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Characterization of planktonic and biofilm fatty acid profiles and evaluation of their trophic transfer potential to Hyalella Azteca

Jerry Chien-Yao Chao
Ryerson University

CHARACTERIZATION OF PLANKTONIC AND BIOFILM FATTY ACID PROFILES AND EVALUATION OF THEIR TROPHIC TRANSFER POTENTIAL TO *HYALELLA AZTECA*

By

Jerry Chien-Yao Chao

HBSc, University of Toronto, 2006

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ABSTRACT

Characterization of planktonic and biofilm fatty acid profiles and evaluation of their trophic transfer potential to *Hyalella azteca*

Master of Science, 2009

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Molecular Science, Ryerson University

Fatty acid (FA) composition between biofilms and batch planktonic cultures were compared for two bacterial species *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Biofilm cultures exhibited decrease in saturated fatty acids (SAFA) that potentially conform to a more fluidic biophysical membrane property. The amount of FA in the biofilms' extracellular polymeric substance was not sufficient to consider it having a major contribution to the observed differences between biofilms and batch planktonic cultures. While biofilm grazing by the amphipod *Hyalella azteca* was evident, only certain bacteria-specific FA appeared to have the potential to be retained (odd-number SAFA and branched-chain FA). *H. azteca* with diet strictly consisted of bacteria biofilms did not demonstrate significant changes in their nutritional condition in terms of ω-3 and ω-6 polyunsaturated fatty acids (PUFA); combined with the results from fasting trials, *H. azteca* appears to have the capacity to retain ω-3 and ω-6 PUFAs up to 10 days.

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Chapter 1: INTRODUCTION

1.1 Background

Bacteria are primarily found in surface associated communities call biofilms, which possesses genetic expression, physiological properties and growth patterns that are markedly different than planktonic bacteria. Biofilm communities are well protected from external stress by multiple layers of cells and an extracellular polymeric substance matrix. The study of biofilm has generated significant research interest in the health and industry-related sciences mainly because of their persistent and detrimental association with chronic infections, contamination, deterioration and bio-corrosion. Conversely, the unique genetic and phenotypic properties in biofilm have also been recognized as a valuable tool in various bio-processes. In the aquatic sediment, bacteria (both the planktonic and biofilm forms) are ubiquitous and abundant and have important role in nutrient cycling and degradation of organic matter in addition to their contribution to the diets of primary consumers.

In recent decades, studies on lipid and fatty acids have grown exponentially, in part because of the recognition of the numerous health benefits of "essential fatty acids". Unlike proteins and carbohydrates that break down into relatively few molecular species of amino acids and monosaccharide units, fatty acids (FA) and other lipids from ingested food exist in many forms and are generally not further degraded as they pass into the circulatory system. For example, polyunsaturated FA (PUFAs) produced by microalgae in the aquatic ecosystem are transferred to primary consumers and are then transferred and retained in consumers at progressively higher trophic levels culminating in humans. The great diversity of FA and other lipids combined with the uneven distribution of biosynthetic capacities for different lipids among

species provides researchers with the potential to use FA signature analysis to assess food web dynamic.

While bacterial FA have been identified in the past, biofilm-specific details of the factors that influence their abundance and distribution, especially from an ecological perspective, are relatively scarce. By means of FA analysis, this study attempted to identify biofilm-specific features in term of FA signatures. The amphipod, *Hyalella azteca*, was then introduced to evaluate the concept of transferrable FA markers as a possible method to monitor the biofilm community. Finally, the nutritional contribution of biofilm in the aquatic food web was also investigated by assessing the FA profile of *H. azteca* raised on a pure biofilm diet.

1.2 Hypothesis

Given the unique properties and physiological importance of certain FA, and based on what is known about FA dynamics in other organisms, it was expected that the FA profiles in *H. azteca* should reflect that of the microbial species in the biofilm grazed by these amphipods, if so, the result would support the feasibility of using *H. azteca* as a natural bioindicator for monitoring certain aspects of the microbial community in the aquatic systems where amphipods occur.

The overall goal was to determine the FA composition of model bacterial biofilms and to evaluate their potential as FA biomarkers using information derived from fatty acid methyl esters (FAME) analyses. Then the bioindicator species *H. azteca* was introduced into the model system to evaluate the potential of these FA signatures for trophic transfer.

1.3 Specific objectives

1) Assess FA composition in biofilm
    - Five different bacteria; *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus megaterium* were tested to confirm FA profiles can be species-specific.
    - *P. aeruginosa* and *S. aureus* were selected as the bacterial model organisms to assess the differences in FA composition between planktonic and biofilm culture.
    - The extracellular component of biofilms was extracted to determine the contribution of EPS to the overall FA profile.
2) Evaluate trophic transfer of bacterial FA to *Hyalella azteca*
    - Transferrable bacteria-specific FA markers were identified to assess the practicality of using *H. azteca* as indicator organism to monitor biofilm community.
    - FA profiles of biofilm fed *H. azteca* were analyzed with particular focus on the long-chain ω-3 and ω-6 fatty acids as a measure of its health condition, which in turn determines the nutritional value of the biofilm.

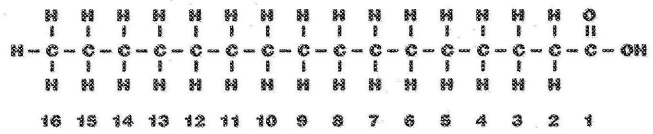
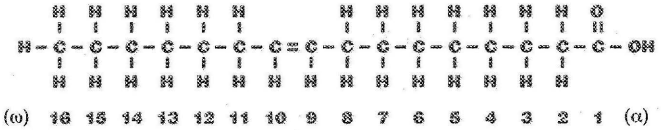
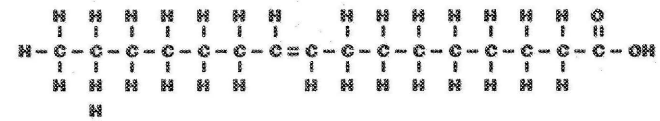
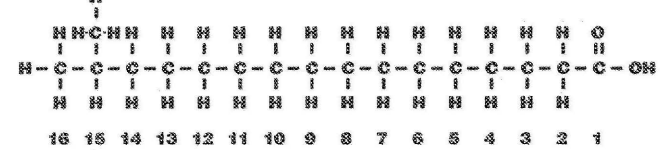
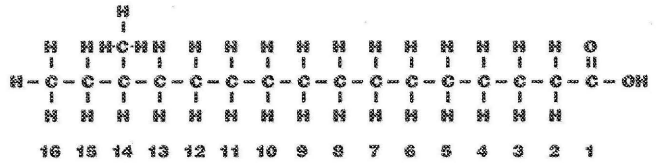
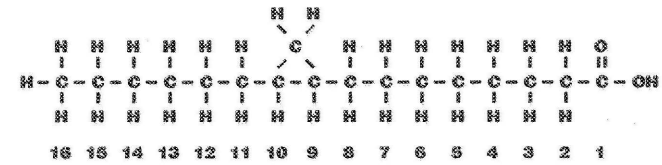
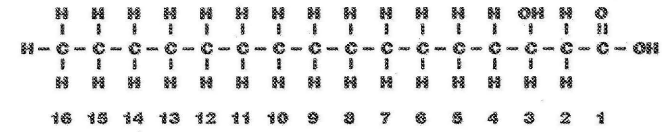
Chapter 2: LITERATURE REVIEW

2.1 Lipids and fatty acids

Fatty acids (FA) are aliphatic acids made up of long hydrocarbon chains with a carboxyl terminal. Saturated fatty acids (SAFA) consist of hydrocarbon chains with no double bonds. FA with one or more double bonds in the hydrocarbon chain are called monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA), respectively. Straight chain FA with an even number of carbons ranging from 14 to 24 are the most common, the number of double bonds typically range between 2 and 6 as a result of this limited chain length. FA with ≤14 carbons are more prominent in prokaryotic organisms and FA with >24 carbons and >6 double bonds only exist in trace amounts in some organisms (Budge *et al.*, 2006).

FA nomenclature indicates the number of carbon atoms in the FA chain with the number of double bonds and any additional functional groups it may contain. For example, a 12 carbon FA with hydroxyl group at the third carbon from the carboxyl terminal is written as 3OH-12:0. Branched FA are denoted with their respective prefixes; *i* for *iso* branch position (methyl group on the second carbon from the methyl terminal) and *a* for *anteiso* branch position (third carbon from the methyl terminal). Cyclopropane FA are denoted by the suffix *cyc* followed by the position of the cyclopropane ring on the alkyl chain (Table 2.1). Polyunsaturated fatty acids (PUFA) have multiple double bonds, but the position of the first double bond from the terminal methyl group (denoted as n or ω) has the greatest metabolic significance. Given that animals tend to have limited ability to alter the first double bond position, particularly at the ω-3 position, PUFA nomenclature describes the location of first double bond from the terminal methyl group. For example, the PUFA eicosapentaenoic acid (EPA) has 5 double bonds with the first at the third carbon from the terminal methyl group and is denoted as 20:5n3.

Table 2.1: Summary of various FA classes with their respective nomenclature and chemical structures

FA class & nomenclatures	Chemical structures
SAFA 16:0	
MUFA *Cis* - 16:1n7c *Trans* - 16:1n7t	 
Branched FA *i*-17:0 *a*-17:0	 
Cyclopropane FA 17:0 cyc (9, 10)	
Hydroxy FA 3-OH 16:0	

Most lipids contain FA as part of their structure; triacylglycerol for example is the long term energy storage lipid. In mammals, FA chains are metabolized via a series of β-oxidations to yield multiple acetyl-CoA units, all of which feed into the citric acid cycle in the mitochondria to generate ATP. FA incorporated into phospholipids (PLFA) are major components of cellular membranes, along with factors such as the type of phospholipid head group and amount of sterol (cholesterol in animal, hopanoid and carotenoid in bacteria), the type of FA that constitutes the

phospholipid can also influence the membrane fluidity (Arts *et al.*, 2009). Highly unsaturated FA have a melting point that approaches -50°C; corresponding to a more fluidic and flexible membrane than SAFA 12:0 and 18:0 that has melting temperature range from +58°C to +77°C. Other important membrane properties such as permeability, elasticity, efficiency of membrane associated enzymes and formation of transport vesicles are in part influenced by PLFA composition (Adams, 1999; Stillwell and Wassall, 2003; Arts *et al.*, 2009). Ultimately, FA's property on the cellular level contributes to the overall health of individual organisms and potentially influences the entire food web in the ecosystem through the hierarchy of consumers (Arts, *et al.*, 2001).

PUFA has an important role in intra-molecular signaling and hormone regulation (Budge *et al.*, 2006). In mammals, omega-6 (ω-6, n-6) FA such as arachidonic acid (ARA; 20:4n6) are precursors to pro-inflammatory hormone-like substances called eicosanoids, prostaglandins in particular participate in modulation of inflammation response, blood pressure, dilation and constriction of blood vessels. Other eicosanoids include thromboxane, protacyclin and leukotriene (Christie, 2007). While ω-6 FA has important biological functions, an excess amount can cause imbalanced inflammatory response due to the pro-inflammatory nature of the particular type of prostaglandin derived from ω-6 FA. This had been associated with development of autoimmune conditions such as rheumatoid arthritis, lupus erythromatous and asthma (Fernandes *et al.*, 2008; Biltagi *et al.*, 2009).

