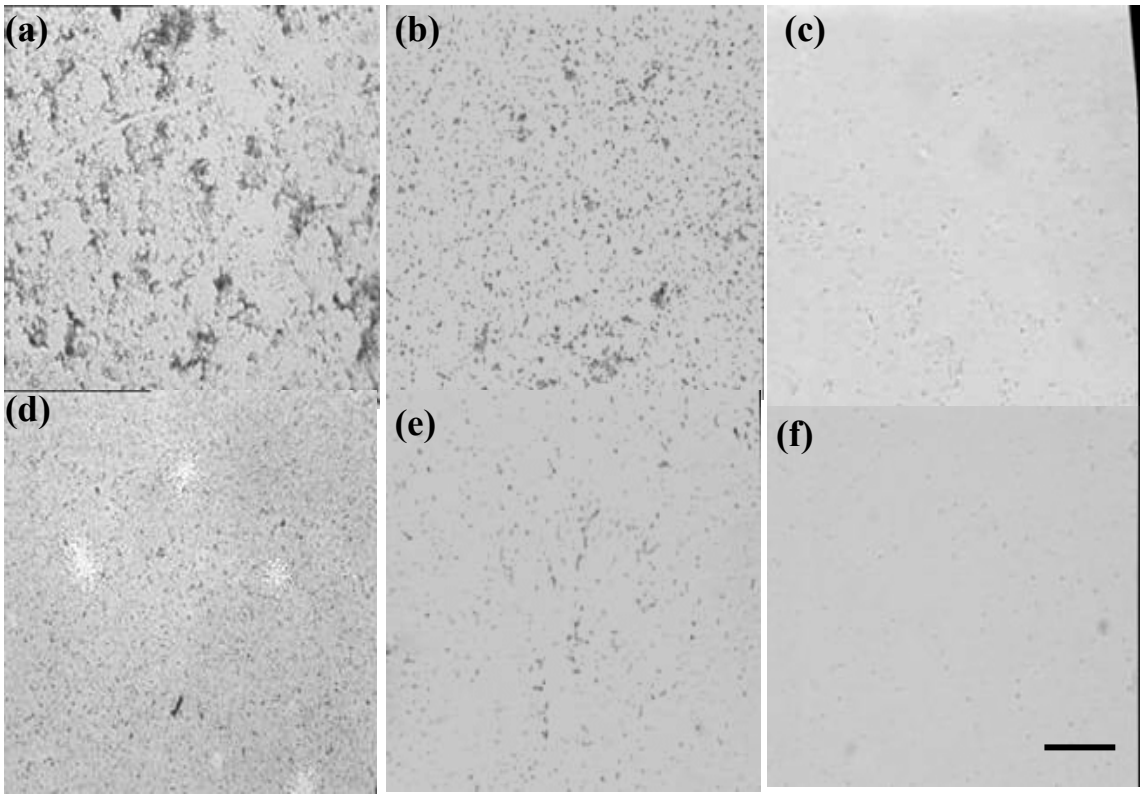

Figure 5.11 Coatings of sylgard® 184 immerged in various bacteria solution after 14 days: (a) Lake Erie bacteria without zosteric acid; (b) Lake Erie bacteria with 50 mg/L zosteric acid; (c) Lake Erie bacteria with 500 mg/L zosteric acid; (d) *P. putida* without zosteric acid; (e*) P. putida* with 50 mg/L zosteric acid; (f) *P. putida* with 500 mg/L zosteric acid, bar =30 μm (Barrios took the pictures).

Table 5.2 Comparison to control for biofilm formation of suspended zosteric acid (ZA) in Lake Erie solution

Lake Erie	Pixels	% Relative
Control	1618	100.0
50 mg/L ZA	121	7.5
500 mg/L ZA	29	1.8

5.2.2 Comparison of the antifouling function of NPAs

The final target for antifouling is to inhibit the aqueous organisms attaching on the surface. Thus, in experimental study, the biofilm formation on the slides should be the primary parameter to compare the efficiency of antifouling function by capsaicin and zosteric acid.

5.2.2.1 Effectiveness of NPAs exposed to LE bacteria

As mentioned in section 5.2.1.5, compared to the biofilm formation on the slide immersed in the LE solution without capsaicin, the biofilm coverage decreased 93.5% when 20 mg/L capsaicin was added, and it decreased 98.5% as the concentration of capsaicin increased to 40 mg/L. It was obvious to see that 20 mg/L capsaicin had a higher efficiency on antifouling than that of 40 mg/L. The biofilm formation decreased by only additional 5% with 40 mg/L compared to 20 mg/L capsaicin present, even though the concentration was double. When zosteric acid exposed to LE system, the higher the concentration of zosteric acid in the solution, the less biofilm formation on the surface was achieved. Compared to control condition, the coverage of biofilm decreased 92.5% and 98.2% as 50 mg/L and 500 mg/L zosteric acid were present in the solution,

respectively. The biofilm formation only further decreased 5.7% but increased ten times of zosteric acid concentration in solution, thus 500 mg/L zosteric acid in bacterial solution had a less efficiency on antifouling than that of 50 mg/L zosteric acid.

5.2.2.2 Effectiveness of NPAs exposed to Pp bacteria

As NPAs exposure to LE bacteria, the biofilm formation on the slides decreased when the concentration of NPAs in the solution increased. For instance, compared to the to the control condition, the coverage of biofilm on the slide decreased 95.2% and 99.6% when 20 mg/L and 40 mg/L capsaicin was present, respectively. It decreased 94.8% and 99% as 50 mg/L and 500 mg/L zosteric acid was respectively dissolved in. As mentioned in section 5.2.2.1, since a large concentration increase only caused a small effect on the biofilm formation, 20 mg/L of capsaicin and 50 mg/L zosteric acid are considered more effective than that of 40 mg/L capsaicin and 500 mg/L zostric acid, respectively, in antifouling.

In addition, the antifouling function of NPAs to Pp bacteria was more effective than that to LE system under a same concentration. For instance, compared to their corresponding control conditions, the biofilm formation decreased 93.5% when 20 mg/L capsaicin exposed to LE bacteria, but it decreased 95.2% for Pp system. Similarly, 50 mg/L zosteric acid decreased 92.5% and 94.8 biofilm formation of LE bacteria and *P. putida*, respectively. As mentioned in section 5.2.1.5, the reason is that the bacterial components of LE system are much more complex than that of *P. putida* system. Some bacteria could resist the effect of NPAs and still attached onto the coating surface.

5.2.2.3 Comparison of effectiveness between capsaicin and zosteric acid

According to the analysis in last two sections, 20 mg/L and 50 mg/L were the relative ideal concentrations for capsaicin and zosteric acid, respectively to inhibit the bacteria attaching onto the surface. Furthermore, these two NPAs at the respective concentration had similar effectiveness on antifouling with the same bacteria strain. For instance, 20 mg/L capsaicin decreased the attachment of LE bacteria and Pp system by 93.5% and 95.2%, respectively; 50 mg/L zosteric acid did by 94.5% and 94.8% to the corresponding species. However, in accordance to section 4.2.2, the EC_{50} of capsaicin and zosteric acid was 5.5 mg/L and 170 mg/L to *P. putida*, 22.6 mg/L and 380 mg/L to the LE bacteria, respectively. Thus, a concentration of four times of the EC_{50} of capsaicin performed a same level of effectiveness on the anti-attachment of *P. putida* as the concentration of about one third of EC_{50} of zosteric acid did. A concentration similar to the EC_{50} of capsaicin had the same effectiveness on antifouling of LE bacteria as the concentration of about one eighth of the EC_{50} of zosteric acid did. It indicated that zosteric acid is more effective than capsaicin as an antifoulant.

5.2.3 Zosteric acid entrapped in the silicone

Zosteric acid was entrapped in the silicone to perform a further attachment study. The reasons for selecting zosteric acid instead of capsaicin are due to 1) zosteric acid is more effective than capsaicin as an antifoulant (section 5.2.2.3); 2) capsaicin poses the poison effect to the Pt-catalyst and hinders the curing of the silicone coating (i.e, Sylgard® 184); 3) The amount of capsaicin leaching into water has to be monitored by

HPLC instead of conductivity. Conversely, zosteric acid has no effect on silicone curing an its presence in water can be easily measured via a conductivity meter. Zosteric acid was entrapped into silicone by 0.3 wt %, 0.5 wt % and 1 wt % and the coated slides were immersed in LE solution for three weeks, the higher concentration of entrapped zosteric acid, the higher conductivity in the solution was observed. However, there was no significant difference (P-value=0.456) of MPN among the different conditions (Figures of aqueous properties change were shown in Appendix B). However, a leach rate study, indicated that 1 wt % was the relative ideal zosteric acid concentration to be entrapped (Barrios, 2004). Thus the next set of experiments used 1 wt % zosteric acid bulk entrapped into the silicone (Sylgard® 184) to further evaluate the effectiveness on preventing bacteria attachment. The slides coated with entrapped ZA were immersed in the LE bacteria solution. Also, the physiochemical and biological characteristics of the solution, as well as the biofilm formation on the coatings were investigated to provide additional evidence to support the antifouling mechanisms of NPA described earlier in this chapter.

5.2.3.1 Change in temperature and pH

The temperature fluctuation throughout this set of experiment was only ±1.8ºC. As mentioned in section 5.2.1.1, this slight change would insignificantly affect the pH and conductivity. Therefore, the impact of temperature can be ignored.

Figure 5.12 presents the pH changing with time for LE bacteria with different concentration of zosteric acid entrapped in the silicon coating. Control means no zosteric acid was entrapped.

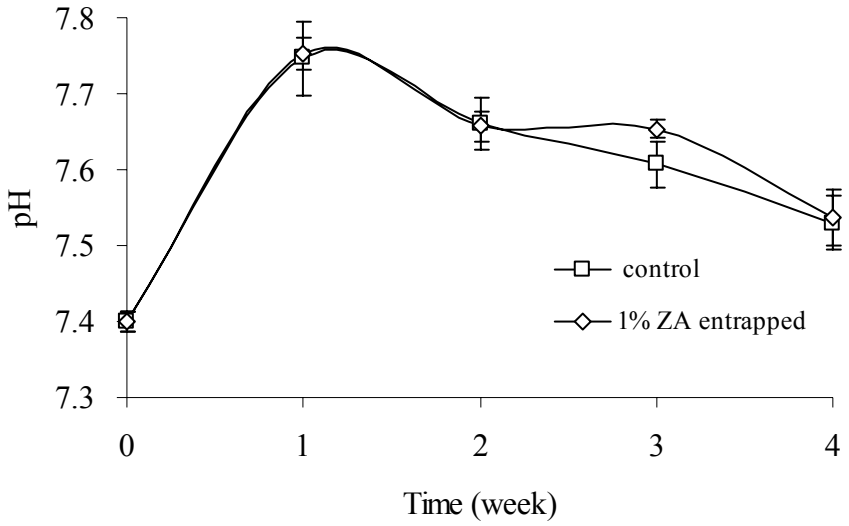

Figure 5.12 pH change with time for LE solution containing coated slides with different concentration of zosteric acid entrapped in the silicon coating (Sylgard® 184), control represents no zosteric acid was entrapped (error bars represent ± standard deviation, n=3)

As shown in Figure 5.12, the trend of pH change was almost the same for both conditions. Biobuffering property was still attributed as the key role in pH change. It increased the pH from the initial value of 7.4 to 7.75 at the end of week one. Afterwards, the pH decreased to about 7.6, which is an optimal pH condition for LE bacteria after 4 weeks. There was no statistical difference (P-value= 0.836) on the pH value between these two conditions. The lack of significance indicated that the entrapped zosteric acid leaching from the silicon didn't affect the pH value in the aqueous solution. As was stated in section 5.2.1.1, low zosteric acid concentration would not affect the pH value, but the

high concentration would (500 mg/L zosteric acid could decrease the pH of LE solution by 0.3). It hinted that the concentration of zosteric acid leaching from the silicon was low. These results were corroborated by the concurrent study that showed zosteric acid leached at a constant rate of 0.1μg/cm^2/day after the coating immersed in water for 4 hours (Barrios, 2004).

5.2.3.2 Change in conductivity

Figure 5.13 shows the change of conductivity in the aqueous solution for the control and 1 wt. % bulk entrapped zosteric acid in coating of Sylgard® 184. For control condition, the conductivity slightly increased by approximately 5 mg/L in the first two weeks. From then on, it decreased to 1014 mg/L. For the condition with 1 wt. % entrapped zosteric acid, the conductivity increased slowly (5 mg/L) in week one, then it increased about 15-20 mg/L until week three. After 3 weeks, a slight decrease was observed, but the final value (1032 mg/L) was still higher than that of the initial value (1017 mg/L). In addition, from the statistical analysis (Appendix D), P-value is 0.003< 0.05, the finial conductivity levels in the aqueous solution of these two conditions are significantly different. The concurrent study indicated that the leached zosteric acid concentration was about 1 mg/L after 2 weeks and about 2 mg/L after 4 weeks. Thus, the reason for the conductivity change was attributed primarily to the microbial activity of bacteria. As mentioned in 5.2.1.2, because the components of bacterial species in LE system were complex, some kinds of bacteria could buffer the conductivity change or

made a slight increase in the first three weeks. As the consumption of inorganic nutrient continues, the conductivity decreased about 10 mg/L after 3 weeks.

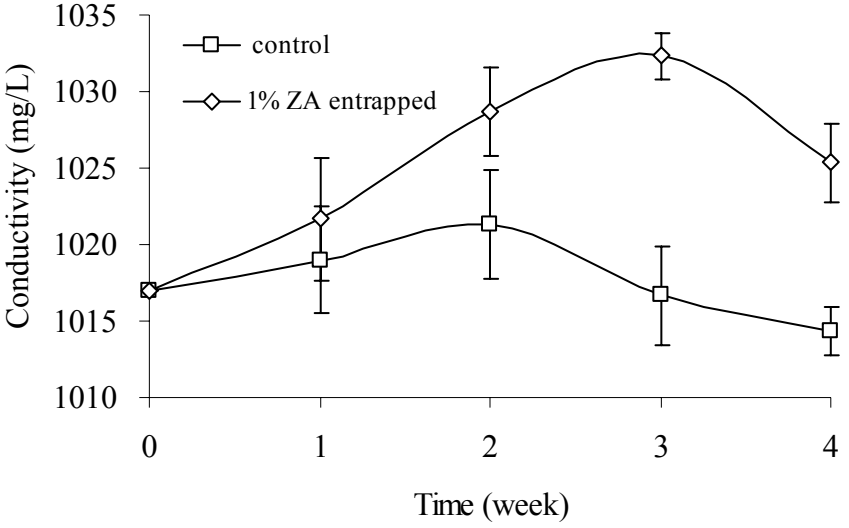

Figure 5.13 Conductivity change with time for LE solution containing coated slides with different concentration of zosteric acid entrapped in the silicon coating (Sylgard® 184), control represents no zosteric acid was entrapped (error bars represent ± standard deviation, n=3)

5.2.3.3 Change in DO

Figure 5.14 is a graph of DO level change with time when the aqueous solution contained LE bacteria and coated slides with different concentration of zosteric acid entrapped in the silicon (Sylgard® 184) coatings. For each condition, the DO level decreased as time increased. As mentioned in 5.2.1.3, this decrease was due to two reasons. One is the temperature. The change (±1.8°C) of temperature caused a maximum DO change of ±0.28 mg/L (The detail calculation was shown in Appendix E). The other reason was the respiration consumption of the bacteria, which were both in the aqueous

solution and the biofilm on the slides. A statistical analysis (P-value =0.293>0.05 in Appendix D) indicated that there was no significant difference in DO level between these two conditions after four weeks.

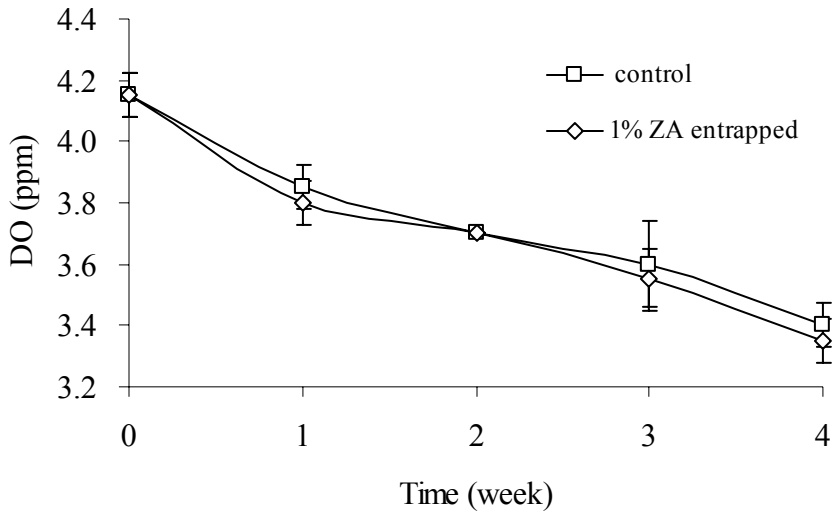

Figure 5.14 DO change with time for LE solution containing coated slides with different concentration of zosteric acid entrapped in the silicon coating (Sylgard® 184), control represents no zostric acid was entrapped (error bars represent ± standard deviation, n=3)

5.2.3.4 Changes in aqueous microbial numbers

Figure 5.15 presents the results of the aqueous microbial activity as determined by MPN for LE solution containing coated slides with or without zosteric acid entrapped. As shown, MPN decreased with time for both conditions. After 4 weeks, the bacteria concentration in the aqueous solution decreased by 73.1±2.1% and 64.2±4.2% for control (no zosteric acid entrapped) and 1 wt % zosteric acid entrapped in the coating, respectively. This decrease can be due to the bacteria attached on the slides immersed in

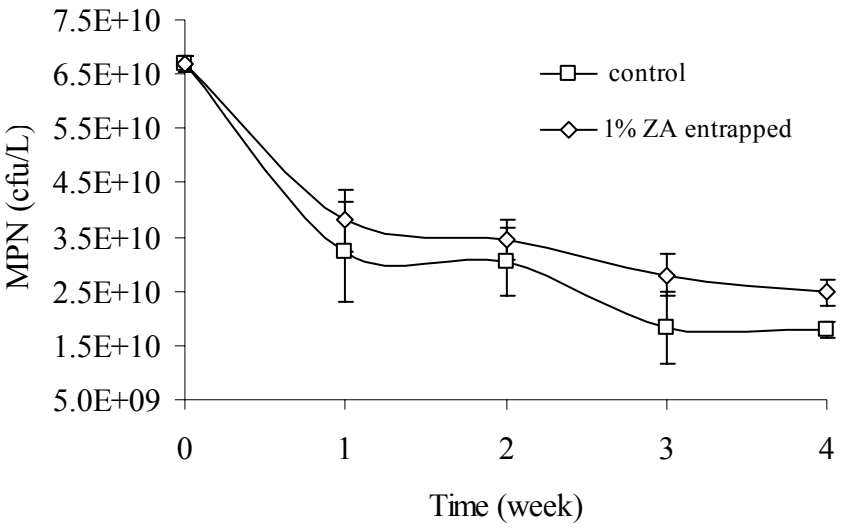

Figure 5.15 MPN change with time for LE solution containing coated slides with different concentration of zosteric acid entrapped in the silicon coating (Sylgard® 184), control represents no zosteric acid was entrapped (error bars represent ± standard deviation, n=3)

the solution or the microbial activity decrease. The MPN in the solution containing entrapped zosteric acid slides was 6.0×10^{9}cfu/L (8.9%) higher than that of control. The statistical analysis indicated (P-value=0.028) that there was a significant difference between them. The most reasonable explanation is that the entrapped zosteric acid leached from the silicone (as expected) and inhibited the bacteria attaching onto the surface of coating, by either posing a lethal toxicity or blocking the specific binding site. The toxicity of zosteric acid was determined to be 380 mg/L. However, a relatively low concentration (1-2 mg/L) of zosteric acid was found in the solution (section 5.2.3.2). Even if the concentration of zosteric acid at the interface of coating was very high, the MPN in the solution should be lower than that of control condition if the bacteria were killed by the leached zosteric acid. Since the bulk entrapped system had a higher MPN than the control, the zosteric acid didn't prevent attachment by killing the bacteria. A

microscope image analysis of biofilm formation on the coated slides provided evidence that the biofilm coverage on the coatings containing zosteric acid was less than that of no zostsric acid entrapped (section 5.2.3.5).

5.2.3.5 Effect of entrapped zosteric acid on biofilm formation

Figure 5.16 shows the representative morphology images of biofilm formation on the silicone coatings with and without entrapped zosteric acid. For each condition, the bacteria attachment to the coating surface increased with time, corresponding to the MPN decreased (stated in section 5.2.3.4). Figure 5.16 (a) and (c) presents the normal growth (no zosteric acid entrapped) of LE bacteria on silicone (Sylgard® 184) coating after 2 weeks and 4 weeks, respectively. Compared to this control condition, the biofilm coverage obviously decreased when 1 wt. % of zosteric acid was entrapped in the silicon coating (Figure 5.16 b and d). For instance, it decreased 94.2 % and 93.8%, respectively at the end of 2 weeks and 4 weeks. It indicated that the entrapped zosteric acid (actually it was releasing from the silicone coating into the solution with time) inhibited the bacteria attaching on the surface to form colonies and the biofilm. Compared to Figure 5.11 (a) and (b), 1 wt % zosteric acid entrapped (91.2% decrease attachment) had the similar effectiveness with that of 50 mg/L zostreic acid suspension (92.5% decrease) on antifouling. Table 5.3 presents the comparison of coverage for biofilm formation. As mentioned in Chapter II, the biofilm formation stage is the key step for biofouling. The characteristics of zosteric acid by preventing the bacteria attaching onto the surface will make it an effective antifouant in the ship industry.

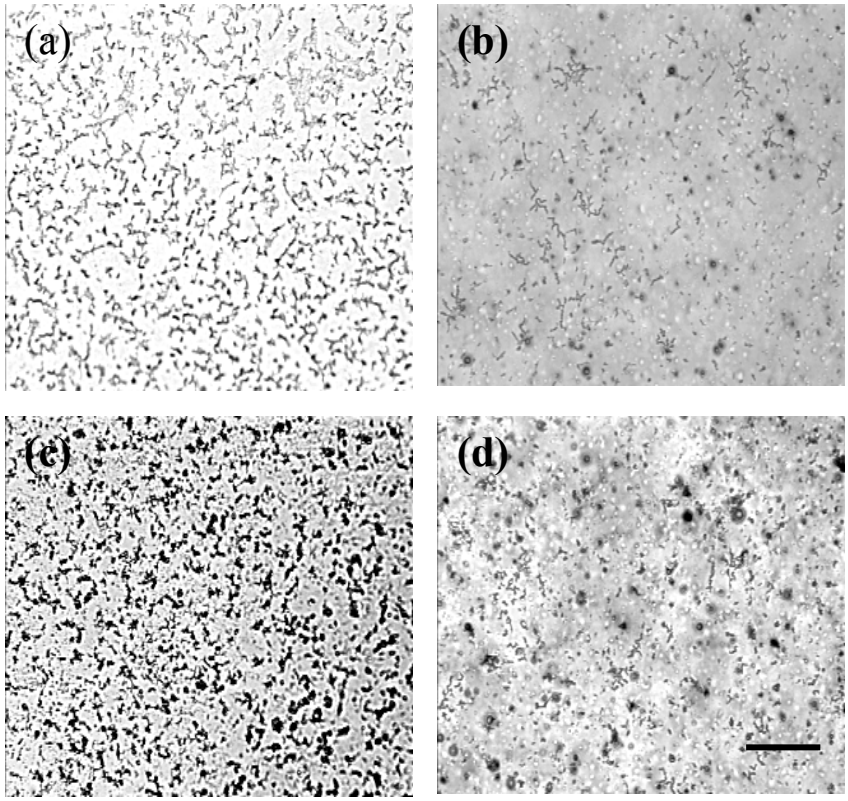

Figure 5.16 Various concentration of zosteric acid entrapped in coatings of Sylgard® 184 immerged in *LE* bacteria solution: (a) without zosteric acid after 2 weeks; (b)with 1 wt. % zosteric acid after 2 weeks; (c) without zosteric acid after 4 weeks; (d)with 1 wt.% zosteric acid after 4 weeks, bar=30 μm (Barrios took the pictures).

Table 5.3 Comparison to control for biofilm formation of entrapped zosteric acid in Sylgard® 184

Lake Erie	Pixels	% Relative
Control (2 weeks)	1018	100.0
1 % ZA entrapped	89	8.8
Contol (4 weeks)	2003	100.0
1 % ZA entrapped	287	14.3

5.2.4 Lower concentration of zosteric acid suspended in solution

Both the leach rate study and the attachment study by bulk entrapped 1 wt % zosteric acid in silicone (Sylgard® 184) coating indicated that the concentration of leached zosteric acid in the solution was in a relative low level. In order to evaluate if a low concentration level zosteric acid in solution still have a satisfactory effectiveness on antifouling, 5 mg/L and 10 mg/L zosteric acid was applied for attachment study in the following set of experiment.

5.2.4.1 Effect on the change of aqueous properties

Figure 5.17 (a)-(d) shows the graphs of aqueous properties (pH, conductivity, DO and MPN, respectively) change with time when different concentration of zosteric acid was present in LE solution. Control means no zosteric acid was dissolved. Due to the function of biobuffering, the pH (Figure 5.17a) increased from the initial value of 7.22 (low concentration of zosteric acid didn't make the initial pH value different) to around 7.55 at week 1, then it decreased to a stable level about 7.5 from week 1 to week 4. Statistical analysis indicated that there was no significant difference (P-value=0.229) in the three different conditions.

Compared to control, the presence of 5 mg/L and 10 mg/L zosteric acid increased the initial conductivity by 4 mg/L and 7 mg/L (Figure 5.17b), respectively. There was 6 mg/L difference between 10 mg/L and control, 2 mg/L difference between 5 mg/L and control at the final week. The microbial activity was the key role in conductivity change. It slightly increased in the first week, and decreased from then on.

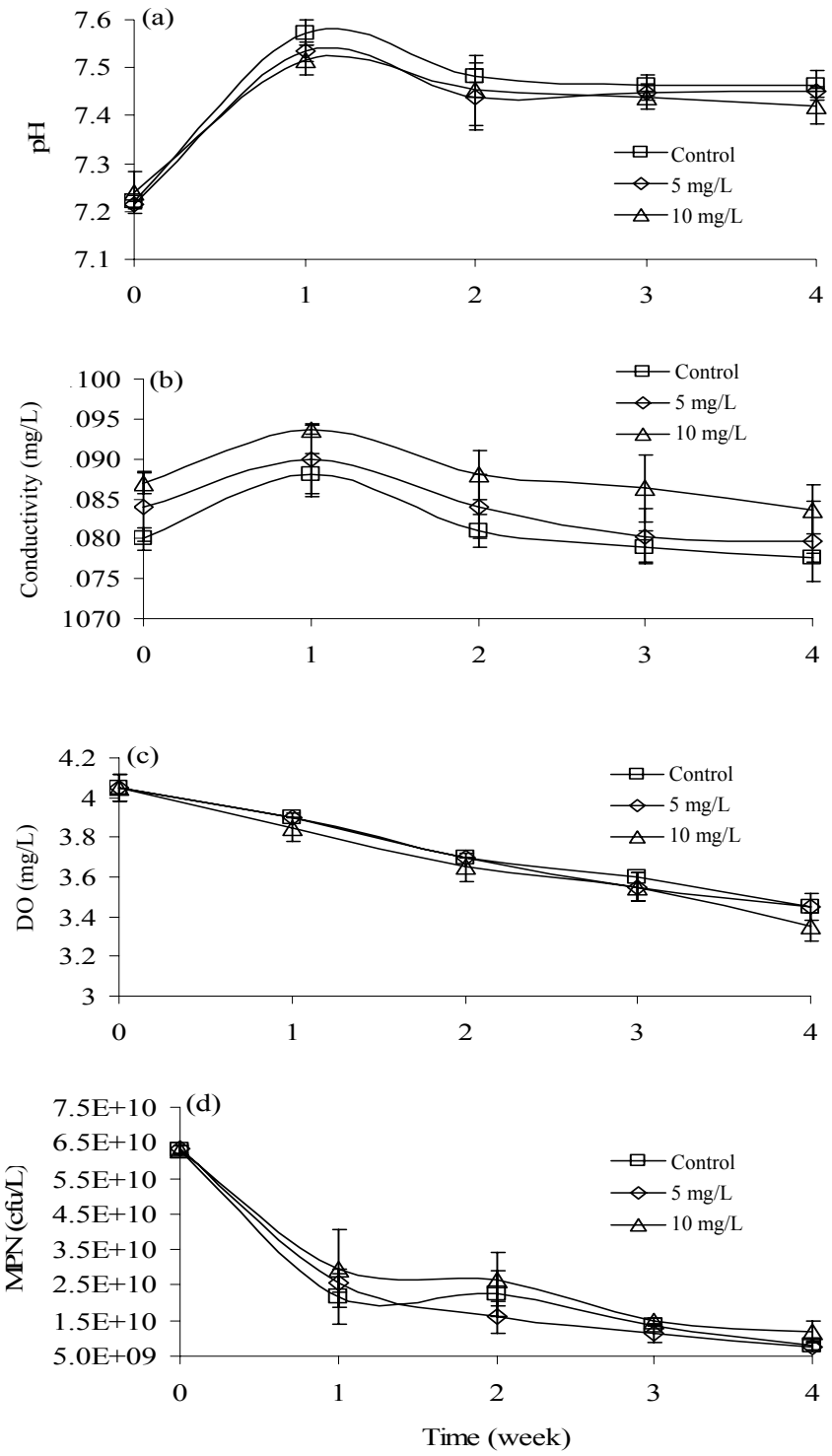

Figure 5.17 Aqueous properties changing with time for *LE* with different concentration of zosteric acid was dissolved, (a) pH changing with time, (b) conductivity changing with time, (c) DO changing with time, and (d) MPN changing with time. Control represents no zosteric acid present (error bars represent ± standard deviation, n=3)

As shown in figure 5.17 (c), DO decreased from the initial value of 4.05 mg/L to average 3.4 mg/L after four weeks. There was no significantly different (P-value=0.385) among three conditions. Because the concentration was very low, the present of zosteric acid could not change the initial DO value. The consumption of oxygen by the LE bacteria was the primary reason that caused the DO level decrease.

As expected, the MPN decreased with time (Figure 5.17d). After 4 weeks, the MPN decreased 88.3%, 88.8% and 84.2%, respectively when 0 mg/L, 5 mg/L and 10 mg/L zosteric acid was present in solution. Thus, there was 2.7×10^{8} cfu/L (0.5%) higher in MPN of 5 mg/L than that of control, and 3×10^{9} cfu/L (3.9%) higher of 10 mg/L than that of control. However, the statistical analysis indicated that no significant difference (P-value=0.369) in MPN between any pair of conditions.

5.2.4.2 Effect of zosteric acid on biofilm formation

Figures 5.18(a)-(c) are morphology images of biofilm formation on the silicone (Sylgard® 184) coatings immersed in LE solution with different concentration of zosteric acid present. As zosteric acid concentration in solution increased, the amount of bacteria attachment (i.e., biofilm growth) to the coating surface decreased. Figure 5.18 (a) shows normal (no zosteric acid in solution) growth of LE bacteria on the surface of silicone coating after 14 days. When 5 mg/L and 10 mg/L of zosteric acid was added in, the biofilm coverage decreased 29.6% (Figure 5.18 b) and 54.3% (Figure 5.18 c), respectively. Comparison of coverage on biofilm formation was given in table 5.4.