The types of eicosanoid derived from omega-3 (ω-3, n-3) FA tend to be anti-inflammatory (e.g. prostaglandin D3) compared to those derived from ω-6 (e.g. prostaglandin D2). Due to enzymatic restriction, many animals are unable to synthesize ω-3 and ω-6 FA precursors (α-linolenic acid and linoleic acid respectively) in sufficient quantities and need to be

acquired from their diet in order to maintain optimal physiology (Arts *et al.*, 2009). Animal and vegetable fat contain high quantities of ω-6 FA, whereas abundant ω-3 FA can be found in aquatic sources such as salmon, herring, anchovy, sardine and other oily fishes. However past FA studies on these aquatic organisms indicated they also do not synthesize PUFA themselves, instead they are obtained from phytoplankton species that accumulated throughout the food web (Nichols & Mancuso-Nichols, 2008); the amount of PUFA in them is also largely influenced by their diet.

2.2 Fatty acid as an assessment tool - trophic transfer of fatty acid profile

FA signature analysis can provide a qualitative estimate of diet composition because the diversity of FA molecules, along with narrow limitations on FA synthesis and storage patterns; the lipid profile of organisms reflect, at least in part, to their dietary lipid and FA. However, it is also important to note that FA composition can be effected by external factors and the physiological condition of the organism in addition to dietary FA (Sushchik *et al.*, 2003). These factors will always contribute to the overall FA profile to a certain degree and need to be cautiously considered when performing FA signature analysis. For example, mobilization and catabolism of FA during starvation and reproduction were found to cause significant alteration in the FA profile of the opossum shrimp *Mysis relicta* (Schlechtriem *et al.*, 2008). Iverson *et al.* (2004) developed an in-depth mathematical model that can provide quantitative diet estimates, which accounted for predator (various species of seal) fat content and their lipid metabolism, but the model cannot be fitted for all predators. While the simplest scenario would be having a single unique FA that can be traced to single prey species, the ubiquitous nature of many of the FA molecules in organisms makes this situation rare, and thus tracer analyses often requires an

expanded level of comparison that looks at ratios of particular FA or groups of FA (Budge *et al.*, 2006).

Transfer of fatty acid between trophic level by feeding is the fundamental basis of the intention to use amphipod *Hyalella azteca* as the bioindicator to assess biofilm community structure, nutritional value of biofilm and nutritional status of the *H. azteca*. It is important to characterize the fatty acid transfer and recognize the metabolic capability of the indicator species in order to ensure its ability to retain the specific dietary FA. Incorporation may lead to detection of specific FA signatures of bacterial origin (Piotrowska-Seget & Mrozik, 2003; Stevens *et al.*, 2004; Hall *et al.*, 2006), or changes in the proportion of the components in the FA profile of the indicator organism (Yano *et al.*, 1997; Nichols, 2003). The copepod *Calanus glacialis* belongs to the same Crustacean subphylum as *H. azteca*; their diet depends more on bacterivorous dinoflagellates – branched-chain FA are abundant in bacterivorous dinoflagellates because their diet mostly consists of bacteria. However, when *C. glacialis* fed on these dinoflagellates, none of the branched FA were observed in *C. glacialis*; indicating loss of FA signal between these two levels of the food chain (Stevens *et al.*, 2004). Unique bacterial FA are irrelevant, from a tracer perspective, if they cannot readily be observed in the predator species' FA profile. MUFA 18:1n7 was found to be a more appropriate signature to infer the FA transfer from bacteria to dinoflagellates and further into another copepod (*Calanus* sp.).

There are unique arrays of FA with various chain length and different number of double bonds in marine organisms. Eighteen carbon FA in the ω-3 and ω-6 family are not readily biosynthesized by heterotrophic organisms and PUFA with more than 18 carbons are often synthesized in amounts that are insufficient to meet an organism's requirements for optimal physiological performance. However, these FA can potentially be used as biomarker/tracer for

marine food web studies to investigate dietary preference and history. Feeding experiments using the cuttlefish (*Sepia officinalis*) demonstrated appreciable differences in the FA profile between *S. officinalis* whose diet consisted of crustacean and those which consisted of fish (Fluckiger *et al.*, 2008). *S. officinalis* fed with crustaceans showed higher levels of 17:1n8, 18:1n9, 18:2n6, 18:1n7, 20:2n6 and eicosapentaenoic acid (EPA; 20:5n-3); the majority of these FA were also found and originated from the crustacean tissue. This trophic relationship between *S. officinalis* and its prey demonstrated that these FA biomarkers can be deposited into predator tissue with little modifications.

Seasonal trends in profundal benthic invertebrates and sediment was investigated by FA analysis. As long chain PUFA are important determinants of growth, survival and reproduction of many organisms, it was an appropriate criteria to use to evaluate the food quality in the sediment (Goedkoop *et al.*, 2000). FA analysis in the sediment showed highest amount of PUFA in the spring and autumn. The FA profiles of profundal invertebrates (e.g. chironomids) were strongly influenced by these seasonal fluctuations. The differences observed in the FA profile reflected the contribution of autotrophic (phytoplankton) and heterotrophic (detrital food web) dietary source. Phytoplankton dominated during spring and autumn, and was considered to be better in term of nutritional quality because of their ability to produce PUFA. As a response to the increase in food quality, when diet consisted of these autotrophic diatoms, chironomids experienced better reproduction rate. With increased reproductive success in chironomids and their overall health, a more efficient energy and nutrient transfer from the base of the food chain to higher trophic level predators (e.g. fish) that feed on chironomids is taking place. On the other hand, detrital feeding consists of mostly bacteria resulted in a lower efficiency of energy transfer across trophic levels. Evident by the accumulation of isoheptadecanoic acid (*i*-17:0) in

chironomids, there was little nutritional value and no impact on optimizing their growth and development. A similar assessment approach was applied in this study where *H. azteca* was used as a test organism to feed on a biofilm-rich system; the FA profile of *H. azteca* should reveal whether biofilms satisfy the nutritional need of the grazing amphipods and, at the same time, provide insight to biofilms' nutritional contribution to the food web.

FA analysis is applicable to various trophic levels in various ecological systems. In a terrestrial system, lipid composition analysis of polychaete annelid (segmented worm) found a broad range of lipid content. Total lipid ranging from 1.6 to 35 μg/mg of wet mass; among the total lipids, 78 to 90% of the lipids are polar lipids. Neutral storage lipids only represent about 5% (Phleger *et al.*, 2005). Looking further in depth at the FA profile, 15 to 19% of the total FA are branched (both *iso* and *anteiso*), odd chain and bacterial specific MUFA that are indicative of strong bacterial dietary input for the annelids. Terrestrial models using earthworm (*Lumbricus terrestris*) as the indicator organism revealed a rather unconventional result; in the feeding experiment, *L. terrestris*' FA profile resembled more toward its intestinal microbiota rather than the microbiota residing within the ingested bulk soil (Sampedro *et al.*, 2006). It appears that the symbiotic intestinal microbiota metabolizes the foodstuff in the bulk soil diet into secondary metabolites, which can then be readily utilized by *L. terrestris*. Thus, the intestinal microbes serve an important role in providing nutrient for *L. terrestris*.

In the trophic interaction of the below-ground system, feeding strategies of the arthropod *Collembola* was determined using FA analysis. Vaccenic FA (ω-7) was the signature FA indicative of bacterial diet. In addition, the presence of branched and cyclopropane FA correlated to the consumption of a greater portion of Gram positive and Gram negative bacteria, respectively. Greater amounts of linoleic acid (18:2n6) in *Collembola* was observed when they

were fed with fungal diets, oleic acid (18:1n9) when they were fed with plants and 20:1n9 when their diet consisted of nematodes (Ruess *et al.*, 2005). Thus, *Collembola* FA profiles correspond to the dietary FA, and the signature FA were evidently conserved, allowing the determination of dietary composition through FA analysis.

Biogeochemistry involves the circulation of carbon, nitrogen and other elements between cells of living organism and their environment. Fecal pellet of zooplankton is a source of rapid vertical transfer and recycling of these biogeochemical elements (Itoh *et al.*, 2007). FA composition analysis was able to indirectly evaluate the state of decomposition of egested fecal pellets in the sediment, provided strong evidence to support the role of microbial decomposition. As the fecal pellets precipitate, the proportion of unsaturated FA rapidly decline, and increase in odd chain and branched-chain FA was observed; implying an increase in bacterial biomass colonization in the fecal pellets.

2.3 Bacterial Fatty Acid Synthesis

Synthesis of SAFA is analogous among all organisms. Animals, plants and bacteria use acetyl-CoA as the universal substrate and synthesis is carried out by an enzyme complex called the FA synthase system, which consists of a series of mono-functional polypeptides (Kaneda, 1991; Thompson, 1992). Synthesis begins with acetyl-CoA carboxylation to form molonyl-CoA. This conversion is a key regulatory step as it commits the acetyl-CoA unit to the production of FA. Regulation of FA synthesis in animals function to facilitate fat production, therefore FA synthesis is activated by an abundance of citrate and inhibited by long chain FA derivatives (Thompson, 1992). Regulation in bacteria however, is controlled by amino acid availability and is more closely coupled to cell growth (Polakis *et al.*, 1973). Deprivation of amino acid lead to

the production of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) which ceases normal protein synthesis. This guanosine derivative has inhibitory effects on cellular activities such as RNA, carbohydrate and phospholipid synthesis, as well as acetyl-CoA carboxylases that are crucial in the initiation of FA synthesis.

Upon activation of acetyl-CoA by binding with the acyl carrier protein (ACP), acetyl-CoA is transferred to the thiol group in the activation site of the FA synthase complex. A molonyl-CoA unit is then recruited and linked to the activated acetyl-CoA. Followed with reduction with ketoacyl-ACP reductase, dehydration with hydroxyacyl-ACP dehydrase and a second reduction with enoyl-ACP reductase forms a hydrocarbon chain by extension of two carbon units. As the hydrocarbon chain continue to grow with repeated addition of molonyl-CoA units, increased chain length reduces its binding affinity to the synthase complex, thereby lead to the termination of FA synthesis. Regulation of the FA synthase complex is determined by the presence of phosphate compounds; an abundance of glucose derivatives such as glucose-1-phosphate, glucose-6-phosphate and fructose-1, 6-diphosphate activates FA synthase. In addition, FA synthesis in *Escherichia coli* is closely coupled with the formation of phospholipids and generally no acetyl-CoA derivatives or free FA are produced under optimal growth conditions (van den Bosch and Vagelos, 1970).

There are two distinct mechanisms of FA desaturation in bacteria: under aerobic mechanism, MUFA synthesis is very similar to mammals or plants; fatty acid synthase I catalyzes the synthesis of straight chain saturated FA, followed by insertion of double bond into the complete saturated FA via desaturase enzyme with oxygen as the electron acceptor. Only one double bond can be inserted though this mechanism and the position vary depending on the species specific desaturase enzyme. *Mycobacterium phlei* and *Bacillus megaterium* for example

has desaturases specific for palmitate at ω-10 and ω-5, respectively (Fulco, 1970). Desaturase synthesis is stalled and their activities are irreversibly lost with increasing temperature (Fulco, 1983). With regulation at both the transcriptional and translational level, the proportion of unsaturated FA can rapidly shift to accommodate an increase in temperature. Desaturase synthesis can also resume rapidly when the condition shift from high to lower temperature, thereby increasing the membrane fluidity with greater proportion of unsaturated FA.

Alternatively, anaerobic desaturation mechanism uses hydroxydecanoyl-ACP dehydrase of type II FA synthase that has the ability to also isomerize *trans* to *cis* unsaturates, therefore directly produce *cis*-unsaturated FA without completing the synthesis of saturated FA nor require insertion of double bonds via desaturase (Garwin *et al.*, 1980). The dehydration step during synthesis of C10:0 saturated FA is the commitment step at which the double bond at the ω-7 position is introduced. Further elongation produces *cis*-palmitic acid (16:1n7c) and *cis*-vaccenic acid (18:1n7c), which are abundant in bacterial organisms (Russell and Nichols, 1999; Thompson, 1992). The enzymatic activity between type II FA synthase and type I FA synthase can regulate membrane FA composition in bacteria. Type II FA synthases are less susceptible to cold inactivation and preferentially elongate unsaturated substrates than type I FA synthase. As growth temperature decrease, enzymatic activity of FA synthase II will remain relatively more active than FA synthase I; this result in an increase in the average FA chain length and unsaturation that improve membrane fluidity to accommodate lowered temperature conditions (Figure 2.1) (de Mendoza and Cronan, 1983).