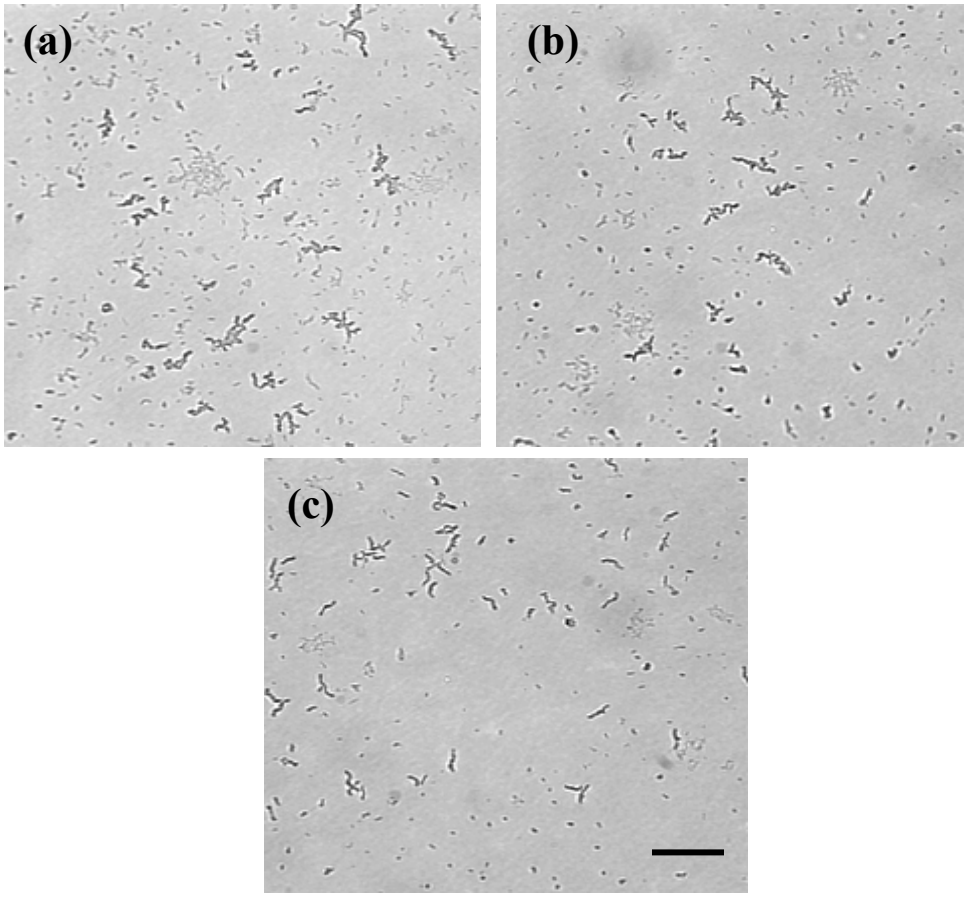

Figure 5.18 Morphology of the attachment of enriched Lake Erie bacteria on Sylgard® 184 silicone coatings in solutions (a) without zosteric acid (b) containing 5mg/L, or (c) 10mg/L of zosteric acid after 2 weeks of immersion, bar=30 μm (Barrios took the pictures).

Table 5.4 Comparison of biofilm formation for suspended zosteric acid in LE solution

Lake Erie	Pixels	% Relative
Control	675	100.0
5 mg/L ZA	476	70.4
10 mg/L ZA	308	45.7

Obviously, compared to the effectiveness of 50 mg/L and 500 mg/L (Figure 5.11), 5 mg/L and 10 mg/L of zosteric acid were less effective on inhibiting the bacteria attachment on surface. For instance, 50 mg/L zosteric acid decreased 92.5% of LE bacteria attachment, and 98.2% decrease of biofilm formation on slides was observed when 500 mg/L zosteric acid was present. The low concentration (5 and 10 mg/L) of zosteric acid also had less effectiveness on antifouling than that of 1 wt % entrapped (decrease 91.2% biofilm formation, section 5.2.3.5), but with a less leached concentration level (1-2 mg/L) in solution. Even with leaching, it hypothesize that the concentration of zosteric acid at the interface of coating was relatively much higher than that in the solution. It was the zosteric acid at the vicinities of the coatings (where the attachment took place) that played a key role in preventing the bacteria attachment. Furthermore, only 29.6% and 54.3% decrease of bacterial attachment means that there were still a huge number of bacteria colonized on the surface. Once attached, these microbes generate daughters to provide a suitable surface for fungi or alga to attach on and further to lead to macrobiofouling (section 2.2).

5.3 Attachment study by using RTV 11 as silicone coating

It was proved that Sylgard® 184 is an effective foul-release silicone to be applied in antifouling industries. In order to evaluate the effectiveness of another popular silicone--RTV 11 on antifouling, attachment study by using RTV 11 coated slides immersed in LE solution was performed. Because the former study indicated that 50 mg/L suspension and 1 wt % entrapped of zosteric acid were the ideal concentrations for

zosteric acid, these two conditions were evaluated for the RTV 11 attachment study, and the control (RTV 11 coated slide immersed in LE solution without zostric acid) was used in a parallel experiment.

5.3.1 Effect on the change of aqueous properties

Figure 5.19 (a)-(d) presents the graphs of aqueous properties (pH, conductivity, DO and MPN, respectively) change with time in LE bacterial solution for the three different conditions of control, 50 mg/L zosteric acid suspended in solution, and 1 wt % entrapped in coating of RTV 11. There was no difference in initial pH between control and the condition of 1 wt % zosteric acid entrapped. However, the presence of 50 mg/L of zosteric acid in solution decreased the initial pH value of 0.1 (Figure 5.19a). Due to the biobuffering, pH increased average 0.3 at the end of week 2 and slightly decreased to 7.6. Physically, after four weeks, the finial pH of three condition had the relationship of control >50 mg/L zosteric acid suspension >1 wt % entrapped. Because zosteric acid would decrease the pH value in solution (section 5.2.1.1), and the higher the concentration of zosteric acid, the lower the pH value measured. However, the statistical analysis indicated that there was no significant difference (P-value=0.086) in pH between 50 mg/L zosteric acid suspension and 1 wt % entrapped. In addition, there was significant difference (Tukey's comparison in Appendix D) between control and 1 wt % entrapped but no significant difference (Tukey's comparison in Appendix D) between control and 50 mg/L of suspension. It seams that the leaching of zosteric acid from RTV 11 did affect the final pH in the LE solution.

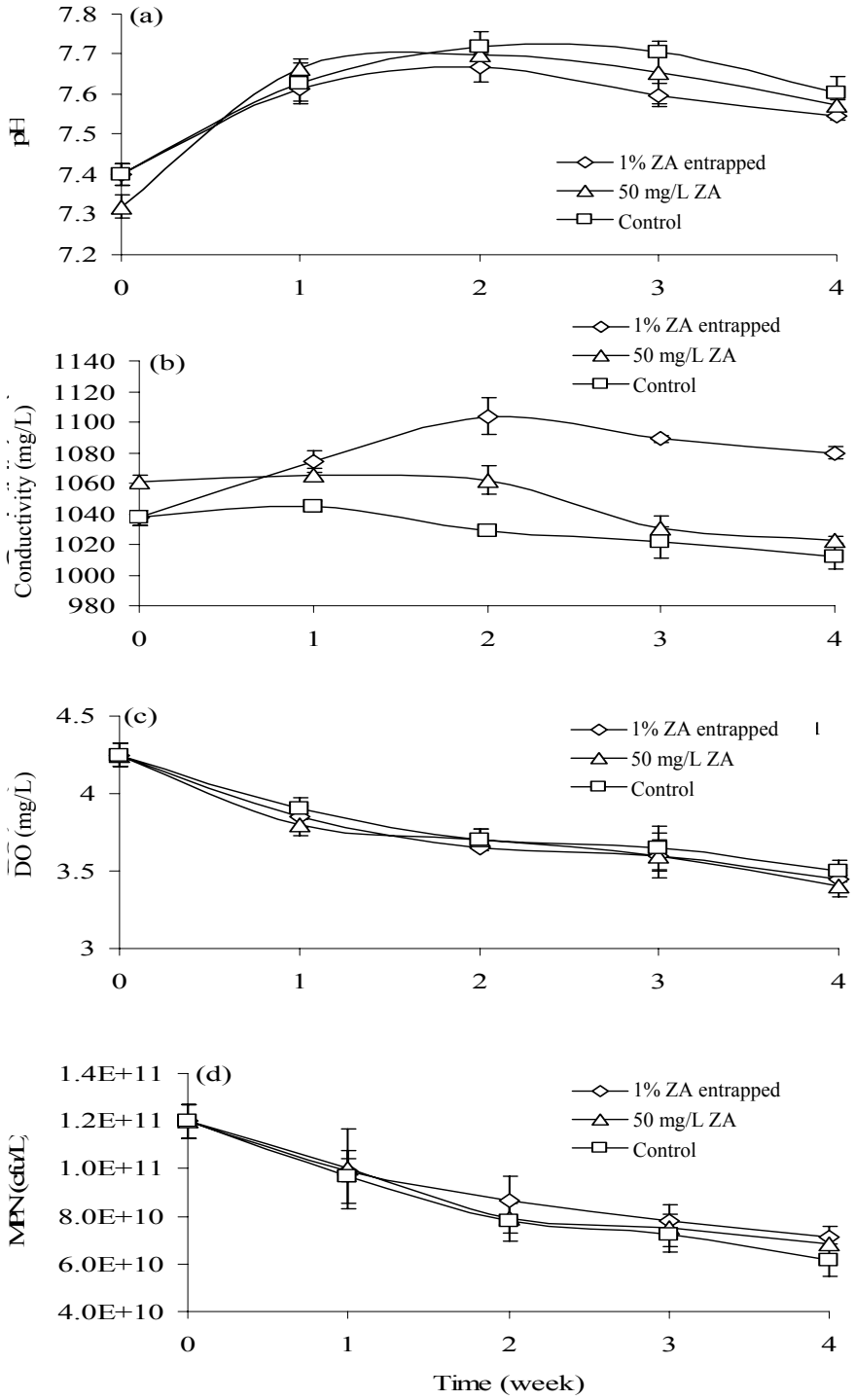

Figure 5.19 Aqueous properties changing with time for *LE* with RTV 11 coated slides immersed in different concentration of zosteric acid dissolved or 1 wt % entrapped in coatings, (a) pH change with time, (b) conductivity change with time (c) DO change with time, (d) MPN change with time. Control represents no zosteric acid dissolved in solution or entrapped in silicone (error bars represent ± standard deviation, n=3)

Figure 5.19 (b) shows that the presence of 50 mg/L zosteric acid made the initial conductivity about 20 mg/L higher than that of the other conditions (no difference between them). As a result of microbial activity, the conductivity slightly increased in the first week and then decreased until week 4 for the condition of control and 50 mg/L zosteric acid suspension. Furthermore, there was no significant difference (Tukey's comparison in Appendix D) in the final value between them, although the value of 50 mg/L was physically higher than that of control. They had the same trend as Sylgard® 184 applied as silicone coatings (Section 5.2.1.2). For 1 wt % entrapped system, the conductivity increased about 70 mg/L after 2 weeks immersion, and decreased about 10 mg/L after that. The concurrent study indicated that the average leaching rate for zosteric acid from RTV 11 was 1 $\mu g/cm^2$/day (Barrios, 2004). Thus, the leached zosteric acid concentration was about 10 mg/L and 20 mg/L, respectively after 2 and 4 weeks immersion. Therefore, the principle reason for the huge increase of conductivity was not due to the leach of zosteric acid from the coating. The reasons maybe were: 1) other compounds (Such as zosteric acid solvents) leached from the coating; 2) bacteria could degrade the compounds into more simple matters in the solution; 3) biobuffering property of LE bacteria. Unfortunately, the exact reason was not clear, further work should be done on this.

The DO level (Figure 5.19c) in three conditions decreased from initial value of 4.25 mg/L to average 3.45 mg/L after 4 weeks. In addition, there was no significant difference (P-value=0.604) in the final DO level among the three conditions.

Figure 5.19 (d) shows that the MPN decreased with time in the three conditions. After 4 weeks, the MPN decreased 48.9±5.7% for control; 42.9±1.8% for 50 mg/L

zosteric acid suspended and 40.8±3.8% for 1 wt % zosteric acid entrapped. Physically, the MPN of 1 wt % zosteric acid entrapped was 2.5×10^9 cfu/L (2.1%) higher than that of 50 mg/L zosteric acid suspension, and was 9.7×10^9 cfu/L (8.1%) higher than that of control. But statistically, there was no significant difference (P-value=0.163) between any pair of conditions.

5.3.2 Effect on the biofilm formation

Figure 5.20 (a)-(f) was the representative morphology of the attachment of LE bacteria on RTV11 silicone coatings in three different conditions of control, 50 mg/L zosteric acid dissolved in solution and 1 wt % zosteric acid entrapped in silicone after two weeks (a), (c) and (e) and four weeks (b), (d) and (f) of immersion, respectively. Because the reflected light mode was used to analyze biofilm formation on RTV 11 coatings, the white domains represented the biofilm on the surface. A lot of black domains on the surface were observed when 50 mg/L zosteric acid was dispersed in the solution. These domains could not be removed by cleaning the surface using a scotch tape or rinsing, it indicated that zosteric acid maybe interacted with the fillers or the polymer matrix of the coating, and causing zosteric acid adsorption-desorption or zosteric acid/filler conjugates from and towards the surface during all the immersion period (Barrios, 2004). The complexity of the interactions of zosteric acid with calcium carbonate fillers, polymer matrix and water environment made it difficult to quantify the bacterial coverage.

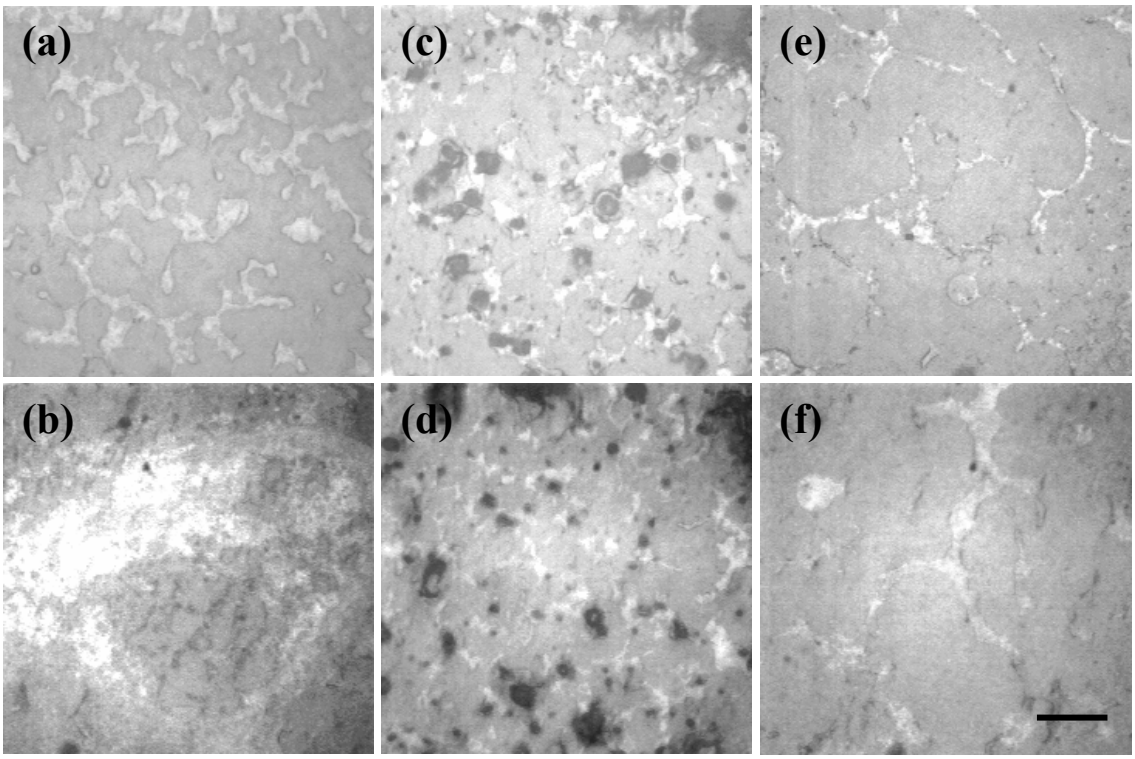

Figure 5.20 Morphology of the attachment of enriched Lake Erie bacteria on RTV11 silicone coatings in solutions (a), (b) without zosteric acid or (c), (d) containing 50 mg/L of zosteric acid, and (e), (f) 1 wt % zosteric acid entrapped in coatings after 2 (a, c e), and 4 (b, d, f) weeks of immersion, bar=30 μm (Barrios took the pictures).

In Figure 5.16 (section 5.2.3.5), 1 wt % entrapped zosteric acid in Sylgard® 184 made 91.2 % decrease of bacteria attachment after 2 weeks of immersion. However, the presence of zosteric acid only reduced the attachment to ~50% when it was entrapped in RTV 11. It is surprising that the leaching rate of zosteric acid from RTV 11 is about 10 times than from the Sylgard® 184. Obviously, change of silicone coatings definitely affected the biofilm formation. The Sylgard® 184-zosteric acid combination had a better effectiveness on inhibiting the bacteria attachment.