Branched-chain FA in Gram-positive bacteria have similar fluidizing effects on membranes that are analogous to that exerted by *cis*-unsaturated FA, and *Anteiso*-branched FA generates greater membrane fluidity than *iso*-branched FA (Silbert *et al.*, 1973). Regulation of

branched-chain FA production appears to be substrate dependent (Buckner *et al.*, 1978); higher availability of malonyl- and acetyl-CoA will induce greater amount of saturated palmitic acid (16:0) diluting the proportion of branched FA. Level of branched-chain derivatives can also be induced when bacteria cells are grown in presence of isovalerate (Conner and Reilly, 1975). Perhaps amount of branched-chain FA can correlate to nutrient availability rather than regulated by the physical need for a more fluidic membrane composition.

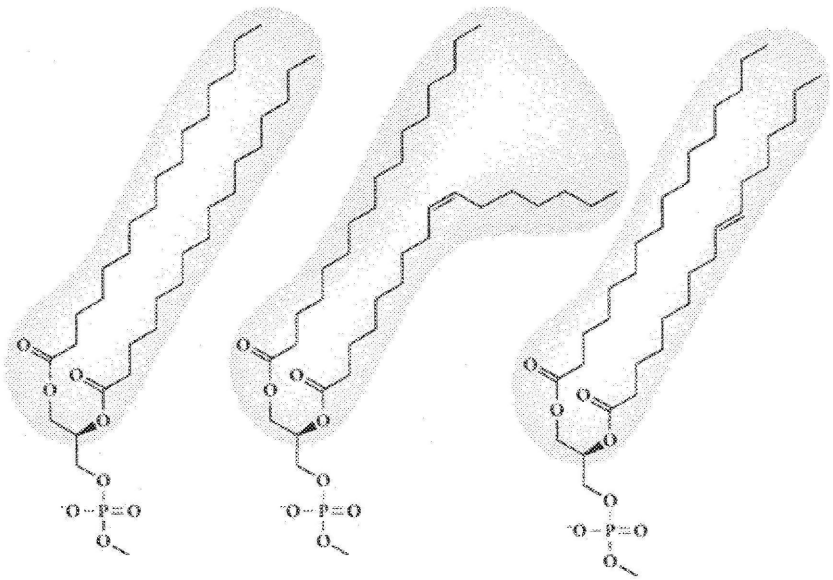


Figure 2.1: Comparison of phospholipid containing (From the left) only SAFA, with one *cis*-MUFA 16:1n7c and one *trans*-MUFA 16:1n7t. Shaded areas indicate the molecular volume occupied by the phospholipid acyl chain. Note how the *cis*-MUFA generated a kink in the molecular structure that will create a more flexible membrane bilayer with the loosely packed structure than SAFA. *Trans*-MUFA isomer has biophysical properties similar to SAFA. Figure modified from Zhang and Rock (2008).

Cyclopropane FA can be found in both Gram-positive and Gram-negative bacteria, but mostly Gram-negative. Cyclopropane FA is formed by cyclopropane synthetase enzyme transferring the methyl group from S-adenosylmethionine to phospholipid-bound MUFA. Cyclopropane FA are present at only trace amounts of in *E. coli* during logarithmic growth phase; however, it increases drastically when the cells shift to stationary phase. As a result, the final

amount of cyclopropane FA in the stationary phase appeared to be dependent on the level of mono-unsaturated FA attained during logarithmic growth (Cronan *et al.*, 1974).

2.4 Fatty acids in bacteria

Classification of microorganisms can be based on their morphological structures, metabolic, serological and toxigenic responses. Conventional methods utilize various biochemical tests to assess these various phenotypic features to make appropriate identification. The difficulties with these methods are the extensive culturing process often required to generate pure culture of species of interest. In addition, optimal growth conditions may be difficult to simulate in laboratory conditions and often leads to gross under-estimation of organism due to this bias of cultural selection. Incubation period for the biochemical tests can also be time consuming, even then, biochemical tests can result in similar reactions between different genuses.

As early as the 1970's, total cellular FA analyses had been proposed as a possible alternative method to provide additional criteria for rapid identification of bacterial cultures (Wayne-Moss and Dee, 1975; Wayne-Moss, 1981). Spore forming food-borne pathogenic *Bacillus* sp. and *Legionella* sp. were always difficult to distinguish by biochemical tests, but their FA profile has distinctive features. *Legionella pneumophila* colonization on copper surfaces can be monitored by the unique 2, 3-dihydroxy FA in their lipopolysaccharide (LPS) (Walker *et al.*, 1993).

Numerous studies have documented the FA profiles of various bacterial species (O'Leary, 1962; John & Perry, 1977; Nichols, 2003; Whittaker *et al.*, 2007). The amount of FA in most bacteria ranges between 2 to 8% of total dry weight (Gillan & Johns, 1986) and the majority of FA (70-90%) in bacteria reside as the acyl constituent of cell membrane phospholipids (Lennarz,

1966; Kaneda, 1991; Nichols & Mancuso-Nichols, 2008). Contrary to FA analysis in higher trophic organisms, prokaryotic organisms are single-celled with no internal compartments. Consequently, their cell membrane is one of the most important components regulating their cellular physiology. It has further been shown that their phospholipid fatty acids (PLFA) can be strongly influenced by environmental conditions such as temperature (Silbert *et al.*, 1973; de Mendoza and Cronan, 1983; Dubois-Brissonnet *et al.*, 2000; Nichols, 2003; Zhu *et al.*, 2005; Zhang and Rock, 2008), pH (Giotis *et al.*, 2007), salinity (Komaratat and Kate, 1975) hydrostatic pressure (DeLong and Yayanos, 1985) and organic solvents exposure (Mrozik *et al.*, 2004; Nielsen *et al.*, 2005).

FA composition in the membrane influences the membrane fluidity and flexibility, which directly contribute to the efficiency of molecule/ion exchange for the bacterial cells. FA constituents in the bacterial membrane phospholipids can correlate to their phenotypic properties. For example, the pathogenic strain of *Streptococcus pneumoniae* has a greater proportion of MUFA; the resulting phenotype has visually more transparent cellular membranes with increased fluidity/flexibility that may assist bacteria attachment and enhance pathogenicity of the particular strain (Aricha *et al.*, 2004). Similarly, given the host cell membrane plays a central role in virus life cycle, membrane composition of bacteria PLFA are likely to affect their susceptibility to bacteriophages as well (Kaneda, 1991).

Hydroxy FA are an integral part of the lipopolysaccharides (LPS) of Gram negative bacteria. 3-OH hydroxyl FA is part of the structural component in the lipid-A portion of the LPS that anchors the polysaccharide on the exterior of outer membrane (Figure 2.2). Gram negative bacteria also have more total FA compare to Gram positive bacteria because of the fact that they have a double membrane. During the stationary phase of microbial growth cycle, the proportion

of hydroxyl FA was found to be slightly lowered from ~18% of total FA to 12%, the reason for this response was unclear (Härtig *et al.*, 2005).

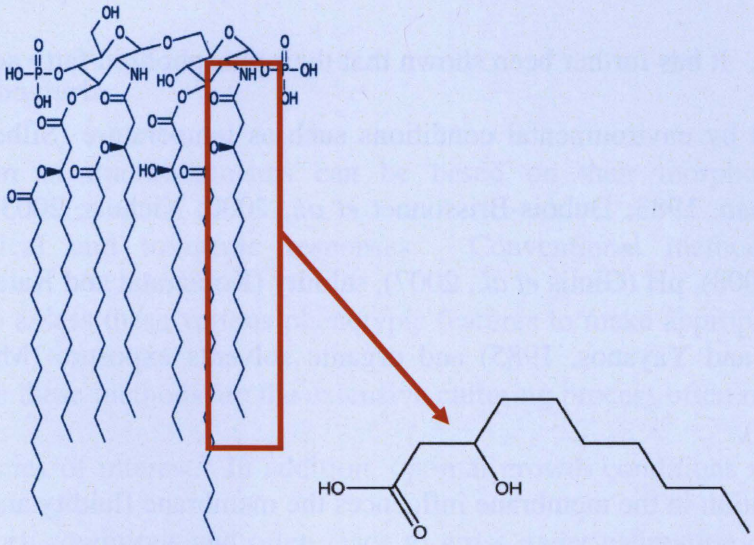


Figure 2.2: Structure of lipid-A of LPS (Left). Hydroxy FA 3-hydroxydecanoic acid; 3-OH 10:0 derived from component of lipid-A (Right). Image modified from Matreya biochemical (http://www.matreya.com)

Iso- and *anteiso*-branched FA are predominantly found in Gram positive bacteria; more than 50% of the total FA in *Bacillus* and *Staphylococcus* species is composed of branched FA (O'Leary, 1962; Keinane *et al.*, 2002; Denich *et al.*, 2003). Branched chain SAFA in Gram positive bacteria provide the mechanism of controlling membrane fluidity as MUFA do in Gram negative bacteria. For example, Gram positive *Listeria monocytogenes* are able to thrive at low temperature because of the greater membrane fluidity provided by the higher proportion of branched chain FA (Zhu *et al.*, 2005). Gram positive *Bacillus subtilis* actually fail to grow when the amount of branched chain FA falls below 28% (Kaneda, 1991). In addition, *anteiso-* and shorter chain FA (C14 and C15) are typically more abundant at lower growth temperature to increase membrane fluidity whereas *iso-* and longer C16 to C18 FA chains that tend to make the membrane more rigid are more abundant at higher temperatures (Zhang and Rock, 2008). Due

to biosynthetic restriction, even numbered *anteiso*-FA are extremely rare, and pairs of saturated branch FA with differences of two carbons can generally be detected in bacteria, for example *i*-15:0 and *i*-17:0 (Kaneda, 1991).

Most bacterial species generally either have hydroxy FA or branched FA, but not both. An exception to this can be found in *Bacteroides fragilis* that possesses the unique branched-chain hydroxyl FA; 3-hydroy-15-methylhexadecanoic acid (3-OH *i*-16:0). In addition, mid-chain branched FA (10-methyl-16:0, 10-methyl-18:0) are characteristics of actinomycetes. Multi-methyl branched FA are common in mycobacterium (Smith *et al.*, 2000) and alkyl ether polar lipids are prominent in Archae (White *et al.*, 1998).

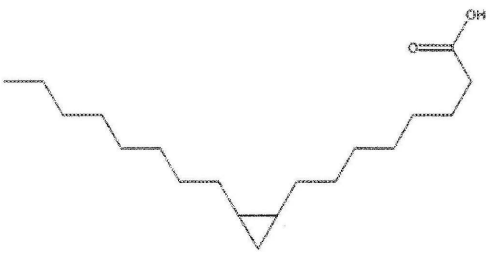


Figure 2.3: Cyclopropane FA cis-9, 10-methyleneoctadecanoic acid; cyc-19:0 (9, 10). Image adopted from Matreya biochemical (http://www.matreya.com)

Another family of FA typically found in bacteria is the cyclopropane FA (Figure 2.3). While phospholipid containing cyclopropane FA has similar biophysical property as *cis*-MUFA, cyclopropane bond is more stable than double bond. Cyclopropane FA stabilizes the phospholipids against turnover and degradation, therefore believed to enhance the viability of slow-growing cells in hostile conditions (Zhang and Rock, 2008). For example, an increase in the proportion of cyclopropane PLFA (cyc-17:0, cyc-19:0) over MUFA (16:1n-7, 18:1n7) was observed during the stationary phase and when the culture was subjected to starvation (*Smith et al.*, 2000; Piotrowska-Seget & Mrozik, 2003; Whittaker *et al.*, 2005). This conversion in the FA profile demonstrates an adaptive response that minimizes membrane degradation under stress

conditions and starvation in order to improve survival rate of the bacterial cells. FA with a terminal cyclic acyl group are characteristic of acidothermophilic *Bacilli* (Zayed, 2004).

PUFA are generally rare in bacterial species. It is reasonable to suggest the detection of PUFA can often be a good indicator for presence of micro-eukaryotes in the sample. Micro-eukaryotes such as algae, zooplanktons and diatoms are major source of PUFA in the aquatic food web because of their ability to synthesize PUFA *de novo* (Arts *et al.*, 2001). However, it became evident there are exceptions; marine extremophiles, deep sea psycrophilic and barophilic bacteria had been confirmed to have the metabolic capacity to synthesize PUFA such as EPA and DHA as well (Yano *et al.*, 1997; Russell & Nichols, 1999; Denich *et al.*, 2003). The EPA and DHA in these organisms may constitute up to 31% and 21% of the total FA, respectively. PUFA's contribution to membrane fluidity is believed to play a significant role in their adaptation to the extreme conditions. In the pelagic ecosystem, PUFA-producing prokaryotes may have roles beyond nutrient cycling and degradation of organic matter; they are potentially an appreciable source of dietary essential fatty acid (EFA) for marine organisms lacking the *de novo* mechanisms to produce them (Nichols, 2003). Although freshwater microbiota may not have the comparable PUFA production, it does illustrate bacterial FA contribution as dietary substance and its transfer in the aquatic food web. While the production of PUFA from algae has been extensively studied, the depth and scope of the bacterial contribution to FA in freshwater food webs is less well understood.

PUFA such as EPA were also shown to have antimicrobial activity against common food borne spoilage pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Shin *et al.*, 2007). Cultures containing 62.5 µg/mL of EPA were sufficient to cause decline in the cell count. Scanning Electron Microscopy (SEM) experiments found wrinkled abnormalities on the

outer membrane of *P. aeruginosa*. The presence of EPA presumably disrupts the structural integrity of bacterial membrane thus exerting a bactericidal effect.

FA in bacteria can exist as a glycolipid component of poly-β-hydroxyalkanoate (PHA). PHA is a carbon and energy storage molecule for bacterial cell that acts as the equivalent of fat in mammalian organism, which is typically synthesized when carbon is abundant (Ayub *et al.*, 2006). Excessive accumulation of PHA can be attributed to the lack of specific essential nutrients necessary to proceed with cell division (White *et al.*, 1998). Energy storage is not considered the primary function of lipids in bacteria; amino acid, peptide and/or carbohydrate are the endogenous reserve material. Neutral lipid constitute minor portion of the total bacterial lipids (Lennarz, 1966).

With increasing understanding in the quantitative and qualitative distribution of FA of different microbial species, comparing the FA profile of biofilms can also be used to assess their nutritional status and community structure. However, further interpretation to identify the members within the community must precede with caution as most bacterial members lack unique FA signatures. Multivariate principal-components analysis of FA profile can generate a clear separation of two different microbial communities, but detailed interpretation of specific community taxonomy still requires further knowledge of FA distribution across a wide range of bacterial taxa (Kidd-Haack *et al.*, 1994; Karczmarski *et al.*, 2002; Antunes *et al.*, 2008) and the effects of growth conditions on their FA composition (de Mendoza *et al.*, 1983; Dubois-Brissonnet *et al.*, 2000; Mozik *et al.*, 2004; Nielson *et al.*, 2005).

2.5 Environmental effects on bacterial membrane fatty acid profiles

Environmental perturbation can upset the optimal physical properties of cell membranes therefore, compensatory adaptive changes will be necessary to modify the membrane to adapt to environmental changes. As prokaryotes lack internal compartments, membrane modification is crucial in maintaining internal cellular properties when it comes to dealing with environmental exposures like temperature, inorganic ions and chemicals (Lennarz, 1966; Thompson, 1992).

Bacillus megaterium membrane lipid does not contain unsaturated FA when grown at 35°C. Transferring them to 20°C induced the production of ω-5 desaturase and caused a rapid increase in amount of MUFA; this observation was reversed when they are returned to high temperature (Fulco, 1972). Psychrophilic *Micrococcus cryphilus* responded to lower temperature by decreasing the FA chain length; the ratio of 18:0 to 16:0 decreased from 3 to 1. It was observed that C2 unit from 18:0 was selectively removed to elongate 14:0 intermediate in order to produce more 16:0 (Russell, 1984). Increase in pressure also showed a similar response to decrease in temperature, as observed in deep sea bacterium *Vibria sp.* (DeLong and Yayanos, 1985).

Multi-cellular organisms ensure constant ionic conditions with an efficient system of ion channels and pumps. The effect of inorganic ions and salts on membrane lipid is likely to have a more explicit effect on unicellular bacteria. Divalent cations such as calcium or magnesium may interact with the negatively charged portion of the phospholipid, creating a more rigid membrane by linking two adjacent phospholipid molecules. *Staphylococcus aureus* exposed to 10% NaCl have increased cardiolipin phospholipid from 10% to 50%, decreased phosphatidylglycerol and lysophophatidylglycerol phospholipid. However, the overall FA profile in the phospholipids showed little change (Yasuhiro *et al.*, 1972). Halotolerant *Staphylococcus epidermidis*

demonstrated less drastic difference in the FA content and little change in cardiolipin was observed until the exposure reached 25% NaCl (Komaratat and Kates, 1975).

E. coli grown in the presence of alcohol with 5-10 carbon length showed higher level of SAFA. Alternatively, the presence of short chain alcohols such as ethanol has shown to increase the amount of unsaturation in its FA profile. The amount of vaccenic acid (18:1n7) in *E. coli* had been found to increase at the expense of saturated FA (Ingram, 1976). *Tetrahymena pyriformis* in 1.6% ethanol were found to have decreased amount of palmitoleic acids (16:1n7) from 23 to 5% and linoleic acid (LIN; 18:2n6) increased from 14 to 25%. Ultimately, ethanol induces a more fluidic membrane than normal cells; however it is uncertain whether it is a homeoviscous adaptation to counter the effect of ethanol or this response was merely manifestation of ethanol toxicity on the FA synthesis enzymes (Nandini-Kishore *et al.*, 1979).

2.6 Fatty acids in biofilms

A biofilm can be defined as a community of microorganisms accumulated at interfaces and are typically held together by a matrix of extracellular polymeric substance (EPS). It is a mode of growth for microorganisms as a multi-cellular community that allows for specialization and cooperation between individual cells (Denkhaus *et al.*, 2007). Multiple layers of cells along with dense EPS provide a protective barrier against physical stress and resistance against anti-microbial treatments, making them a problematic issue in environmental and health-related fields. Although biofilms are often associated with bacteria because of their ability to produce EPS and initiate biofilm formation, biofilm may contain different types of organism such as algae, protozoa and fungi.

Biofilms are an important link of the food web because they coat virtually every surface in aquatic systems and are heavily grazed by aquatic invertebrates. Recent evidence found higher vertebrates feeding on biofilm as well, this suggest biofilms may have more direct influence on the higher trophic species than originally expected and greater importance of biofilms to ecosystem processes. For example, macrofauna (prey > 0.5 mm) alone is not a complete diet for Western Sandpipers, *Calidris mauri*. Video images of feeding behavior, stomach content and stable isotope analysis displayed surficial intertidal biofilm grazing that accounted up to 60% of their total diet; which work out to be nearly 50% of their daily energy budget (Kuwae *et al.*, 2008). From the lipid and FA perspective, bacterial biofilm is generally considered of low nutritional value, but the rich source of carbohydrate in EPS is perhaps a good immediate source of consumable energy. The ubiquitous presence of biofilms in large quantity may also compensate for its low nutritional value. On a separate note, a Sandpiper can ingest up to 190 g wet mass of biofilm material per day; therefore an average flock of 100,000 Sand pipers can consume an immense amount of biofilm. Potential threats to microbial biofilm due to coastal development, atmospheric changes or competitive pressure from invasive foreign species could potentially disrupt biofilm availability as food source for local invertebrates, as well as higher vertebrates like the Western Sandpipers.

Considerable efforts are devoted to correlating the genetic expression of the biofilm with the planktonic stage of the bacterial life cycle (Kuchma & O'Toole, 2000; Mack *et al.*, 2000; Blankenship & Mitchell, 2006; Perry *et al.*, 2008; Tsang *et al.*, 2008). It has been confirmed that bacteria in the biofilm community are very different in terms of their behavior, metabolism and gene expressions when compared to the same organism in the planktonic state (O'Toole *et al.*, 2000). Biofilms tend to have a reduced growth rate and different gene expression when

compared to their planktonic counterparts (Denkhaus *et al.*, 2007). Change in genetic expression during biofilm formation is often related to adhesion, cell-surface and/or cell-cell interaction phenotypic properties. For instance, the *pgaABCD* operon of *Escherichia coli* expression is responsible for the production of adhesion protein in biofilms (Goller *et al.*, 2006). Also, the 15-gene *psl* operon encodes for polysaccharide has important role in *P. aeruginosa* initial adhesion and maintaining biofilm structure (Ma *et al.*, 2006). The cell membrane is the vital barrier that control extracellular and intracellular interaction in bacteria playing a central role in the complex regulation of planktonic-biofilm transition. It is reasonable to suspect the membrane FA profile will differ as a result of change in genetic expression; shifts in metabolic rate should also affect the FA profile in other lipid types. This comparison is rarely considered in most biofilm studies.

Bacterial FA analysis in the context of biofilm had been looked at in several studies; analysis of phospholipid FA in particular is an effective method to characterize biofilm community composition. Droppo *et al.* (2007) utilized phospholipid FA analysis as one of the criteria to assess the contribution of microbial community to biofilm stability, and ultimately determined that the continuous biofilm development and decay were linked to the temporal oscillation of sediment stability in freshwater environment exposed to shear stress.

Lipid analysis on biofilms enabled detection of shifts in the biofilm community structure induced by stress of amphipod grazing. Increased short chain and branched chain saturated FA suggests that the microeukaryotic community in the biofilm was replaced with a bacterial dominated community containing more EPS (White & Findlay, 1988). Analyzing components of cells (in this case the lipid) and/or EPS provide a chemical measure of biomass, nutritional status and metabolic activity of the biofilm; viable biomass of the biofilm can be measured by phospholipids because they are rapidly hydrolyzed and lost upon cell death. This technique is an

alternative to indirect methods such as ATP analysis or direct cell count. Comparing the ratio of PLFA to PHA can relate viable biomass to the amount of nutrient available.

FA profile comparison between glass adhering *Listeria monocytogenes* cells and planktonic cells found variability in the proportion of branched chain FA. Planktonic cells showed greater proportion of *iso-* and *anteiso*-15:0 and 17:0 (Gianotti *et al.*, 2008). Abundance of branched FA was previously acknowledged to contribute to the increased membrane fluidity that supports the psychrotrophic nature of the Gram positive *L. monocytogenes*. However, membrane FA composition rapidly changed to nearly total prevalence of straight chain FA during initial phases of biofilm formation. This elevated rigidity caused by the straight chain FA was suspected to be the response to provide membrane phospholipid structural stability along with other membrane proteins to support contact with adhesion surfaces. Comparison of PLFA in attached *Pseudomonas atlantica* cells to free-flowing cells in flow chambers also found greater proportion of SAFA (44.5% from 35.6%), *trans*-MUFA (12.7% from 4.1%) and lowered *cis*-MUFA (41.2% from 58.7%) (Tunlid *et al.*, 1989).