5.4 NPAs exposed to marine bacteria

The former study identified that the capsaicin and zosteric acid could effectively inhibit the freshwater bacteria attaching on the surface, thereby preventing the biofilm formation. However, the most common biofouling problem still happens in the marine environment. Thus, it is necessary to evaluate the antifouling property of NPAs with marine bacteria. The antifouling effectiveness of capsaicin and zosteric acid on marine bacteria was only investigated by dissolving the NPAs into the aqueous solution containing two typical marine bacteria, *Vibrio parahaemolyticus* (Vp) and *Vibrio natriegens* (Vn). The effectiveness of Vn-capsaicin combination will not be discussed in the following section, since that experimental set was completed by another person. However, the representative figures of aqueous properties changing with time were shown in Appendix B.

5.4.1 Change in temperature and pH value

The temperature was still one of the factors that could change the aqueous properties. However, the temperature in this set fluctuate only ±1.5°C. The slight effect on the pH and conductivity can be neglected. Figure 5.21 presents representative graphs of how pH levels changed with time when the aqueous microbes were exposed to different concentration of zosteric acid (based on toxicity analysis) in solution. Control still means no zosteric acid contained. As shown in Figure 5.21, the pH trend for all of the conditions evaluated was similar. The pH value increased from initial time to week 2 for all concentrations in each bacteria system. After two weeks, the pH value trend was

constant. As mentioned previously, biobuffering adjusted the pH to the optimal growth condition by releasing of exudates to the external surrounding environment. For the Vp system (Figure 5.21a), the optimal pH value with zosteric acid was 8.6 for three concentrations evaluated.

For Vn system (Figure 5.21b), because the EC_{50} of zosteric acid to Vn bacteria was relative low (7.4 mg/L), three concentrations at 5 mg/L, 10 mg/L and 20 mg/L of zosteric acid were evaluated. The optimal pH value for Vn growth was around 8.5. This was evident by the pH value for all of the conditions were very close to 8.5 by end of eight weeks. No significantly statistical difference (P-value=0.764, and 0.778, for Vp and Vn, respectively) was found for the different conditions of each system. It represented that the present of zosteric acid didn't affect the pH change in both marine bacterial system.

Capsaicin exhibited the same trend in pH change, except that it increased from the initial time to week 3, then it was constant (Figure 5.22). The ANOVA table (P-value=0.46) indicated that there was no statistical difference among the three evaluated conditions. By the end of the eighth week, all the three treatments were stable at 8.4 for Vp systems. Compared with Figure 5.21 (a) (final pH value was 8.6), capsaicin and zosteric acid had different effect on the biobuffering of Vp in pH change.

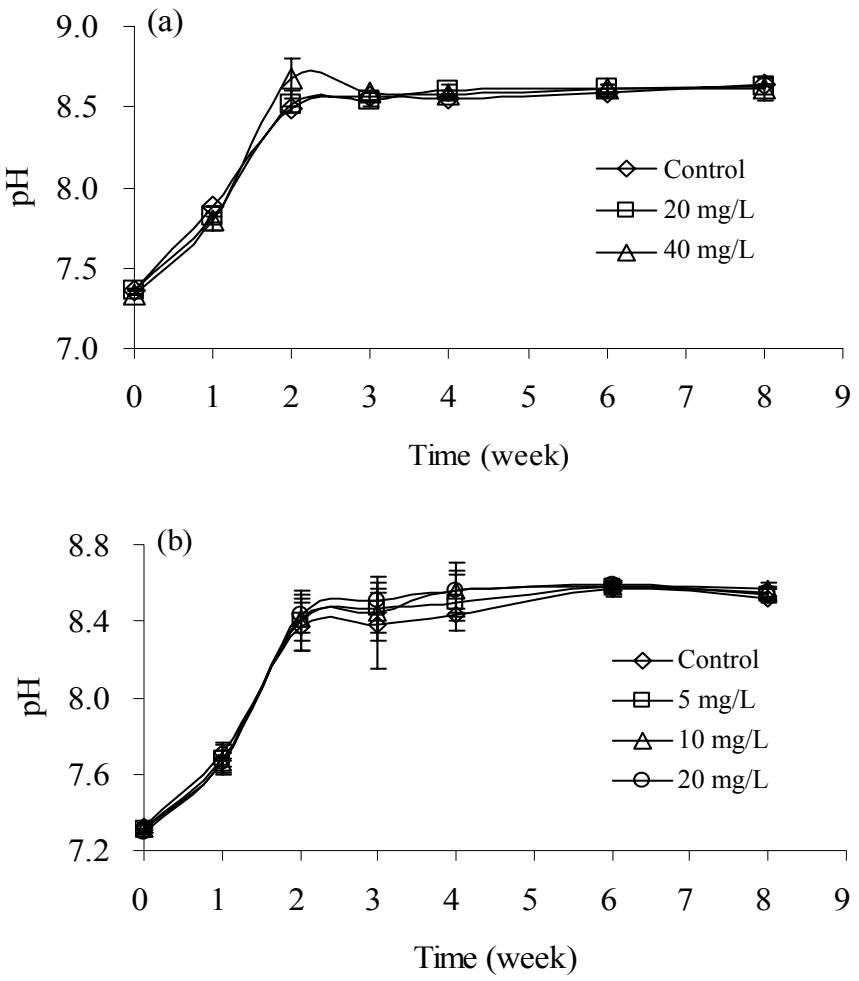

Figure 5.21 pH changed with time in the aqueous solution containing different concentration of zosteric acid with marine bacteria *V. parahaemolyticus* (Vp) (a) and *V. natriegens* (Vn) (b), control represents no zosteric acid in solution (error bars represent ± standard deviation; n=3).

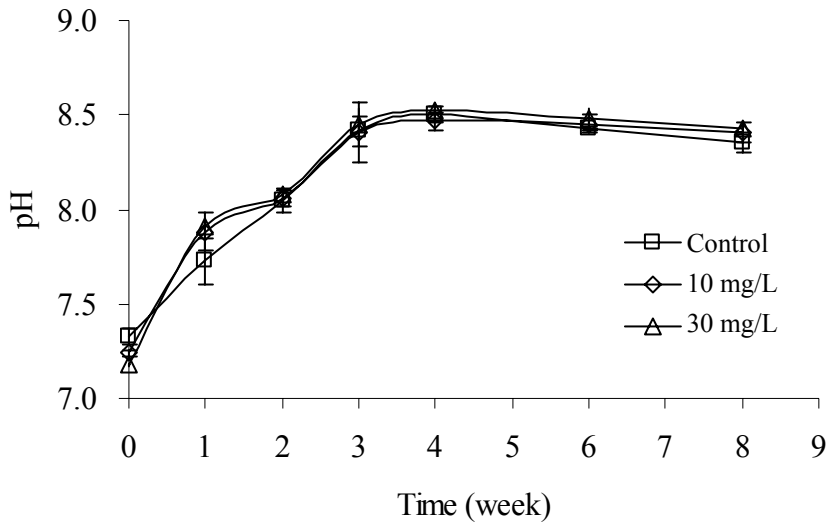

Figure 5.22 pH changing with time in the aqueous solution containing different concentration of capsaicin with marine bacteria *Vibrio parahaemolyticus* (Vp), control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

5.4.2 Change of conductivity

The initial characterization ascertained that the conductivity of nutrient solution for Vp and Vn was 15.2×10^3 mg/L and 13.5×10^3mg/L, respectively. Transferring 3 mL of batch solution (with 16.5×10^3 mg/L conductivity for Vp and 15.5×10^3 mg/L for Vn) to sterilized nutrient solution would result in an initial conductivity in the bottles prior to adding any NPAs of 16.6×10^3 and 14.7×10^3 mg/L. As shown in Figure 5.23, they were the initial conductivity values for the control flasks of Vp and Vn system, respectively.

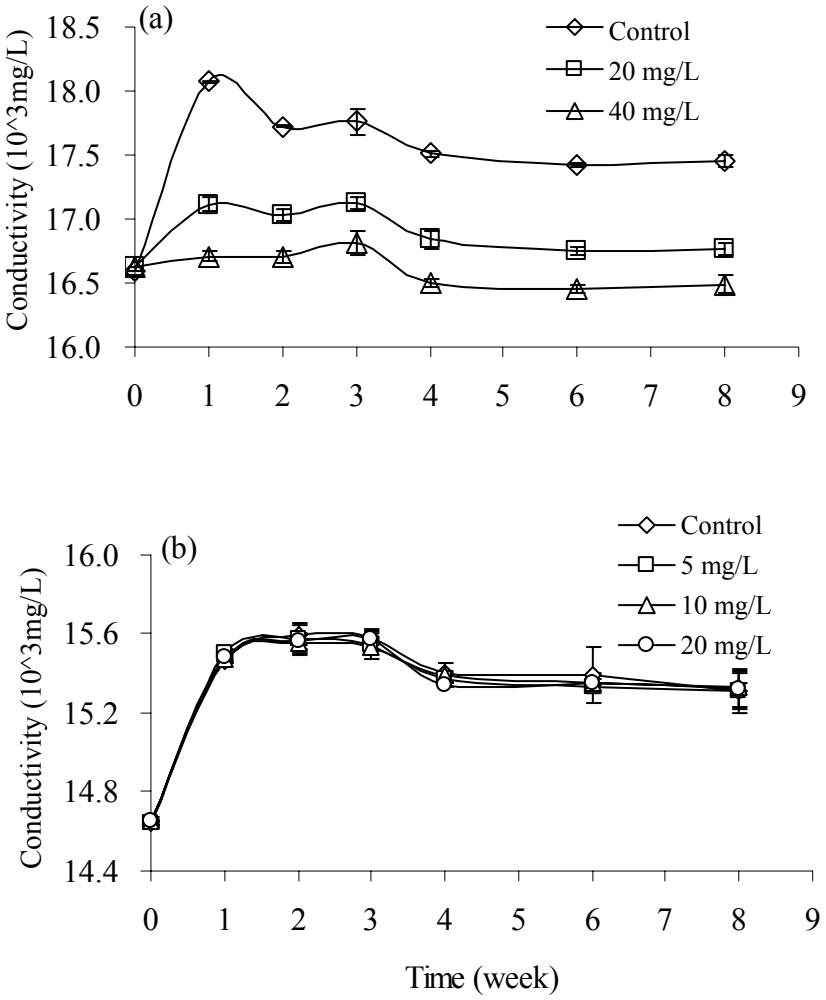

Figure 5.23 Conductivity changed with time in the aqueous solution containing different concentration of zosteric acid with marine bacteria *Vibrio parahaemolyticus* (Vp) (a) and *Vibrio natriegens* (Vn) (b) control represents no zosteric acid in solution (error bars represent ± standard deviation; n=3, 4 for Vp and Vn system, respectively).

Large initial conductivity readings were expected due to the nature of marine environment. Compared to the high initial conductivity levels, any increase due to presence of NPAs can be neglected. For control of Vp system (Figure 5.23a),

conductivity increased from the initial time of 16.6×10^3 mg/L to 18×10^3 mg/L at the end of week 1, and then decreased to be a stable level at 17.5×10^3 mg/L. This was directly related to microbial activity. At the beginning of the experiment, the bacteria were at the exponential growth stage, the metabolism was very active. The deamination of peptone (5 g/L) and yeast extract (1 g/L) by the bacteria produced an extend amount of ammonia in the solution. This caused a significant increase of conductivity in the solution. For instance, Gallert (1998) found that the ammonia concentration increased about 250 mg/L after deamination of 5 g/L peptone in only one day. So, it was not surprising that the conductivity increased so much after one week. Simultaneously, as inorganic nutrients were consumed during cellular maintenance, conductivity levels decreased. After one week, the conductivity only increased 0.5×10^3 mg/L and 0.1×10^3 mg/L when 20 mg/L and 40 mg/L zosteric acid was present in solution. The most reasonable explanation was that zosteric acid (EC_{50} was 18 mg/L to Vp) inhibited the microbial activity of Vp, and reduced the ability of metabolic deaminitation on peptone and yeast extract. Furthermore, it caused a significant difference (P-value almost equals zero) between the final value.

For capsaicin presence in Vp solution (Figure 5.24), the trend of conductivity change was similar with time going for three conditions. Due to the microbial activity, it increased about 400 mg/L after four weeks and was constant at about 16.5×10^3 mg/L after that. In addition, there is no statistical difference (P-value=0.12) in the final value. Thus, the presence of capsaicin didn't affect the metabolic process of Vp bacteria.

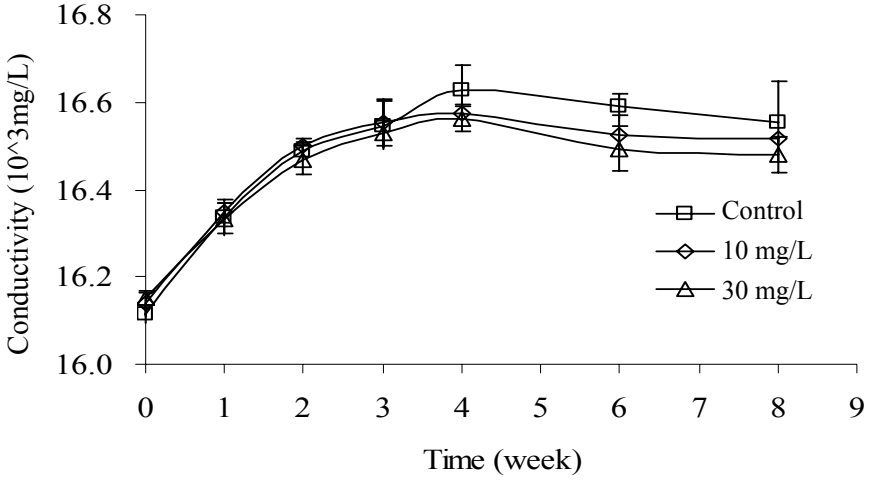

Figure 5.24 Conductivity changing with time in the aqueous solution containing different concentration of capsaicin with marine bacteria *Vibrio parahaemolyticus* (Vp), control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

5.4.3 Changes in dissolved oxygen (DO) levels

As shown in Figure 5.25, DO level decreased with time for all of the conditions in both marine bacteria systems. By the end of eight weeks, it had decreased from 3.0 mg/L to 0.8 mg/L for the Vp system (Figure 5.25a) and from 3.2 mg/L to 0.8 mg/L for the Vn system (Figure 5.25b). According to Coyer (1996) and the U.S. Food and Drug Administration (2001), *V. parahaemolyticus* and *V. natriegens* are facultatively anaerobic, but prefer a microaerobic environment. So, both of them could survive in a low DO level. Furthermore, Shuler and Kargi (2002) stated that "anaerobic condition" could actually be considered as DO level reduced to 5%-10% of its saturated value at a given temperature. Based on Herry's law, the saturated DO level at 25°C is about 8.9

mg/L. A 5%-10% level is 0.45~0.89 mg/L. Thus, the marine bacteria could be considered in aerobic condition.