Biofilm formation is widely found in drinking water distribution systems, its formation is a cause for concern because it can protect pathogenic organisms from water treatment strategies, at the same time biofilm is a potential inoculum for periodic re-infestations. FA analysis has been shown to be an effective alternative to assess water conditions. Disinfection by chlorine causes formation of epoxide FA from monounsaturated phospholipid FA in bacteria. Epoxide FA are normally not found in bacteria, thus a potential lipid biomarker to assess chlorine content in the water (Smith *et al.*, 2000). Another study of biofilm in drinking water by Keinane *et al.* (2002) observed that greater availability of phosphorus in the system lead to increased proportion of 3-OH FA; signifying changes in the biofilm community towards Gram negative bacteria.

When FA profiles of water samples containing planktonic bacteria was compared with the biofilm, the planktonic cells had lower percentage of saturated FA, which coincide with the situation observed in *L. monocytogenes*. The authors also speculated that the differences may be due to selection pressure favoring the more rigid saturated FA to provide structural integrity in the biofilm. Presence of EPS or the possibility of it contributing to the FA profile was not mentioned.

The effectiveness of remediation strategies in contaminated soil has been determined by monitoring the progression of microbial community structure; a similar assessment strategy should also be applicable in the aquatic ecosystems. White *et al.* (1998) established the correlation between contaminant/toxicity disappearances to the return of viable biomass, community composition and nutritional status. A combination of signature lipid biomarker analysis and nucleic acid probes were used as molecular 'fingerprints' to monitor microbial community development and metabolic activity.

Microbial FA analysis can also be applied on biofilms involved in industrial bioprocesses: With increasing prevalence of using bioreactors to assist production of bio-fuel, cosmetic, food and etc., there is the necessity for quick and accurate surveillance methods to maintain culture consistency. Using cultures capable of forming biofilm allows for a self-maintaining continuous reactor, but biofilm can also be a contaminant source in the production pipeline. FA analysis had been applied to characterizing the biofilm community in a beer brewery bottling plant, ensuring the bioreactor culture remain consistent and free of spoiler growth contamination (Timke *et al.*, 2005).

2.7 Fatty acid in extracellular polymeric substances

Extracellular Polymeric Substances (EPS) are gel-like, highly hydrated extracellular material that can be removed from microorganism without disrupting the cells (Lazarova & Manem, 1995). EPS makes up 50-90% of total organic matter in biofilm and is involved in the determination of biofilm structural, functional integrity and organization of the biofilm community. It has an important role in cell adhesion/aggregation, cell-to-cell communication and provides a protective barrier (Zhang & Fang, 2001; Denkhaus *et al.*, 2007). Large quantities of EPS can be found during stationary phase and/or when nutrients such as nitrogen, phosphorus, sulfur and potassium are limited (Sutherland, 1982; Manca *et al.*, 1996). The amount of EPS is also enhanced by physical factors like osmotic stress and temperature (Nichols *et al.*, 2005). Production of EPS requires a significant metabolic investment, which may appear to be counter-productive in situations where EPS is more abundant, but the long term protective advantage should offset the cost to the organisms. EPS is composed of a wide variety of organic materials such as polysaccharides, proteins, nucleic acids and lipids; among which polysaccharides is the dominant component (≥65% of total EPS). These organic materials may be the result of active secretion from the cells, cell lysis and/or shredding of cell surface material. However, the precise constituents and the contribution of each component in the EPS remain uncertain. This may be attributed to its dependency on specific microorganisms and the environmental condition they are exposed to, as well as the lack of efficient techniques to isolate and study EPS (Zhang & Fang, 2001).

In the attempt to examine the effect of temperature on activated sludge EPS extraction procedures by Goodwin & Forster (1985), lipids were observed in the form of triglyceride and acyl esters. The presence of lipid or FA in EPS has rarely been considered in biofilm research

to-date; finding evidence to suggest EPS consist of FA will open a new and unknown area of biofilm research, prompting speculations on the source of lipids and FA in EPS, its role in the EPS matrix, overall biofilm integrity, cell-to-cell signaling and its nutritional relevance to the ecosystem.

A recent study by Davies and Marques (2009) proposed the presence of a cell-to-cell extracellular signaling molecule structurally similar to *cis*-2-decenoic acid (10:1n8). Addition of extracellular 10:1n8 to *P. aeruginosa* biofilm cultures were able to encourage cell dispersion in silicon tube reactors. Dispersion mechanism was believed to be a response to overcrowding or starvation within the biofilm population, this allows migration to a more suitable environment and thin out the original population. Biofilm developmental conditions can thus potentially be monitored by the extracellular FA derived from signaling molecules. Specific mechanism of action of 10:1n8 was not available, but it may be reasonable to suspect this FA homologous signaling molecule would accumulate in batch planktonic cultures. Therefore, biofilm cultures may lack specific FA markers compared to planktonic cultures.

Rhamnolipids are extracellular amphiphilic molecules characteristic of *P. aeruginosa*, they are constructed of fatty acid moiety and rhamnose sugars. Rhamnolipids without sugar moiety are called 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), which are also released by bacteria (Figure 2.4). Alkyl chains of rhamnolipid and HAA range from C8 to C12 and with single unsaturation in some cases, but saturated C10 is most common (Soberón-Chávez *et al.*, 2005). Natural functions of rhamnolipids are still highly speculative, but they have been suggested to act as surfactant to solubilize and promote hydrophobic substrate uptake and enhance lipidic signaling molecule-mediated cell-to-cell communication. More recently, both rhamnolipid and HAA had been shown to participate in the swarming motility related to biofilm

development and it structural formation (Lequette and Greenberg, 2005). Perhaps 3-OH hydroxy FA derived from rhamnolipid and HAA can be found in excess in the extracellular component of biofilm culture.

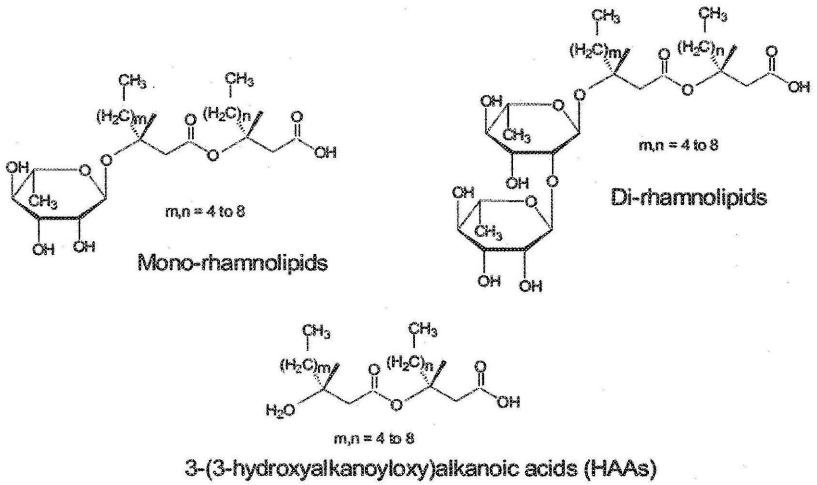


Figure 2.4: Chemical structure of rhamnolipids and HAA. Figure adopted from Soberón-Chávez *et al.* (2005)

Currently there is no single universally standardized method of EPS separation. Mild extraction methods are unable to efficiently extract all EPS, whereas harsh extraction method may rupture the cells and contaminate EPS extract with intracellular materials. The harsh condition may also cause deterioration to the molecule of interest in the EPS. For example, the study by Liu *et al.* (2002) to extract EPS from sludge samples found the highest amount of DNA when EDTA was used. It was suspected that EDTA's cation binding property is more likely to disrupt cell membranes, causing cell lysis and increasing the amount of cellular DNA in the EPS extract. So it is rather challenging to be certain whether trace components such as DNA, and perhaps lipid found in EPS extracts, are truly part of the EPS component, or simply an artifact caused by the various extraction methods. Glucose-6-phosphate-dehydrogenase (strictly intracellular enzyme) assay had been suggested to test for cell integrity and detect cytosol leak after EPS extraction (Liu & Fang, 2002). EPS separation techniques commonly involve physical

and/or chemical means. The common physical isolation method uses high speed centrifugation or ultrasonification. NaCl and formaldehyde solvent can be added to enhance efficiency of EPS extraction (Mojica *et al.*, 2001). Chemical isolation methods can be accomplished using solvent mixtures such as NaOH-EDTA, ethanol-water, phenol-water or by chromatography through ion-exchange resin (McSwain *et al.*, 2005; Zhang & Fang, 2001). Even then, for the purpose of analyzing FA in EPS, there are no evidence in literature that guarantee the preservation of lipid for further FA analysis and proper separation of EPS at the same time.

2.8 *Hyalella azteca* – natural indicator organism

Hyalella azteca (aka scuds) are freshwater epibenthic amphipods. Their length ranges from 3 to 8 mm and their entire life cycle is carried out beneath the water surface with no pupal stage. Development of *H. azteca* can be categorized into 4 stages: juvenile, adolescent, nuptial and adult (Geisler, 1944). External secondary characteristics begin to develop at the adolescent stage; where the male develops enlarged subchelate gnathopods and lamella brood pouch in the female. Nuptial stage is considered sexually mature. Sexual maturation begins at about 24 d (Nelson and Brunson, 1995), but development also depends on other environmental conditions such as space and food. Individually raised *H. azteca* were found to have retarded growth rate that were smaller in size and fail to develop appropriate secondary characteristics at 67 d old (Geisler, 1944).

H. azteca are omnivorous deposit-feeding surface grazers that feed on filamentous algae, fungi and other decaying organic materials over almost any surfaces (Hargrave, 1970). However, there are suggestions of more preferential feeding on bacteria and algae that adhere to sediment particles (Environment Canada, 1997). Schmitz and Scherrey (1983) provided a well described

study on *H. azteca* digestive system: The digestive tract is straight and dived into three sections; foregut, midgut and hindgut. The foregut consists of crushing and pressing mechanisms to break down food particles. The midgut consists of nine caeca; four anterior caeca called hepatopancreatic caeca are the primary sites of digestion and absorption. The hindgut contains a folded wall that contract and expand to a pumping action that takes in water to flush fecal material out of the anus.

Historically classification by morphology suggested *H. azteca* was a single species, but recent genetic analysis have revealed there are at least seven different genetic divergences, but with little morphological differentiation (Witt & Hebert, 2000). They are the most broadly distributed amphipod in North America. The widespread distribution and close association with freshwater sediment makes *H. azteca* an ideal model organisms for ecological studies. In addition, *H. azteca* has high fecundity and brood size; producing multiple generations per year with average of 15 broods over a 5 months period. Containing appreciable amount of SAFA and PUFA, *Hyalella* are a vital source of essential fatty acids for higher freshwater organisms like many species of fish, waterfowl, wading bird and other large invertebrates; therefore, an important link in food web of North American freshwater ecosystems.