The temperature and microbial activity are still the main factors that could facilitate a decrease in DO. The change of temperature was not significant (±1.5°C) and would have had a maximum change in DO of ±0.25 mg/L. The most significant decrease

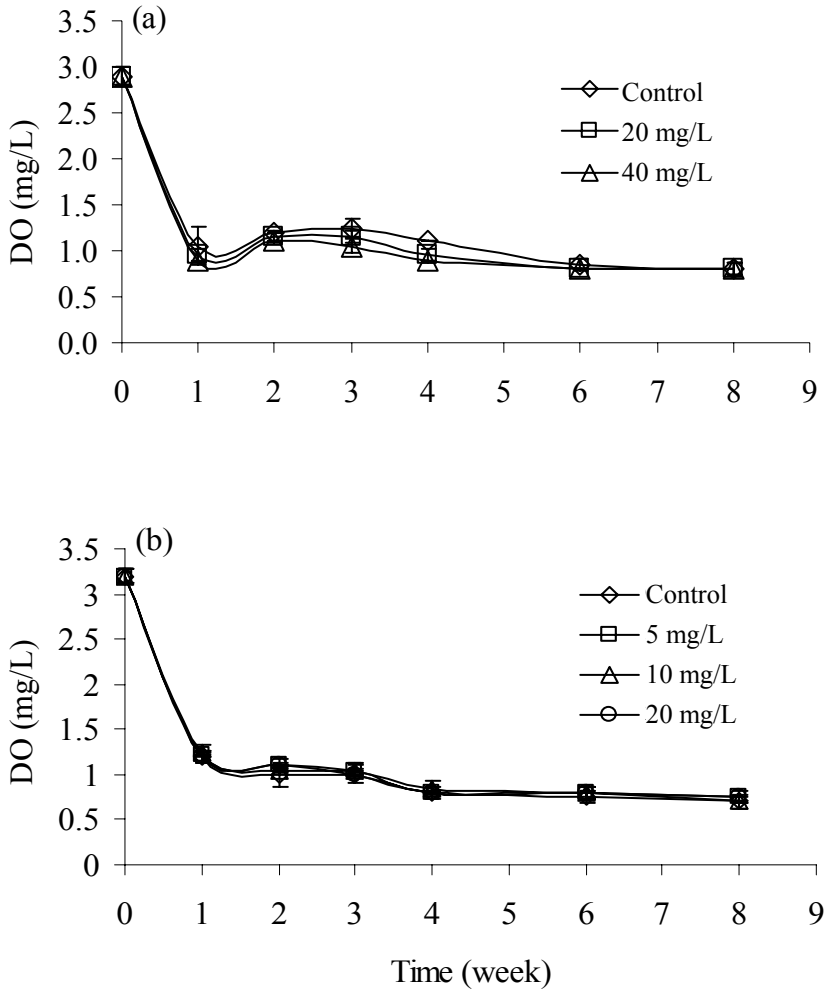

Figure 5.25 DO changed with time in the aqueous solution containing different concentration of zosteric acid with marine bacteria *Vibrio parahaemolyticus* (Vp) (a) and *Vibrio natriegens* (Vn) (b) control represents no zosteric acid in solution (error bars represent ± standard deviation; n=3).

occurred in the first week. The reasons were due to 1) DO consumption by bacterial respiration in the first week was large, implying that the bacteria were in their exponential growth phase (an evidence of conductivity also increased dramatically in the first week). Both the aqueous bacteria culture (MPN result) and the biofilm formation on both the slide and the surface of solution are indications of microbial activity. As bacteria numbers increase, more DO will be needed for the respiration; 2) bacterial production of polysaccharide combining with some bacteria formed a thick film on the surface of solution (section 5.4.4) blocked the oxygen in the headspace of bottles transferring into the solution. Compared to the freshwater systems (section 5.2.1.3), the initial DO level in both of the systems was about 1 mg/L lower. The reason is due to the high salinity in the marine nutrient solution. The high salinity of solution (3 wt. % for Vp solution and 2 wt. % for Vn solution) would decrease the DO level by 1.4 mg/L and 1.0 mg/L, respectively (Detailed calculation process in Appendix E). Statistically, there is no difference (P-value= 0.465 for Vp and 0.792 for Vn) among the evaluated conditions for each system.

Figure 5.26 shows DO changed with time in the aqueous solution containing different concentration of capsaicin with Vp bacteria. The trend of DO change was the same as when zosteric acid was present in the solution. It decreased from 3.0 mg/L to 0.7 mg/L at the end of eight weeks. Also, the obvious decrease happened in the first week. It represented that Vn consumed lots of oxygen for respiration at the exponential growth phase. In addition, there was no statistic difference (P-value=0.437) among the conditions in this system. The reasons for the decrease and the low level at the initial time are the same as mentioned previous.

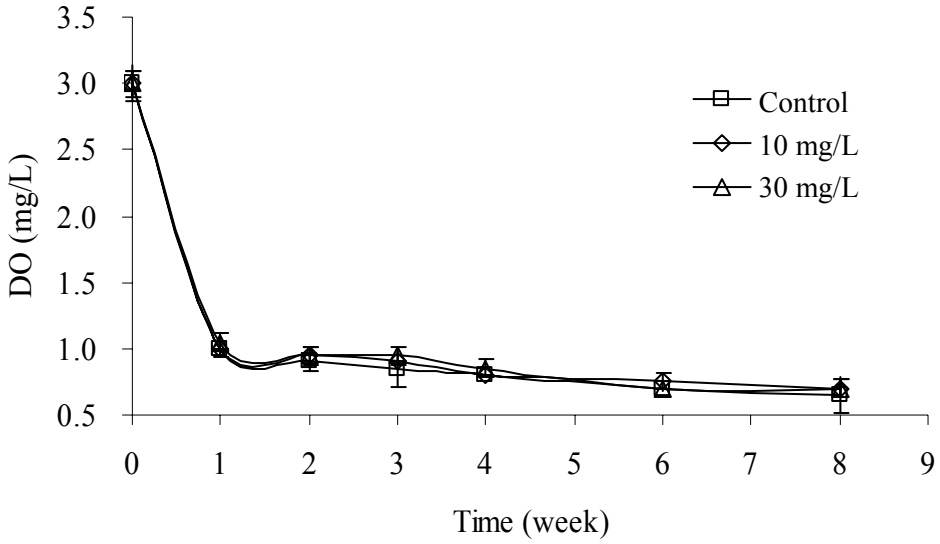

Figure 5.26 DO changing with time in the aqueous solution containing different concentration of capsaicin with marine bacteria *Vibrio parahaemolyticus* (Vp), control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

5.4.4 Changes in aqueous microbial numbers due to the presence of NPAs

Figure 5.27 contains the results of the aqueous microbial activity as determined by MPN for both cultures in the presence of different concentration of zosteric acid. It was found that the marine bacteria (Vn and Vp) started to form a thin biofilm on the surface of the solution after the experiments were started. As time gone, more bacterial production of polysaccharide combining with some bacteria in the solution adhered on it to make the film thicker and thicker. After 2 weeks, the thickness of surface-biofilm was about 2.5 mm and 3 mm for Vp and Vn, respectively. An example of the biofilm formation on the solution surface was shown in Figure 5.28. In order to observe the biofilm clearly, transparent bottles were used instead of amber bottles.

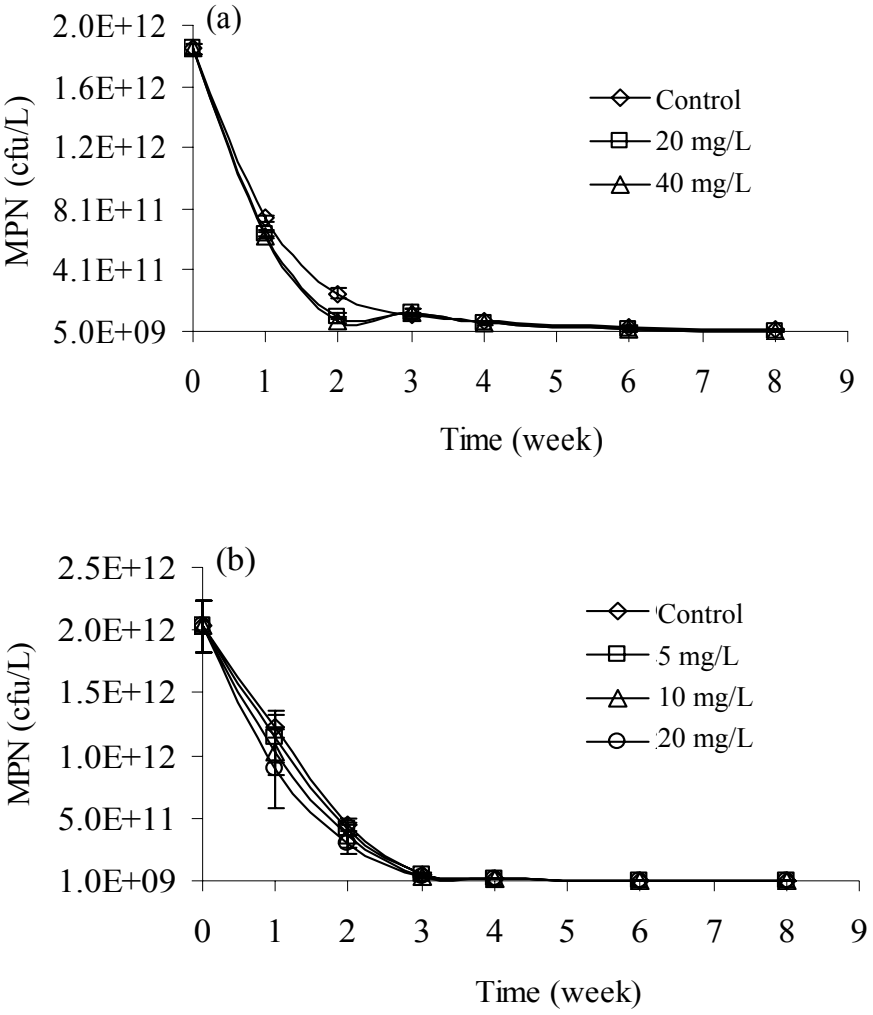

Figure 5.27 MPN changed with time in the aqueous solution containing different concentration of zosteric acid with marine bacteria *Vibrio parahaemolyticus* (Vp) (a) and *Vibrio natriegens* (Vn) (b) control represents no zosteric acid in solution (error bars represent ± standard deviation; n=3, 4 for Vp and Vn system, respectively).

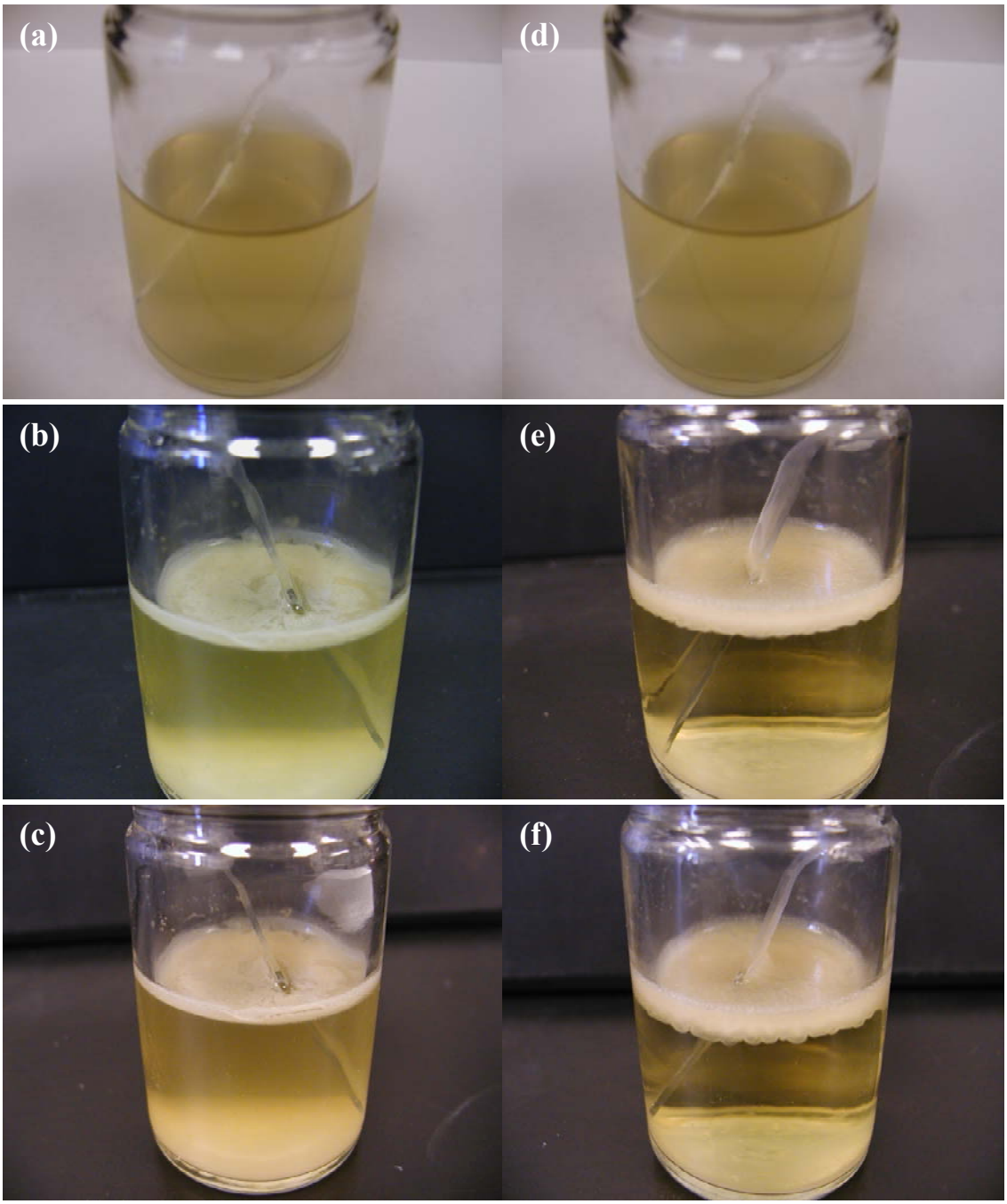

Figure 5.28 Images of marine bacterial biofilm formation on the surface of the solution for V.parahaemolyticus (a), (b) and (c) and V. nartriegens (d), (e) and (f) at the initial time (a) and (d), week 1 (b) and (e), and week 2 (c) and (f).

The biofilm formation on the surface of solution makes the concentration of bacteria in the solution decrease significantly. As shown in Figure 5.27, a sharp decrease of MPN in the aqueous solution happened. After 2 weeks (for Vp) or 3 weeks (for Vn), an equilibrium of attachment almost reached between the surface of the biofilm and the aqueous solution. So, the decrease of the MPN in the solution exhibited to be constant. Fewer bacteria (compared to those formed biofilm on the surface of the solution) attached on the surface of slides in the solution. After a microscope image analysis, the coverage of biofilm formation on the slides has increased from week 1 to week 8. Therefore, it was obviously that the biofilm formation on both the solution and slide surface caused the decrease of MPN in the marine aqueous solution.

Because the biofilm formation at the aqueous/air interface was the main factor on the decrease of MPN in the solution, there was no statistical difference (P-value=0.58) of MPN in the solution between any conditions (presence or absence of zosteric acid, low or high concentration of zosteric acid) in both of the bacteria systems. However, the antifouling effectiveness of zosteric acid to marine bacteria can be observed by the image analysis of biofilm formation on the slides.

Figure 5.29 presents the representative graph of how MPN change with time for Vp-capsaicin combination. As mentioned previously, the biofilm formation on the surface of solution made the MPN a sharp decrease in the first two weeks. After the attachment and detachment of bacteria onto the surface trend to be equilibrium, the MPN was constant. After eight weeks, the MPN of Vp decreased 98.6±0.4%, 98.8±0.1% and 99.0±0.02% for control, with 10 mg/L and 30 mg/L capsaicin, respectively. The

statistical analysis indicated that there was no significant difference (P-value=0.45) between them. Even with a large decrease of MPN in solution, a normal concentration range of Vp bacteria was still in the solution. The effectiveness of capsaicin on preventing bacterial attachment was achieved by the image analysis of biofilm formation on the slides.

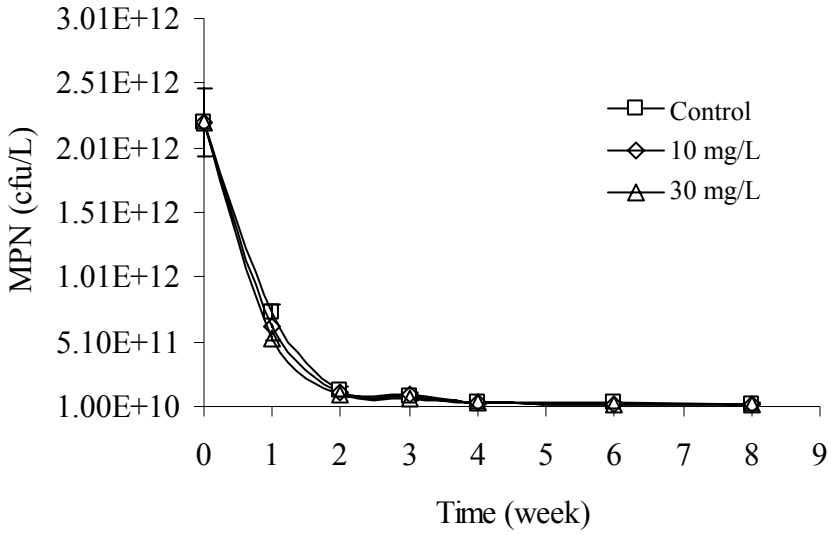

Figure 5.29 MPN changing with time in the aqueous solution containing different concentration of capsaicin with marine bacteria *Vibrio parahaemolyticus* (Vp), control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

5.4.5 Effect of NPAs on biofilm formation

Figures 5.30 (a)-(c) are some representing morphology images of biofilm formation on the plain silicone (Sylgard® 184) coatings with and without the present of zosteric acid in the solution containing marine bacteria of *V. parahaemolyticus*. It is obvious that the biofilm formation on the coated slides decreased as the concentration of

zosteric acid increased. Figure 5.30 (a) shows normal growth of Vp bacteria on silicone coating after 6 weeks in absence of NPAs. When 20 mg/L of zosteric acid was added, only 27.5% (compared to control) colonies were found to have attached after 14 days (Figure 5.30b). With 40 mg/L zosteric acid, the surface almost clear (only 1.8 % compared to control) were found (Figure 5.30c).

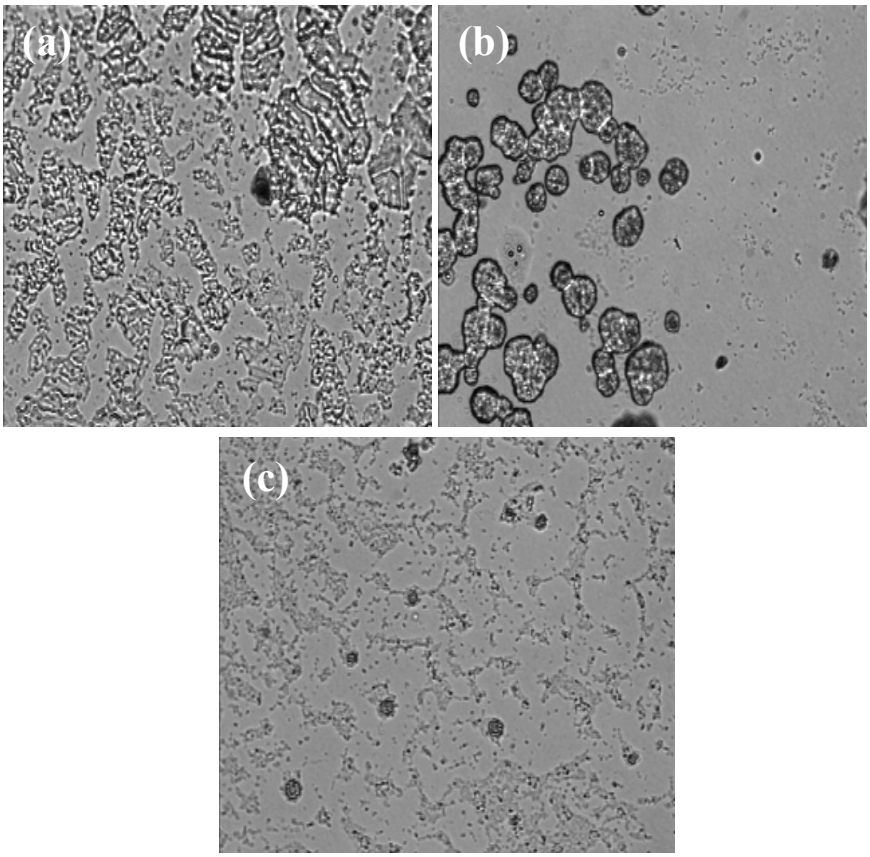

Figure 5.30 Coatings of Sylgard® 184 immerged in marine bacteria *Vibrio parahaemolyticus* (Vp) solution after six weeks: (a) Vp solution without zosteric acid; (b) Vp solution with 20 mg/l zosteric acid; (c) Vp solution with 40 mg/l zosteric acid, bar= 30 μm (Barrios took the pictures).

Comparison of biofilm coverage on the slides was shown in table 5.5. It indicated that zosteric acid inhibited bacteria colonies attaching on the coating surface. Because no significant difference in MPN among the different conditions (section 5.4.4), it is hard to tell the possible mechanisms of the NPA affect on the biofilm formation on the surface.

Table 5.5 Comparison of biofilm formation on the slides for zosteric acid suspended in marine bacterial solution (Vp)

V.parahaemolyticus	Pixels	% Relative
Control	1987	100.0
20 mg/L ZA	546	27.5
40 mg/L ZA	36	1.8

For Vn-zosteric acid system, as the concentration of NPA increased, the coverage of biofilm on the coating slides was not significantly different (Figure 5.31 (a)-(d)). Actually, compared to the bacteria formed the biofilm on the surface of the solution, the colonies attached on the coatings were very few. However, it was seen that the size of colonies definitely decreased as the concentration of zosteric acid increased. It means that the presence of zosteric acid effect the colonization process of the bacteria on the surface. Although, the mechanisms of this action of zosteric acid still unknown, it provided a room for a further research in this field.

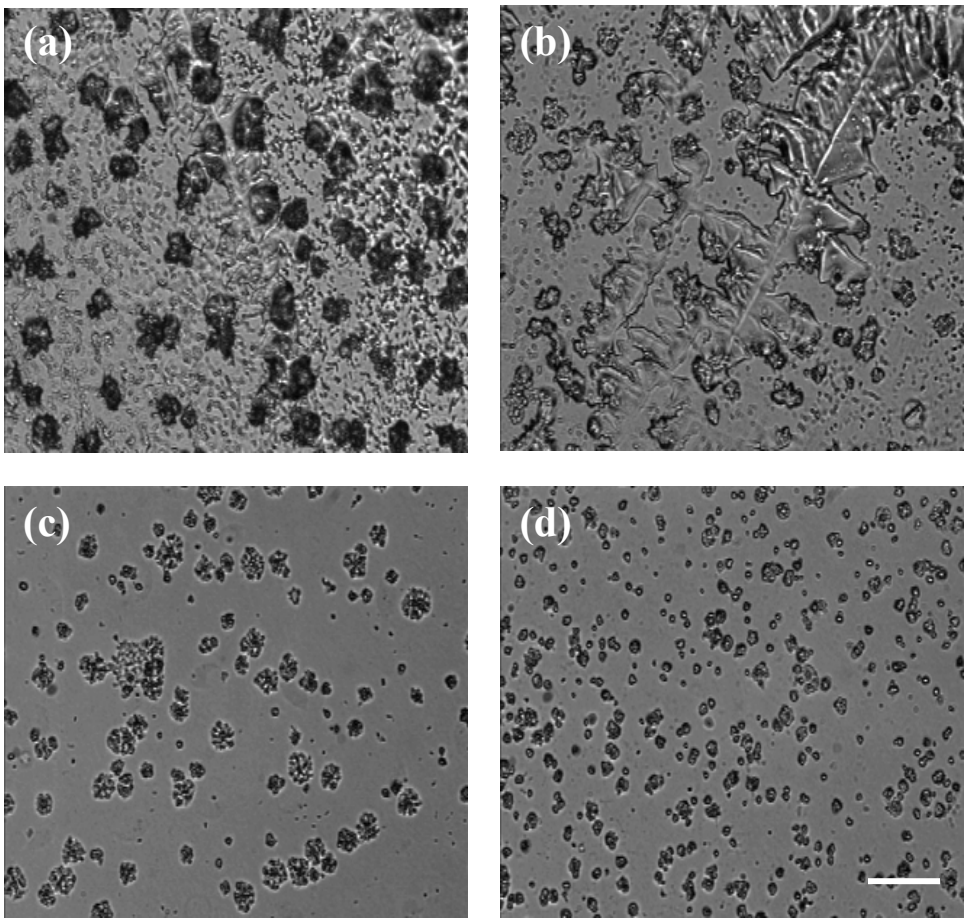

Figure 5.31 Coatings of sylgard® 184 immerged in marine bacteria *Vibrio natriegens* (Vn) solution after six weeks: (a)Vn solution without zosteric acid; (b) Vn solution with 5 mg/l zosteric acid; (c) Vn solution with 10 mg/l zosteric acid; (d)Vn solution with 20 mg/L zosteric acid, bar = 30 μm (Barrios took the pictures).

As shown in Figure 5.32 (a)-(c), morphology images represented the biofilm formation on the plain silicone (Sylgard® 184) coatings immersed in the marine bacteria of *V. parahaemolyticus* solution containing different concentration of capsaicin. Maybe the bacteria in solution would rather to attach on the biofilm formed on the surface of solution rather than to attach on the coatings. The colonies on the coatings were very few.

The presence of capsaicin had not significantly affected the bacteria to attach on the coatings after six weeks of immersion. The comparison of biofilm formation on slides was given in table 5.6.

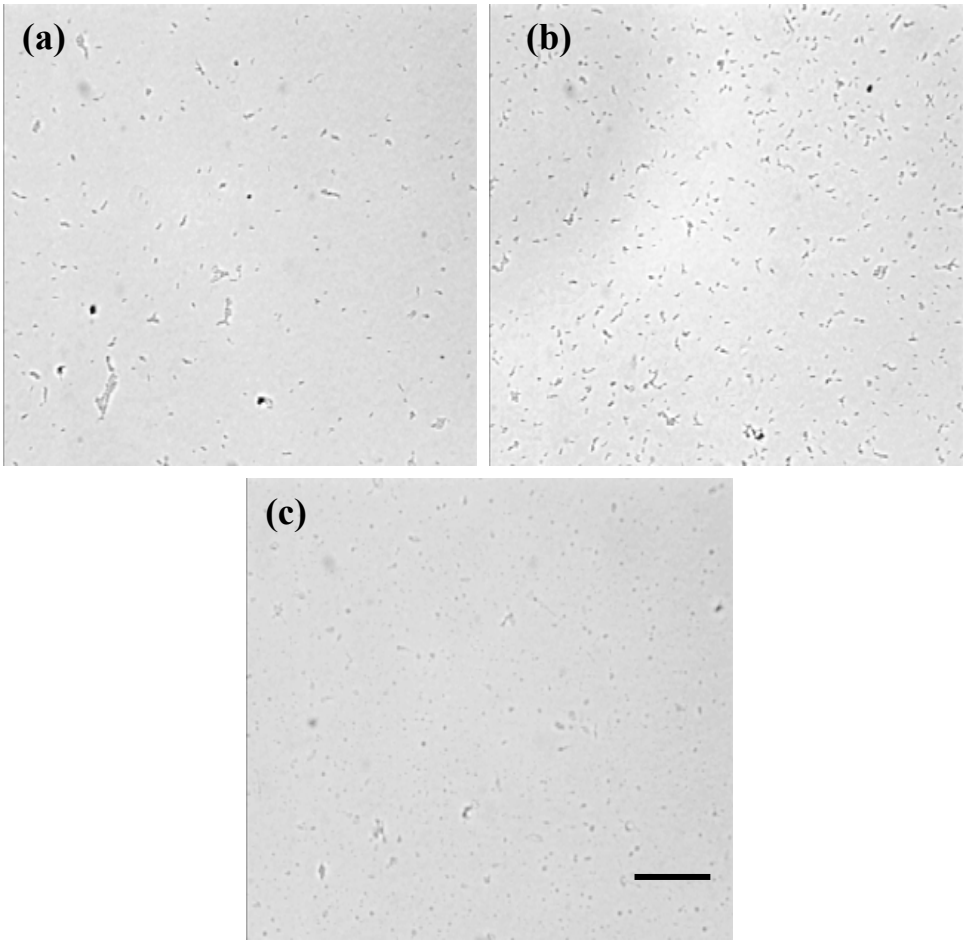

Figure 5.32 Coatings of Sylgard® 184 immerged in marine bacteria *Vibrio parahaemolyticus* (Vp) solution after 6 weeks: (a) Vp solution without capsaicin; (b) Vp solution with 10 mg/l capsaicin; (c) Vp solution with 30 mg/l capsaicin, bar= 30 μm (Barrios took the pictures).

Table 5.6 Comparison of biofilm formation on slides for capsaicin dissolved in Vp solution

V.parahaemolyticus	Pixels	% Relative
Control	153	100.0
10 mg/L ZA	296	193.0
30 mg/L ZA	124	81.0

5.4.6 Comparison of NPAS to marine bacteria attachment

Generally speaking, the biofilm formation on the surface of the solution was the primary reason for the MPN decrease for both marine bacteria systems. This made it difficult to evaluate the effectiveness of NPAs on preventing the bacterial attachment. For Vp system, the presence of zosteric acid did reduced the attachment on coatings and inhibited the metabolic process of bacteria. However, the effectiveness of capsaicin was not significant. For Vn system, colonization decreased on the coatings as the concentration of zosteric acid in solution increased.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The main objective of this research was to evaluate two Natural Product Antifoulants (NPAs) zosteric acid and capsaicin, on preventing bacteria attachment onto the surface. Two secondary objectives were to ascertain the optimal concentrations of NPAs, as well as try to discover the mechanisms of how they act against fouling. In order to achieve these objectives, a series of tasks were performed. They included the toxicity assessments for the evaluation of potential application of capsaicin and zosteric acid as antifoulants; the attachment studies to obtain the effectiveness on fouling by NPAs either suspended in solution or entrapped in silicone coatings (Slygard® 184 or RTV11).

The freshwater bacteria of enriched Lake Erie (LE) consortium and *Pesudomonas putida* (Pp), and two kinds of marine bacteria *Vibrio parahaemolyticus* (Vp) and *Vibro natrigens* (Vn) were used as the model bacteria species in the experiments. The following sections highlight the key findings and verify the realization of the objective.

6.1.1 Toxicity assessments for capsaicin and zosteric acid

Both qualitative (Microtox assay) and quantitative (statistic toxicity assessment) methods were applied to obtain the EC_{50} of these two NPAs. The Microtox assay (5

minutes test) indicated that the EC_{50} of capsaicin and zosteric acid was 11.75±1.02 mg/Land 442±100 mg/L, respectively. It was seen that capsaicin was more toxic than zosteric acid. The Microtox EC_{50} provided a reference concentration range for statistic toxicity assessment. The statistic toxicity assessments showed that the EC_{50} values of capsaicin were 22.6 mg/L, 10 mg/L, 17.0 mg/L and 15.6 mg/L, respectively to LE bacteria, Pp, Vn and Vp. The EC_{50} of zostric acid to the corresponding species listed above were 376 mg/L, 242 mg/L, 7.4 mg/L and 18.0 mg/L. Obviously, in this method, capsaicin was still more toxic than zosteric acid to each (except Vn) specific bacteria.

The capsaicin and zosteric acid were substantially less toxic than the currently used antifoulants such as TBT and DBT. For instance, TBT has a 72 h-EC_{50} ranging from 0.33 to1.03 μg/L to many marine organisms, 48 h-EC_{50} of DBT on oyster larva was 0.1-0.2 mg/L. Therefore, the relative low toxic property of capsaicin and zosteric acid made them ideal non-toxic antifoulants.

6.1.2 The optimal suspension concentration of NPAs to freshwater bacteria

Attachment studies indicated that the presence of capsaicin and zosteric acid performed obvious inhibition of the bacteria attachment. For instance, after the silicone coated slides immersed for 14 days, the LE system depicted 93.5% and 98.5% less biofilm coverage when 20 ppm and 40 ppm capsaicin was present, respectively. For capsaicin, 20 ppm was half the other treatment, but performed a similar level (both were over 90% decrease) in preventing bacteria attachment. Thereby, the more effective and economical concentration of capsaicin was 20 ppm.

With zosteric acid, Biofilm formation was reduced by 29.6% and 98.2%, respectively, as the concentration increased from 5 ppm to 500 ppm. The 92.5% biofilm decrease with 50 ppm zosteric acid was obviously more effective than the concentration of 500 ppm. Compared to 5 ppm (29.6% decrease) and 10 ppm (54.3% decrease), 92.5% decrease of biofilm formation with 50 ppm was a satisfied antifouling level that was expected. Therefore, the optimal concentration of zosteric acid was 50 ppm. It's important to recall that the EC_{50} of capsaicin and zosteric acid was 10 mg/L and 242 mg/L to *P. putida*, 22.6 mg/L and 378 mg/L to the LE bacteria, respectively. According to these EC_{50} values to the same bacteria species, comparison of the NPA effectiveness indicated that zosteric acid was more effective than capsaicin as an antifoulant.