H. azteca are widely used as bioassay tool to assess environmental condition, water, sediment quality and contamination (Borgmann *et al.*, 2007). To determine the effectiveness of anaerobic degradation eliminating oil contamination in sediment, viability of *H. azteca* at the remediation site was one of the assessment criteria (Li *et al.*, 2007). *H. azteca*'s survival rate had been used to measure chronic toxicity of the biocide tributyltin in freshwater samples (Bartlett *et al.*, 2004). Indirect effect of chronic copper exposure in aquatic food web was monitored using *H. azteca*. While survival and reproduction of *H. azteca* were greatly weakened by high aqueous

copper concentration, their molecular compositions such as proteins and total lipid did not appear to be significantly effected (Morris *et al.*, 2003). Traditionally, toxicity/contaminant response studies with *H. azteca* use physical growth, development and reproductive success as evaluation parameters (Nelson and Brunson, 1995). Some have also used biochemical composition (i.e. protein, glycogen and lipid) to assess the health and condition of model amphipods (Dutra *et al.*, 2007; Dutra *et al.*, 2008; Gering *et al.*, 2009), but few have considered evaluating specific fatty acid composition, which should also has great potential to assess their nutritional and health status. Although *H. azteca* had been a well defined model organism, information on the stress response associated with starvation was surprisingly scarce. McNulty *et al.* (1999) used the concept of reference toxins to find that *H. azteca* under starvation exhibit greater sensitivity to certain chemical exposure (carbaryl and sodium pentachlorophenate) compared to well-fed organisms. FA analysis provided an alternative criterion to assess its starvation associated stress response.

H. azteca can have more than 0.5 μg of total FA per mg of sample dry weight (Arts *et al.*, 2001). Triacylglycerol (TAG) to phospholipid ratio of *H. azteca* actively feeding on detritus and microbiota is about 0.70; this ratio drop to about 0.13 after a week of starvation, suggesting that lipid and FA profile are indicative of dietary patterns in *H. azteca* (White & Findlay, 1988). *Dikerogammarus villosus* are recent foreign amphipod invaders that thrived in the European freshwater ecosystems, they are suspected to be the next successful foreign invader to the Great lakes region. FA analysis found predominance of palmitic acid (16:0) and oleic acid (18:1n9c) in these amphipods. PUFA such as EPA, linoleic acid (LIN), arachidonic acid (ARA), α-linolenic acid (ALA) and docosahexanoic acid (DHA) were in relatively high amounts as well (Maazouzi *et al.*, 2007). Central and Western European freshwater Gammarids such as

Pontogammarus robustoides and *Dikerogammarus haemobaphes* also showed significant amount of PUFA, particularly ω-3 PUFA that represented up to 11 to 23% of total FA (Kolanowski *et al.*, 2007). The amount of EPA, DPA and DHA in these Gammarids can range up to 16%, 2.4% and 4.2%, respectively.

Further assessment of FA profile in the Gammarids found significantly higher levels of long chain PUFA (EPA and DHA) in the marine species than their freshwater counterparts (Makhutova *et al.*, 2003; Maazouzi *et al.*, 2007). This difference can be explained by the different diet available in the freshwater and marine system, as well as their adaptation to saline condition. The sum of odd number carbon and branched chain FA can be used as indicator of bacterial diet, which was found to be relatively minor in *D. villosus* (Maazouzi *et al.*, 2007). The FA profile in freshwater Gammarids found marked level of bacterial FA markers, representing about 5% of total FA. Bacterial FA markers were absent in marine Gammarids (Makhutova *et al.*, 2003). With such high level of EPA in the amphipods, it has been suggested that farming them may be a potentially innovative source of n-3 PUFA (Kolanowski *et al.*, 2007). Alternative ω-3 PUFA source can counter the rapid depletion of sea fish communities, provide alternative source of rich oil for nutritional, pharmaceutical purposes and animal feed.

Because of *H. azteca*'s surface feeding strategy complements biofilm growth pattern, it is a preferable choice of model indicator organism to study. In addition, they are abundant in the freshwater system, sensitive to their environmental conditions and a vital component in the aquatic food web.

2.9 Time frame of FA profile changes and retention

H. azteca's bacterial FA signal accumulation capacity is yet to be determined. The amount of feeding required to generate discernible changes in the FA profile determines the feasibility of using *H. azteca* as the indicator organism to monitor biofilm community. In order to be certain the FA profile changes are the direct outcome of dietary influence, the time required for *H. azteca* to incorporate its dietary FA signals and the retention time of these signals are also important factors to consider.

In order to be able to accurately describe *H. azteca* FA profile changes according to diet, caution must be taken to ensure the FA signals are not the product of accumulated gut content. Although the gut content contributes to a small amount of total body weight in *H. azteca*, it had been shown to cause significant overestimation in metal contamination tests (Neumann *et al.*, 1999). After feeding trials that include a cadmium spike, cadmium concentration in *H. azteca* typically exhibit a biphasic decline. The first phase of decline was a more rapid loss that lasted from 4 to 6 h corresponding to the effect of gut clearance. The second phase of decline in cadmium concentration was slower, which was the result of gradual excretion from the body. Therefore, depuration time of approximately 6 h should be taken into consideration when experimenting in feeding trials with *H. azteca*. For *Mysis relicta*, a crustacean widely found in the Great Lakes region that are very similar to *H. azteca*, fasting experiment showed that 3 to 6 weeks of controlled laboratory conditions are required to clear all trace of dietary FA to simulate basal FA composition (Schlechtriem *et al.*, 2008). The lengthy dietary FA depuration period may be attributed to the restricted activity and slowed metabolic rate in that controlled feeding experiment.

A feeding study by Hargrave (1970) showed that ingested sediment passes through the gut of *H. azteca* with a rapid rate; producing 18 µg to 20 µg dry weights of feces per h for a 700 µg adult amphipod. This rapid rate suggests *H. azteca* empties its gut twice per h and only small portion of ingested content is truly digested. An adult *H. azteca* has an estimated feeding rate of 21.1 µg of sediment per h, but it can be influenced by the surface texture of the food particle. Further observation through radio-active labeling showed free organic molecules such as cellulose and lignin are not readily digestible, *Hyalella* obtain these molecules by consumption of bacteria that are able to metabolize them, perhaps synonymous to the symbiotic relation between the intestinal microbiota and the earthworm.

Like most amphipod species, *H. azteca* feeds continuously, possibly an indication of non-selective feeding. An alternative example can be seen in *Diporeia* sp., they have intermittent feeding pattern to exploit high quality food available during planktonic blooms in the spring and feed sparingly during other times, demonstrating a more selective feeding pattern (Quigley & Vanderploeg, 1991).

2.10 -Fatty acid extraction and fatty acid methyl ester (FAME) analysis

FA analysis requires isolation of FA from sample tissue and converts it to its ester derivative called fatty acid methyl ester (FAME). This process allows the FA to be easily volatilized in the injector septum of a gas chromatography (GC) machine. If the FA from a specific lipid source is of interest, different lipid types will need to be fractionated by thin-layer chromatography (TLC) prior to derivitization. Identification of FA can be done by comparison to known standards, precise structural information will need confirmation by mass spectroscopy

(Härtig, 2008) and the different isomers (*cis*, *trans*) can be determine using infrared spectroscopy (Kaneda, 1991).

Distinctive FA signatures of different bacterial species combined with advances in gas chromatography technology allow FA analysis to provide an efficient and rapid alternative for routine microbial identification (John & Perry, 1977; Zayed, 2004; Whittaker *et al.*, 2007). For example, *Enterobacter sakazakii* is a Gram-negative food borne pathogen that can potentially cause sepsis, meningitis or enteritis in infants and immune compromised adults. Current identification methods require more than 5 days of multiple enrichment steps and it is not strictly selective for *E. sakazakii*. Cellular FA analysis using gas chromatography (GC) equipped with flame ionization detector (FID) were able to identify the major FA in *E. sakazakii*; these include 12:0, 14:0, 15:0, 16:1, 18:1 and a more unique 17:0*cyc* (7, 8) (Hoffmann *et al.*, 2008).

FID is one of the many detectors available for gas chromatography. By burning and ionizing the analytes to induce electrical current change in the detector, FID is one of the most sensitive detectors of hydrocarbons and organic compounds. Analytes are separated in the GC by their specific retention time on the capillary column. FAME analysis in higher trophic organisms with emphasis on PUFA is best resolved with polar capillary columns. Bacterial FA in comparison typically has no PUFA and consists of short chained saturated FA that is best resolved with a relatively non-polar capillary column. Agilent Ultra-2 (19091B-102) capillary column is relatively non-polar with 0.33 µm thick (5%-phenyl)-methylpolysiloxane stationary phase. Considerably short at 25 m in length, it is the recommended column for bacterial FAME analysis.

Sample preparation based on the method described by Microbial ID Inc. (MIDI, Newark, Delaware, U.S.A.) was widely used in bacterial FAME analyses in literature (Kidd Haack *et al.*,

1994; Schutter & Dick, 2000; Steger *et al.*, 2003; Zhu *et al.*, 2005; Härtig *et al.*, 2005; Hoffmann *et al.*, 2008). The greatest advantage in using this method is its ability to preserve short-chain saturated, branched cyclopropane FA and hydroxyl FA that would otherwise be lost using the typical FAME preparation method that originated from Bligh and Dyer (Bligh & Dyer, 1959) for larger organisms. The MIDI system uses the unique FAME profile and 16S rRNA sequencing to provide rapid microbial identification service. Identification is done through the proprietary Sherlock® pattern recognition software that compares the results to a database of FAME profile and DNA sequence. This FAME preparation method, which was originally developed by Moss (Wayne-Moss, 1981), consists of 5 steps: sample harvest, saponification, methylation, extraction and base wash.

The original method proposed by MIDI required 40 mg wet weight of bacterial cells in order to obtain adequate signal for FAME analysis, but without active culturing it is unlikely to be able to obtain such sample size of bacteria samples. Various GC techniques had been tested in attempt to reduce the required sample size while maintaining optimal detection sensitivity. Combination of split-less and cold trap injection was able to reduce the sample size to a single colony from an agar plate (Buyer, 2002; Buyer, 2006). Five to ten mg of dried bacterial cells had been shown to be sufficient in attaining detectable FA signals (Härtig *et al.*, 2005). Saponification involves high concentration of sodium hydroxide in methanol to lyse the cells and free up FA chains from lipid molecules. Transesterification (methylation) without saponification leads to underestimation of cyclopropane FA and less effective at releasing hydroxyl FA from the lipids (Härtig *et al.*, 2005).

Free FA are derivitized via acid catalysis with hydrochloric acid and methanol as the nucleophile for transesterification reaction. Acid catalyzed transesterification is inhibited by the

presence of water, so the samples should be freeze-dried to remove all traces of water. Direct transesterification of FA without extraction of total FA is generally less efficient, but efficiency of direct transesterification increases significantly with increase in the methylation reaction time. For microorganisms, studies have found increasing reaction time can have better methylation efficiency than protocols that includes an initial total FA extraction step (Lewis, *et al.*, 2000). However, acid catalysis can also induce partial degradation in certain FA such as cyclopropane FA into branched FA if the transesterification reaction is carried on for too long (Zayed, 2004), therefore the temperature and duration of the reaction is crucial.

FAME products after transesterification reaction are isolated by solvent phase separation with (1:1) hexane-methyl tert-butyl ether (MTBE). FAME will be partitioned into the organic phase with the hexane. FAME extract will then be washed with sodium hydroxide-water to remove any residual reagents and cellular debris to preserve the GC system; particularly the injector port, capillary column and detector. After a chromatography run, peak identification can be done manually by comparison to known FAME standards. Long chain odd number FA such as 21:0 and 23:0 can be used as internal standard for quantification by relative comparison of the peak area in the chromatograms (Goedkoop *et al.*, 2000; Ballantyne *et al.*, 2003; Kolanowski *et al.*, 2007). These FA are generally not found in nature.

The commercial microbial identification system Sherlock® is available from MIDI Inc. The system uses external FAME standards composed of C9 to C20 SAFA as reference. Unknown peaks in the sample are identified by matching the nominal retention time, or the 'Equivalent Chain Length' (ECL) value. The ECL is determined by retention time of the specific compound and its relative position to the closest SAFA. The resulting FA profile is compared with an extensive bacterial FA profile database by series of automated covariance

matrix, principal component analysis and pattern recognition algorithm to identify unknown bacterial sample (Sasser, 2001). The system works for pure culture and its capacity to identify the unknown sample depends on the library of FA profiles available.