6.1.3 The optimal entrapped concentration of zosteric acid to LE bacteria

A leaching rate study of (0.3 wt %, 0.5 wt % and 1 wt %) zosteric acid bulk entrapped in silicone Sylgard® 184 indicated that 1 wt % had a reasonable leaching rate of 0.1mg/cm^2/day. The attachment studies demonstrated that the 1 wt % by bulk entrapped zosteric acid had the similar effectiveness (91.2% decrease of attachment) on antifouling as that of 50 ppm in suspension (92.5% decrease). Changes in conductivity, DO and MPN exhibited the similar trends as well.

In order to evaluate the impact of silicone coating on the antifouling effectiveness of zosteric acid, 1 wt % zosteric acid was bulk entrapped in RTV 11. The study indicated that zosteric acid leached much easier from RTV 11 than Sylgard® 184. However, the biofilm formation on the coatings reduced only ~50% when zosteric acid was bulk entrapped in RTV 11 while the attachment reduced ~90% when zosteric acid was

entrapped in Sylgard® 184. It illustrated that the zosteric acid is much effective on antifouling if it was entrapped in Sylgard® 184 than RTV 11. The reasons were maybe due to 1) more zosteric acid leached from RTV 11 and increased the roughness of the coating surface, making it easier for bacteria to attach; or 2) zosteric acid reacted with RTV 11 and reduced the antifouling property of this foul-release coating.

6.1.4 The optimal suspension concentration of NPAs to marine bacteria

Two target marine bacteria *Vibrio parahaemolyticus* and *Vibrio natriegens* formed biofilm on the water surface. After 2 weeks, the thickness of biofilm was 2.5 mm and 3 mm for Vp and Vn, respectively. This made it very difficult to evaluate the antifouling effectiveness of NPAs on marine bacteria. The bacteria appeared to prefer to form the biofilm on the surface of the solution than on the surface of slides. Therefore, the optimal concentration could not be accurately determined. However, it was still seen that the colonization of bacteria on the slides was inhibited as the concentration of NPAs increased.

6.1.5 Assessment of the antifouling mechanisms of the NPAs

In attachment studies, it was important to note that the higher concentration of NPAs present, the more MPN in the solution and a corresponding less biofilm formation on the slide. This implied that the antifouling performance of capsaicin and zosteric acid was not simply to kill the bacteria, but to block the bacteria's active site of their chemosensory mechanism or inhibit the synthesis of exopolysaccharides for forming the polysaccharide matrix. Either mechanism would successful prevent biofim formation.

6.2 Recommendations

This research investigated the performance of two natural product antifoulants of capsaicin and zosteic acid. However, there are four aspects in which further research is warranted.

- Investigation of the antifouling mechanisms of NPAs in a micro-scale, such as by using Atomic Force Microscopy (AFM). AFM could map a surface in a nanometer-sized, making it possible to analyze the organisms' process of adhesion and attachment onto the surface.
- Additional evaluation of the antifouling performance of zosteric acid when bulk entrapped. The study could either use another type of foul-release coating or investigate other solvents to increase the leaching rate from Sylgard® 184.
- Reevaluation the antifouling performance of capsaicin and zosteric acid. Other sources of marine bacteria could be used, which did not form surface biofilm on the water. In addition, the use of real ocean water instead of artificial nutrient solution would obtain a real marine environment and possible to achieve more accurate effectiveness of NPAs on marine antifouling.
- Evaluation of the antifouling performance of other natural product compounds or non-toxic antifoulants, such as benzoic acid or sodium benzoate. Once completed, comparison with zosteric acid and capsaicin could verify which was more effective as antifoulant.

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APPENDICES

APPENDIX A

FIGURES FOR TOXICITY ASSESSMENT

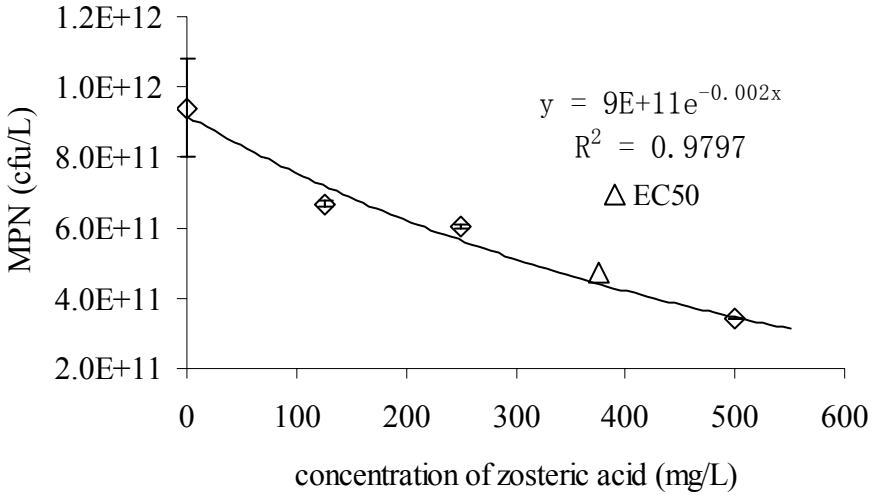

Figure A1 Static toxicity assessment of the MPN of Lake Erie bacteria changing with zosteric acid concentration after exposed 7 days (Error bar represents the ± standard deviation, n=3). Results yield an EC_{50}= 376.4 mg/L.

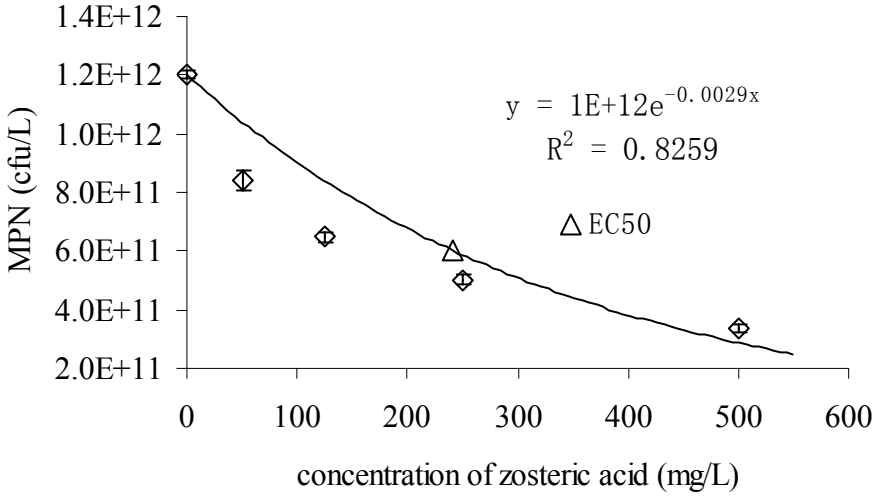

Figure A2 Static toxicity assessment of the MPN of *P. putida* bacteria changing with zosteric acid concentration after exposed 7 days (Error bar represents the ± standard deviation, n=3). Results yield an EC_{50}= 241.5mg/L.

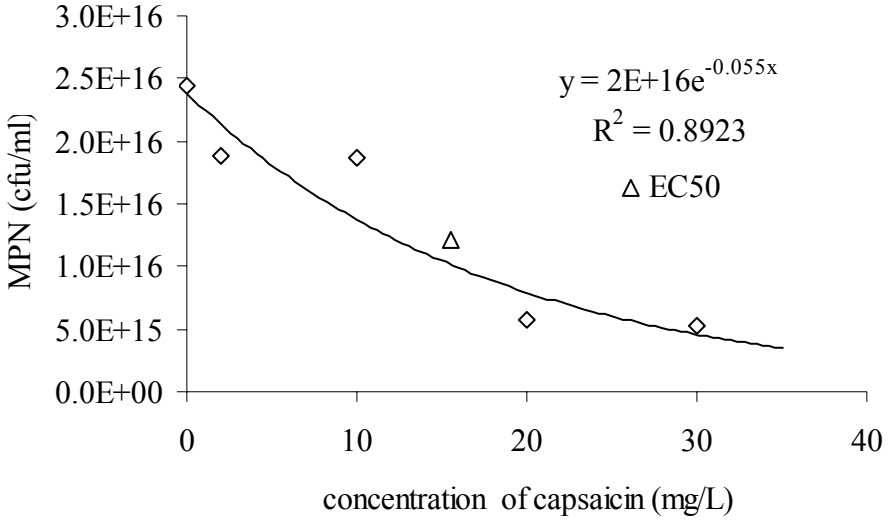

Figure A3 Static toxicity assessment of the MPN of *V. parahaemolyticus* changing with capsaicin concentration after exposed 7 days. Results yield an EC_{50}=15.6 mg/L.

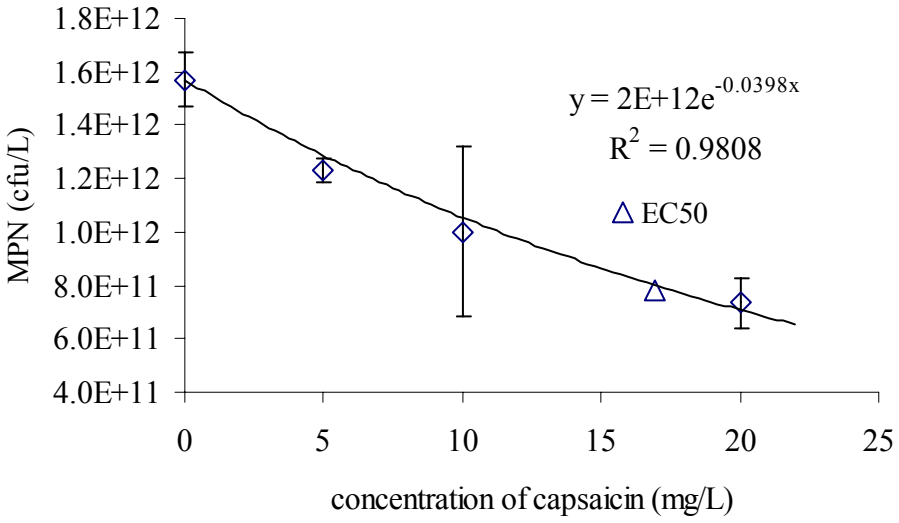

Figure A4 Static toxicity assessment of the MPN of *V. natriegens* changing with capsaicin concentration after exposed 7 days (Error bars represent the ± standard deviation, n=3). Results yield an EC_{50}=17 mg/L.

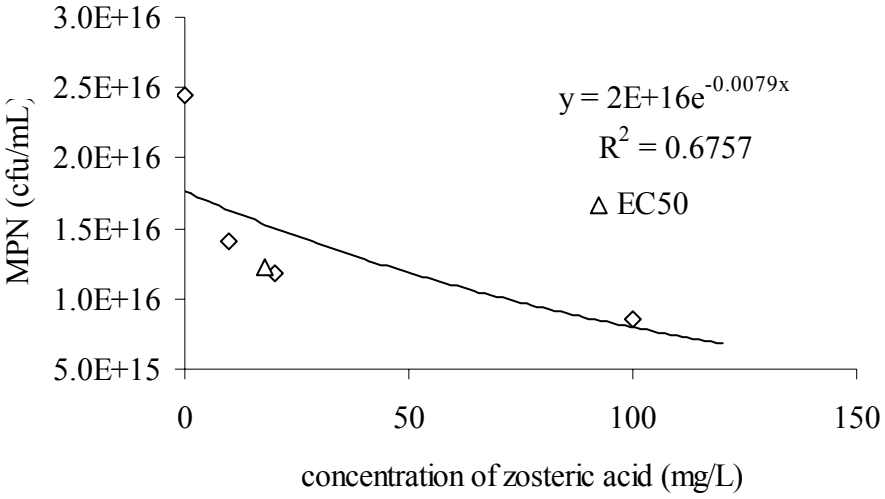

Figure A5 Static toxicity assessment of the MPN of *V. parahaemolyticus* changing with zosteric acid concentration after exposed 7 days. Results yield an EC_{50}=18 mg/L.

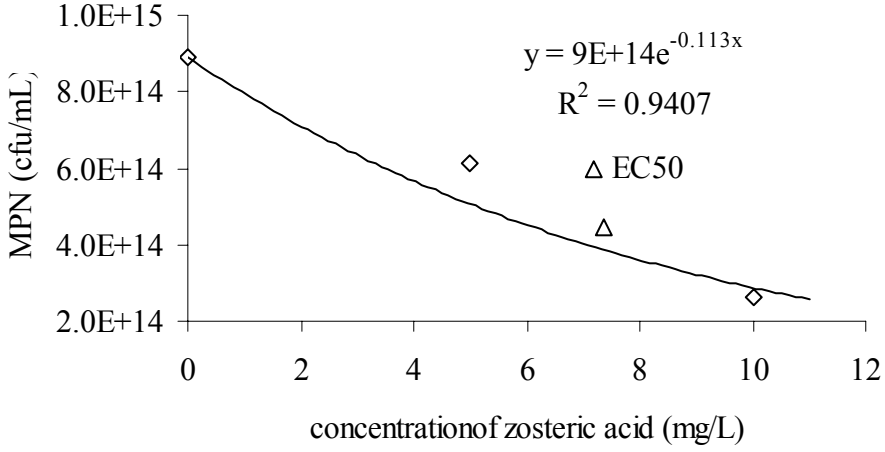

Figure A6 Static toxicity assessment of the MPN of *V. natriegens* changing with zosteric acid concentration after exposed 7 days. Results yield an EC_{50}=7.37 mg/L.

APPENDIX B

ADDITIONAL FIGURES FOR ATTACHMENT STUDY

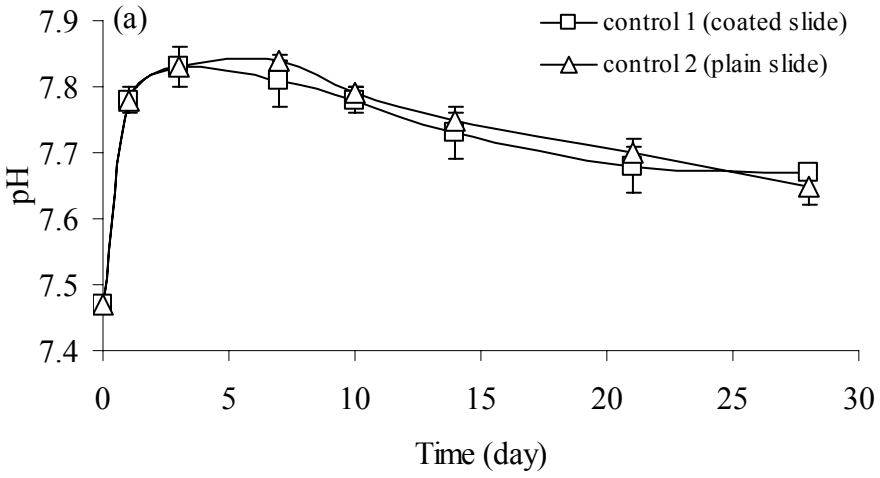

Figure B1 pH change with time for two control slides immersed in LE solution without NPAs, control 1 represents slides coated with silicon Slygard® 184, control 2 represents plain slides (error bars represent ± standard deviation, n=3)

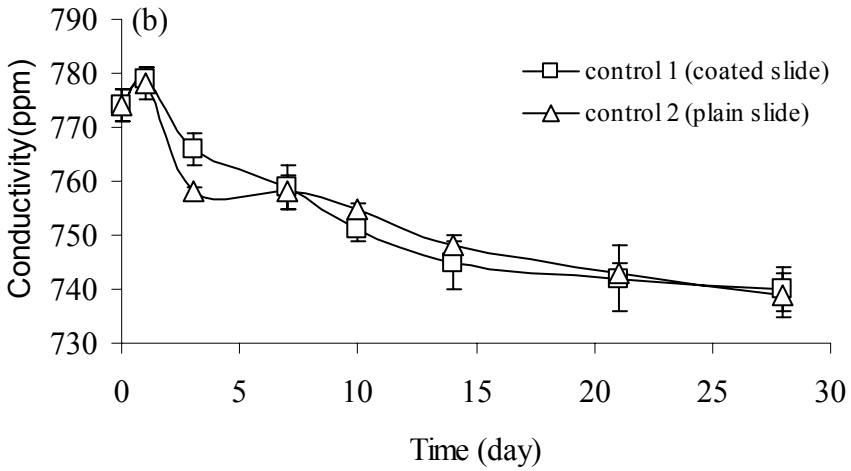

Figure B2 Conductivity change with time for two control slides immersed in LE solution without NPAs, control 1 represents slides coated with silicon Slygard 184, control 2 represents plain slides (error bars represent ± standard deviation, n=3)