There are some important issues that must be considered during FA extraction and derivitization (Budge *et al.*, 2006): (1) Short-chain FA are soluble in water and highly volatile; their FAME derivatives are even more volatile. Samples should be kept at low temperature and minimal exposure to air during sample preparation to minimize this problem. This issue is particularly vital for bacterial FA analysis. (2) Unsaturated FA may be oxidized when they are exposed to elevated temperatures, so transesterification reactions should be carried out under a nitrogen atmosphere. This can be achieved by flushing the reaction vessel with nitrogen before incubation in the water bath. (3) Not all lipid types can be esterified to yield free FA by acid catalysis; dimethyl acetal (DMA) derived from plasmologen and fatty alcohols derived from wax esters both cannot be directly methylated, consequently they are poorly resolved and often show up as a broad peak in the GC chromatogram. In order to resolve the FA derived from these lipid types, extracted total lipid will need to be fractionated by thin-layer chromatography (TLC), followed by oxidation reactions to form the respective free FA (Budge *et al.*, 2006). Most bacteria have simple lipid and majority are found as phospholipids, so the issue with extracting FA from different lipid types should not be too much of a concern.

Chapter 3: MATERIALS AND METHODS

3.1 Bacterial strain selection

Biofilm-forming *Pseudomonas aeruginosa* and *Staphylococcus aureus* were chosen as the primary bacterial model organisms for the experiments because their difference in Gram reaction ensures that they have fundamentally different cell wall structures and thus distinctly different FA profiles. Other bacterial species used in species-specific FA profile comparison experiment include; *Escherichia coli*, *Bacillus subtilis* and *Bacillus megaterium*. All the strains were obtained from Ryerson University. Green fluorescent protein (GFP) labeled *Pseudomonas aeruginosa* (CTO7::*gfp-2*) (Bester *et al.*, 2009) was used for the microscope imaging experiment to confirm *H. azteca* grazing on biofilm.

All the strains were sub-cultured over night and stored in 1.5 mL Eppendorf micro-centrifuge tubes with 40% glycerol at -80°C as stock cultures. In order to prevent mutation and preserve strain consistency, inocula used for all experiments came from overnight cultures (10% TSB at 37°C) derived from these cryogenically-frozen stock cultures (70 µL).

3.2 Bacterial culture media

Trypic soy broth (TSB) medium, 30 g of powder TSB stock (EMD# 1.05459.0500) in 1 L of distilled water, was used to cultivate bacteria. The media were sterilized at 121°C for 20 min prior to use. TSB powder was subjected to FA analysis to verify that there were no FA present.

3.3 Bacteria culturing methods

3.3.1 Batch planktonic cultures

Batch culturing took place in 100 mL of sterile 10% TSB in 250 mL Erlenmeyer flasks. The cultures were grown at room temperature (~25 to 27°C) and shaken at 250 rpm to reduce biofilm formation and/or cell aggregation. The batch culture was pipetted into 50 mL conical centrifuge tube and centrifuged for 20 min at 3000 rpm. The resulting pellet of cells was aseptically transferred into a 2.0 mL cryogenic vial (VWR #16001-102) with a ethanol-washed spatula then stored (-80°C) until FA extraction.

3.3.2 Biofilm cultures

A continuous flow system fed by a peristaltic pump was set up at room temperature (~25 to 27°C) to culture bacterial biofilm samples (Figure 3.1A): The growing substrate for the biofilm was provided by 50-cm long large diameter silicon tubing (~9.5 mm i.d., vol = ~35 mL/tube; VWR# 60985-736) (Figure 3.1C). Small diameter silicon tubing (~1.6 mm i.d., VWR# 60985-714) was connected to the two ends (Figure 3.1A & B); one end was connected with a peristaltic pump that feed in 1% TSB as growth medium at the rate of ~15 mL/h while the other end provided an outlet for effluent (waste). The silicon tubing were sterilized by autoclave, followed by flushing with 10% bleach, sterilized distilled water and then 1% TSB to remove any residues before inoculation.

A sterile 1 mL syringe was used to inoculate the flow system with 1 mL of overnight culture (see above). The inoculum was injected directly into the growth compartment through the silicon tubing ~10 cm from the front; this was to avoid back-growth into the medium reservoir. The flow was turned off during inoculation and kept off for 1 h to allow cell attachment.

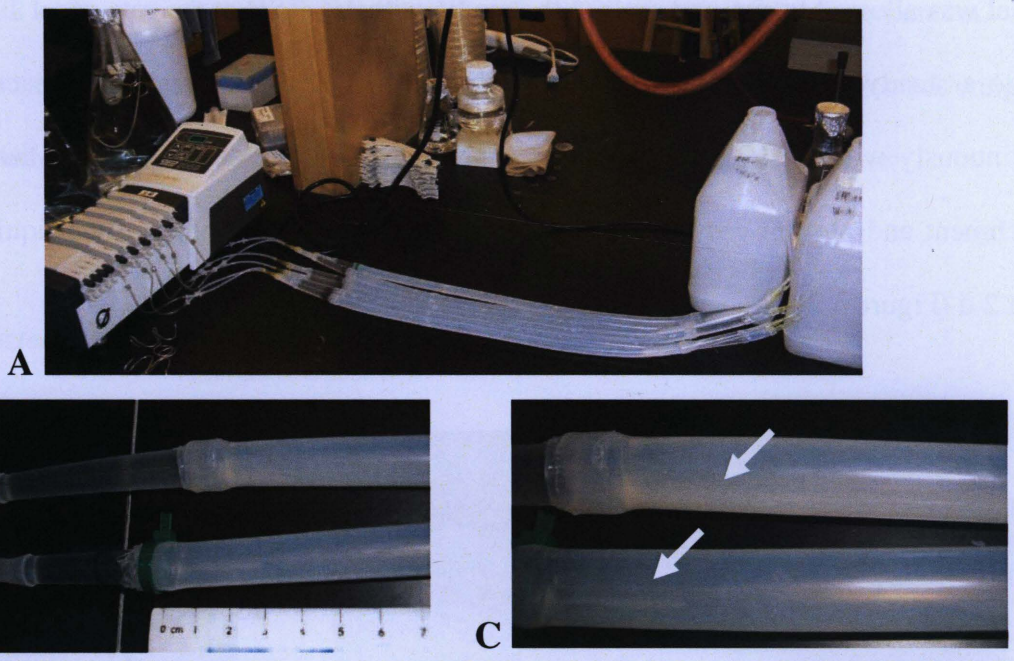


Figure 3.1: Setup of the continuous flow system to culture bacterial biofilm samples. A) Growth medium in 4 L flasks feed into the system via smaller diameter silicon tubes and peristaltic pump, then tapered to the larger diameter silicon tube (growth compartment) seen in B). C) Shows traces of *P. aeruginosa* biofilm growth in the growth compartment two days after inoculation (see arrow).

The flexible nature of silicon tubing allows harvesting of biofilms by physical agitation and squeezing to release the biofilm matrix into the aqueous phase prior to sample collection. The collected material was poured into 50 mL conical centrifuge tubes and centrifuged for 20 min at 3000 rpm. The cell pellet was aseptically transferred into 2 mL cryogenic vial with a spatula and stored (-80°C) until FA extraction.

3.3.3 Biofilm cultures on cotton gauze (for H. azteca feeding experiments)

A 3 L vessel (Sartorius Biostat® A-Plus bioreactor) was used to make a continuous, pure culture, bioreactor system (Figure 3.2A). To initiate the culture, 100 mL of *P. aeruginosa* or *S. aureus* overnight culture was added to the bioreactor which already contained 2 L of sterile 10% TSB. The culture in the vessel was allowed to grow overnight so that it reached a high cell density, thereby reduce the likelihood of contamination. The peristaltic pump connected to the

vessel was allowed to run and replace the media with 1% TSB at the rate of ~1 L/day for 2 d to create a steady-state cell count of 10^8 CFU/mL in the liquid phase. The reactor was stirred continuously with a magnetic stirrer. Sterilized cotton cheese cloth, as the substrate for cell attachment and biofilm growth (~3.0 cm by 5.0 cm), was suspended in the liquid phase for at least 2 d (Figure 3.2B).

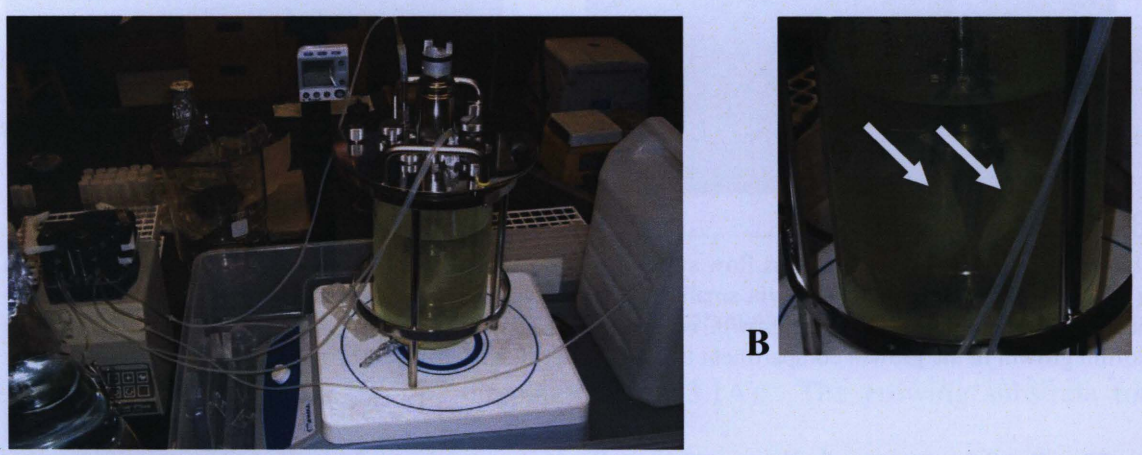


Figure 3.2: Setup of continuous bioreactor to culture bacterial biofilm on cotton gauze for *H. azteca* feeding trials. A) Shows the 3 L bioreactor vessel on a magnetic stirrer. The media feeds into the reactor with the peristaltic pump on the left, the outflow tubes were also connected to the same peristaltic pump, but in reverse direction. B) Cotton gauze (see arrow) suspended in the bioreactor inoculated with *P. aeruginosa*.

3.4 Fatty acid in extracellular polymeric substance

Biofilm samples, harvested after a 6 d growth period in the flow system were centrifuged for 15 min at 4000 rpm. The supernatant from three different samples were filtered through a 0.22 µm polyethersulfone filter (Millipore Stericup® #SCGPU05RE) to remove any trace of cells. Effluent from the filtration (EPS extract) was capped and stored in the Stericup® then kept at -80°C until FA extraction. Pelleted cells were also similarly stored for FA analysis.

The EPS extracts had to be freeze-dried for 5 d due to their high water content. The powder-like substance accumulated at the bottom of the container (EPS) was collected, weighed and analyzed with the bacterial FA extraction/derivitization method described below.