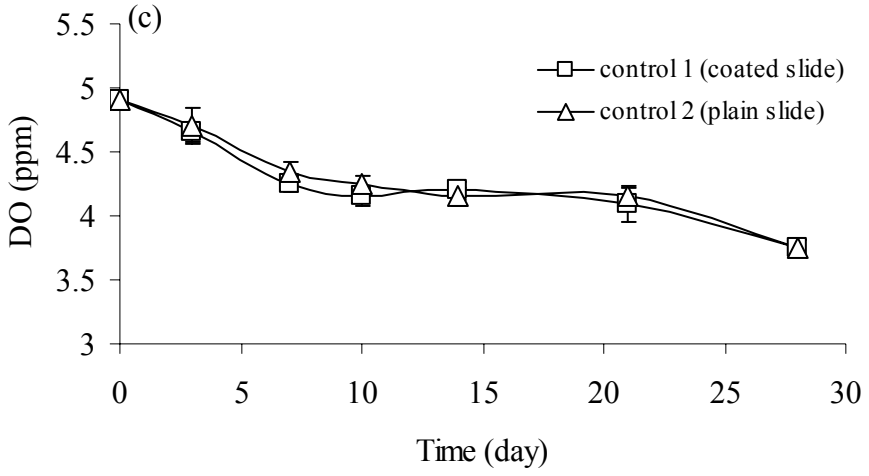

Figure B3 DO change with time for two control slides immersed in LE solution without NPAs, control 1 represents slides coated with silicon Slygard 184, control 2 represents plain slides (error bars represent ± standard deviation, n=3)

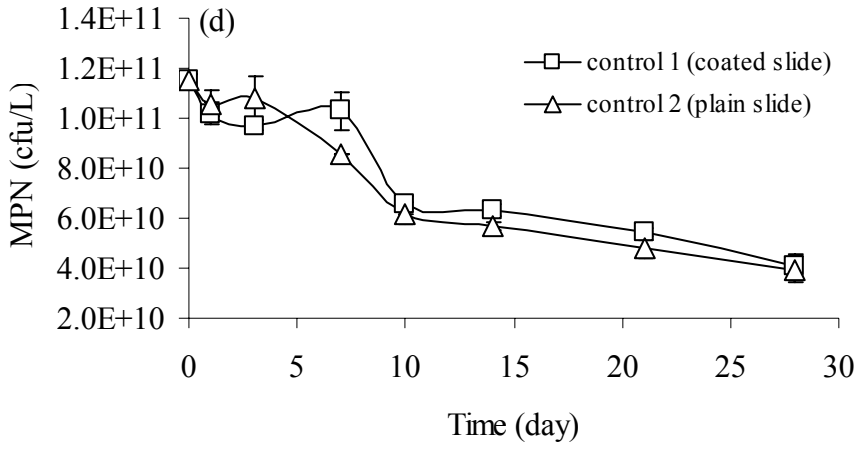

Figure B4 MPN change with time for two control slides immersed in LE solution without NPAs, control 1 represents slides coated with silicon Slygard 184, control 2 represents plain slides (error bars represent ± standard deviation, n=3)

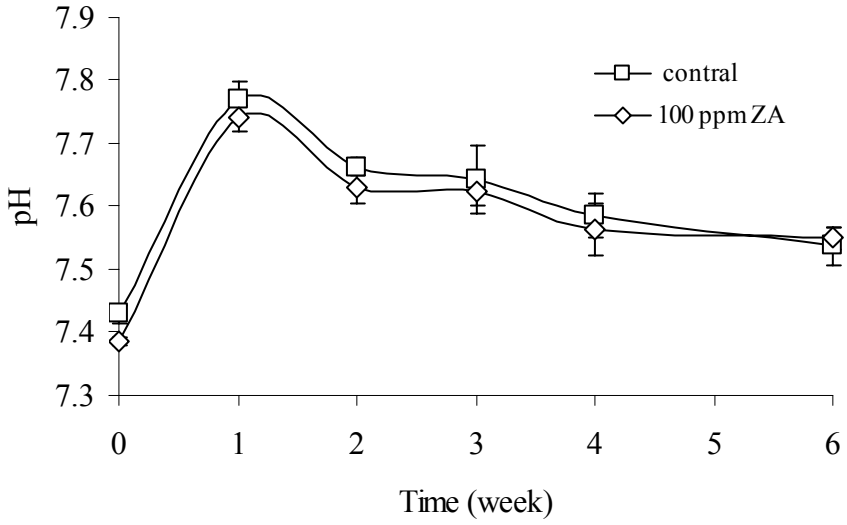

Figure B5 pH change with time in LE solution containing different concentration of zosteric acid, control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

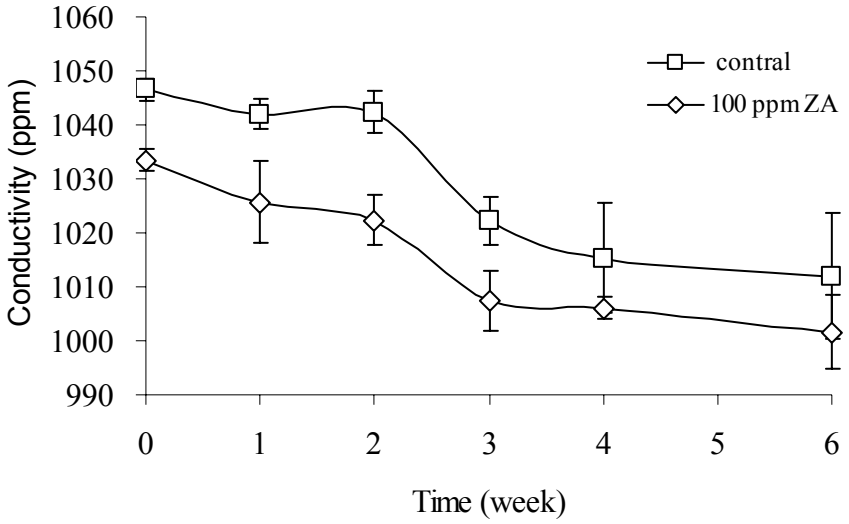

Figure B6 Conductivity change with time in LE solution containing different concentration of zosteric acid, control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

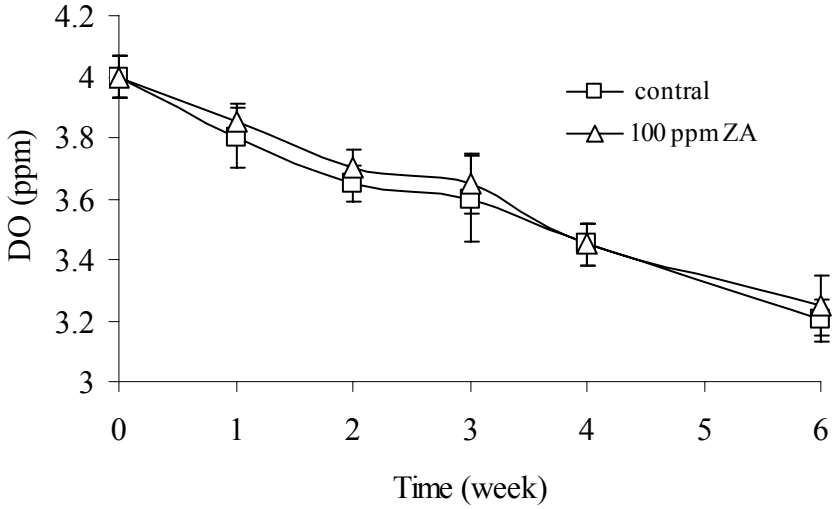

Figure B7 DO change with time in LE solution containing different concentration of zosteric acid, control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

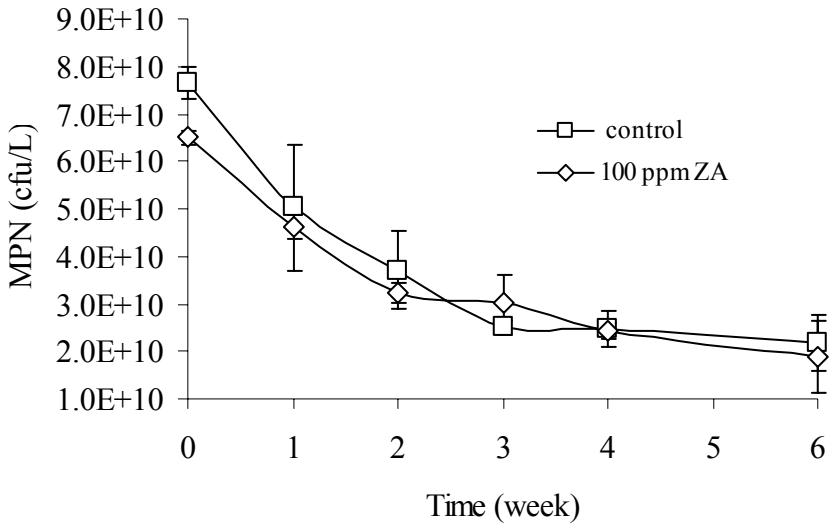

Figure B8 MPN change with time in LE solution containing different concentration of zosteric acid, control represents no zosteric acid in solution (error bars represent ± standard deviation, n=3)

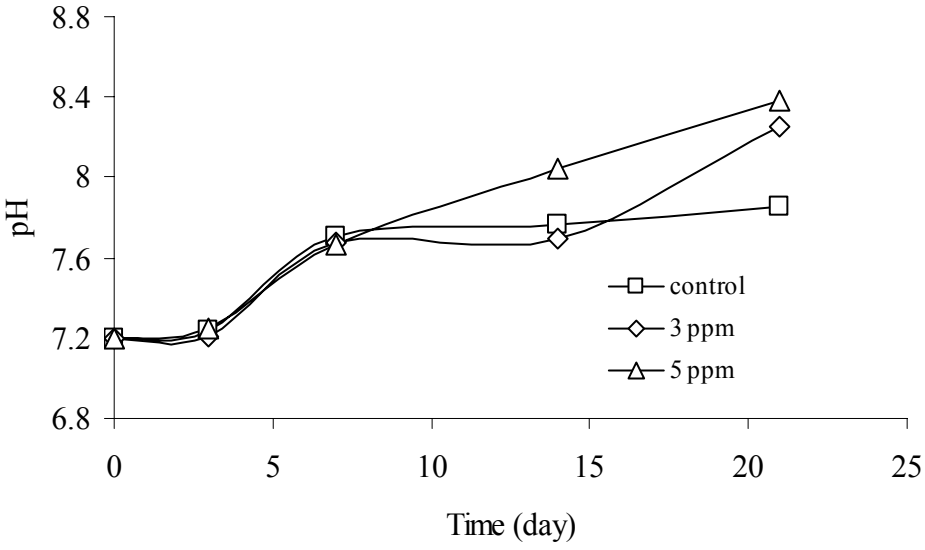

Figure B9 pH change with time in *Vibrio natriegens* solution containing different concentration of capsaicin, control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

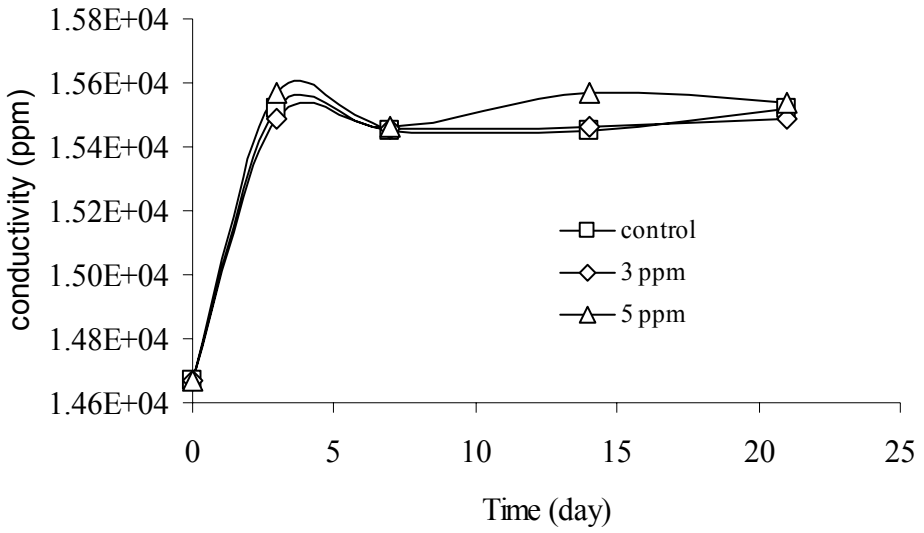

Figure B10 Conductivity change with time in *Vibrio natriegens* solution containing different concentration of capsaicin, control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

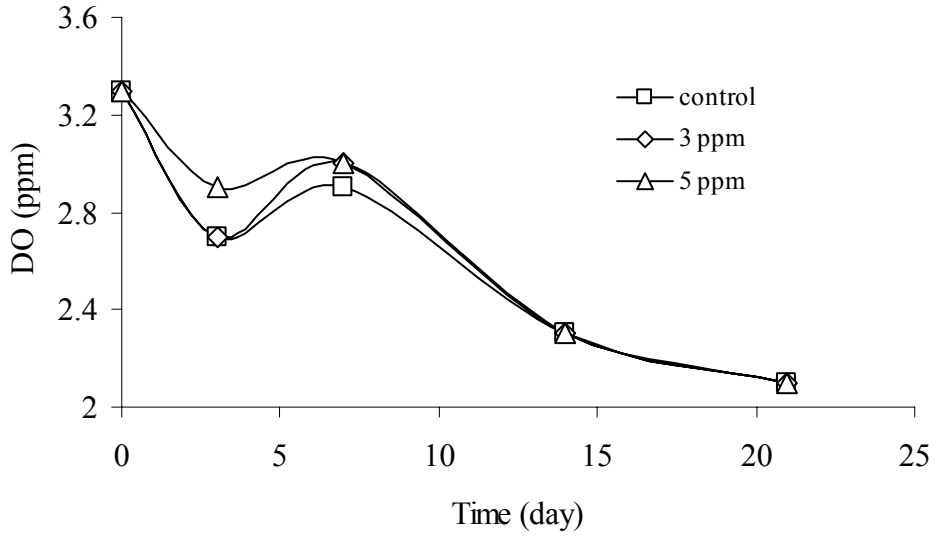

Figure B11 DO change with time in *Vibrio natriegens* solution containing different concentration of capsaicin, control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)

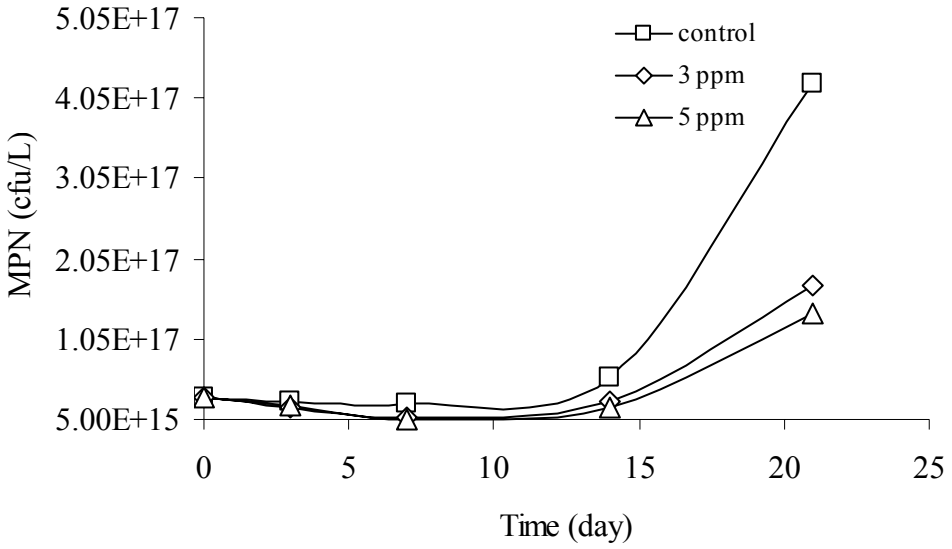

Figure B12 MPN change with time in *Vibrio natriegens* solution containing different concentration of capsaicin, control represents no capsaicin in solution (error bars represent ± standard deviation, n=3)