3.5 *Hyalella azteca* culturing methods

3.5.1 Hyalella azteca stock culture

Original *Hyalella azteca* mating pairs were obtained from Dr. Warren Norwood's laboratory at The Canada Centre for Inland Waters (CCIW, Burlington, ON). The stock culture was grown in a ~10 L aquarium lined with cotton gauze and pebbles at the bottom (Figure 3.3), and then filled with de-chlorinated tap water (vigorous aeration for at least one week). Commercial algae wafer (Spirulina algae, corn gluten, wheat flour and yeasts) and ground fish food flakes (Tetramin®) were added as food for the *H. azteca*. The tank was exposed to natural sunlight and photo-period by keeping it close to a windowsill. The water temperature was maintained at room temperature (i.e. 23 to 25°C). Every week, ½ the water in the tank was siphoned out and replaced with fresh clean de-chlorinated tap water. ~0.5 mg of Tetramin® was added after each water change. *H. azteca* were handled using long stem disposable polyethylene transfer pipettes which were trimmed to enlarge the openings at the tips so as to accommodate the *H. azteca*.

3.5.2 Hyalella azteca feeding trials

For each individual experimental trial, 20 *H. azteca* were transferred to a ~2 L semi-translucent container (VWR #44333-008) 1/3 filled with fresh de-chlorinated tap water (Figure 3.4A). The amphipods were selected based on size (~3 mm long). Because gravid females have

significantly different FA profiles (Hyne *et al.*, 2009) they were removed and returned to the stock culture tank during the experiments. Gravid females were differentiated by the presence of orange spots near the ventral thoracic cavity. Any *H. azteca* that died during the experimental trials were also removed to prevent other amphipods from consuming the decomposing carcasses.

A B

Figure 3.3: *H. azteca* stock culture in ~10L tank. A) The bottom of the tank lined with cotton gauze and pebbles, along with sediment accumulation. B) Closer look at the bottom of the tank where some of the *H. azteca* can be seen (circled in red).

A B

Figure 3.4: A) *H. azteca* feeding/starvation trials in separate 64 oz containers. B) *H. azteca* on clean cotton gauze in the starvation trials.

For the feeding trials, biofilm-covered cotton gauze from the bioreactor was provided to the *H. azteca* as a substrate and food source. Feeding trials were for every 2 d interval and lasted up to 10 d. Clean sterilized cotton gauze was used for the fasting trials; fasting tests were done

for periods of 6 h, 2, 4, 6 and 10 d (Figure 3.4B). The amphipods were transferred to a clean container with fresh de-chlorinated water with the respective cotton gauze type every 2 d.

Prior to sample collection, the amphipods were transferred to a smaller container with clean, fresh de-chlorinated water and allowed to depurate for 6 h. The amphipods were then collected into 2.0 mL cryogenic vials with a disposable pipette. Samples were stored at -80°C until FA analysis.

3.6 Epifluorescent microscopy to demonstrate biofilm grazing by *H. azteca*

In a 250 mL Erlenmeyer flask, 100 mL of 10% TSB, along with a small piece of sterile cotton gauze, was inoculated with 70 µL of *P. aeruginosa* CTO7::*gfp-2*. This culture was incubated for two days at room temperature without agitation to promote cell attachment and biofilm formation.

Eight *H. azteca* were taken from the stock culture tank; four *H. azteca* were transferred into a small container along with the CTO7::*gfp-2* cotton gauze and clean de-chlorinated tap water. After one day, the *H. azteca* were viewed with a Leica dissection microscope (model MZ-FLIII) equipped with a UV lamp, green fluorescent filter, and Leica camera (model DFC350 FX). A fluorescent signal in the intestinal tract and mandible was taken as a verification that *H. azteca* was feeding on the biofilm. As negative control, the other four *H. azteca* were prepared for viewing under the microscopy immediately without exposure to CTO7::*gfp-2* biofilm. Threads of the cotton gauze were also viewed to confirm green fluorescent signal of CTO7::*gfp-2* and biofilm accumulation on the cotton gauze.

3.7 Amphipod fatty acid analysis

FA in *H. azteca* was extracted using a modified Bligh and Dyer method (Zellmer *et al.*, 2004; Hebert *et al.*, 2009; Bligh and Dyer, 1959)

3.7.1 Fatty acid extraction

All *H. azteca* samples were freeze-dried at -62°C under vacuum for one day. For each individual sample, roughly 8 freeze-dried *H. azteca* (~1 to 2 mg) were weighed out using a Sartorius microbalance (1µg precision). A surrogate standard (50 µL of 1.0 mg/mL 5α-cholestane; Sigma #150320 in chloroform), used to estimate percent recovery of the entire extraction procedure, was added to the *H. azteca* samples, which were placed in 10 mL glass culture tubes, after which 2 mL of 2:1 chloroform-methanol was added. The sample was then homogenized using a Teflon® pestle Glas-Col® variable speed homogenizer. Lipid partitioned into the chloroform-methanol was separated from the debris (fragments of carapace) by centrifugation for 10 min at 3300 rpm. Each sample was extracted 3X, the lipid extract was transferred to a 15 mL centrifuge tube and made up to 8 mL total volume with additional chloroform-methanol.

A salt wash (1.6 mL of 0.9% NaCl) was added to remove water-soluble non-lipid material suspended in the extract. After centrifugation (8 min at 3300 rpm) to ensure proper phase separation, the upper salt-water-methanol layer was pipetted off and discarded. The remaining chloroform-lipid phase was dried completely using a nitrogen evaporator (N-EVAP 111).

3.7.2 Gravimetric analysis

In order to determine the amount of total lipid in each sample, the dried lipid extract was re-dissolved with 2 mL of chloroform-methanol. Two aliquots of 100 µL were transferred to separate, pre-weighed miniature tin cups using a micro-syringe. The solvent in the tin cups was allowed to evaporate overnight and the cup was then re-weighed to determine the mass of total lipid in the 100 µL aliquot. The remaining lipid extract (1.8 mL) was re-dried using the nitrogen evaporator before proceeding with methylation.

3.7.3 Methylation of extracted fatty acids

After gravimetric analysis, the remaining FA extract was re-dissolved using 1 mL of toluene. Acid catalyzed methylation was done using 1% concentrated sulfuric acid in methanol; 2 mL of this methanol-sulfuric acid reagent was added to the lipid extract, flushed with nitrogen, capped and briefly vortexed. Samples were incubated overnight for 16 h in 50°C water bath. Fatty acid methyl esters (FAME) were collected into 5 mL of 1:1 hexane-diethyl ether plus 0.1% butylated hydroxytoluene (BHT). After centrifugation for 2 min at 1500 rpm, the upper organic layer was transferred to another centrifuge tube with a Pasteur pipette. This process was repeated with an additional 5 mL of 1:1 hexane-diethyl ether to the original tube. The total FAME extract was dried completely using a nitrogen evaporator then re-dissolved in 2 mL of hexane in GC vials for analysis using an Agilent 6890GC with programmable temperature vaporization (PTV) inlet (Appendix A).

A 37-component FAME standard mix (Sigma #47885-U) spiked with 5α-cholestane was used as external standard to determine the retention time of individual FA. A four-point linear calibration curve was generated for quantification of the resulting peaks by creating 4 stock

solutions of the 37-component FAME + 5α-cholestane standard. All GC data were analyzed with Agilent ChemStation (B.04.01) software.

Each FAME extract was re-analyzed using a Varian CP-3800 GC with Galaxie software (see below) in attempt to look for bacteria-specific FA, particularly hydroxy FA, cyclopropane FA and terminal-branched FA.

3.8 Bacterial fatty acid analysis

All bacterial samples were freeze dried at -62°C under vacuum for 1 d then ~5 to 10 mg of the freeze dried sample was weighed and transferred into a 15 mL glass centrifuge tube with Teflon-lined cap for FA extraction. Cholestane surrogate standard (50 μL of 1.0 mg/mL 5α-cholestane in chloroform) was also added prior to extraction. The bacterial FA extraction method was adopted from MIDI Inc. (Steger *et al.*, 2003; Hoffmann *et al.*, 2008) with minor modifications; these include the saponification reaction temperature was changed from 100°C to 80°C, 1.50 mL of hexane-methyl tert-butyl ether (MTBE) was used for phase separation instead of 1.25 mL and an additional extraction step with 1.0 mL of hexane.

The saponification reaction used 1 mL of sodium hydroxide (3.75 mol/L) in 1:1 methanol-water. Samples were flushed with nitrogen, capped and incubated in an 80°C water bath for 30 min; the samples were vigorously vortexed every 5 min. After the samples were cooled completely, 2 mL of 10% hydrochloric acid in methanol was added for acid catalyzed methylation. This reaction was carried out in the 80°C water bath for 10 min. The incubation period for this step was critical to the recovery efficiency, 10 min incubation period was found to have the optimal recovery percentage for all classes of FA; see "Bacterial fatty acid methylation efficiency".

FAMEs were collected by phase separation using 1.5 mL of 1:1 hexane-MTBE. Samples were gently shaken for 10 min at ~200 rpm, followed by centrifugation for 2 min at 1500 rpm to ensure proper phase separation. The lower aqueous layer containing water and methanol was pipetted off and discarded along with any precipitated debris.

A 3.0 mL aliquot of 0.3 mol/L sodium hydroxide in distilled water was added to the FAME extract to remove residual reagents and debris. The mixture was tumbled on the shaker for 5 min at ~200 rpm then centrifuged for 2 min at 1500 rpm. The upper organic layer containing the FAME product with hexane was transferred to a separate clean centrifuge tube. To ensure maximum yield, the extraction was repeated with an additional 1.0 mL of hexane added to the remaining aqueous phase. The final extract was adjusted to 2.0 mL by adding extra hexane or evaporated down with nitrogen evaporator, then transferred into GC vials for GC analysis on a Varian CP-3800 GC (Appendix A).

Bacterial acid methyl ester (BAME) standard (Sigma #47080-U) was used for qualitative and quantitative assessment of individual peaks. However, the BAME mix does not provide precise amount of each component, therefore 21:0 standard (Sigma #H3265) was added as the reference standard to quantify the individual components in the BAME mix. A four-point linear calibration curve was then constructed with the BAME + 21:0 standard mix to quantify the peaks for each sample. Retention time for other FAME that were not included in the BAME mix were determined with the help of Dr. Claus Härtig (Helmholtz Centre of Environmental Research, Leipzig, Germany) or tested separately with individual standards, whenever available. All GC data were analyzed with Varian Galaxie software.

3.9 Bacterial fatty acid methylation efficiency

A FA standard mix was made with known amount of four different FA standards: 2OH-10:0 (Sigma #H6271), 18:1n7c (Matreya #1266), 18:1n7t (Matreya #1262) and 19:0*cyc* (9, 10) (Matreya #1822). The FA standard mixture underwent the entire MIDI FA extraction method but with a series of different methylation incubation periods (5 min, 10 min, 20 min, 40 min, 1h 20 min and 2 h) to determine the optimal reaction time. FAME was quantified with Varian CP-3800GC to determine the methylation efficiency of the MIDI FA analysis method by comparing detected amount to the BAME standard + 21:0 reference standard mix.

3.10 Statistical analysis

Results from the GC were adjusted for the methylation efficiency and recovery percentage of the FA extraction process, and then normalized to the original dried sample mass to obtain final values in concentration unit (μg FA per mg of dried sample mass).

All statistical analyses were performed with SigmaStat software for Windows (ver. 3.5) based on 95% confidence levels; $p < 0.05$ was considered significant. Pair-wise comparisons were analyzed using two-sample t-tests. Comparisons with multiple treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test if ANOVA comparison indicated significantly difference in the results. Alternative non-parametric Kruskal-Wallis (KW) test was used for non-parametric data. All error bars and variability were expressed as standard error (SE). 'ANOVA sample size' functions from SigmaStat software based on $\alpha = 0.05$ and desire power of 0.80 was used to estimate the sample size necessary for sufficient statistical power in future studies.

Chapter 4: RESULTS AND DISCUSSIONS

4.1 Methylation efficiency of MIDI FA analysis method

Standard MIDI FA analysis protocol did not provide methylation efficiency for quantitative analysis and since it was not reported in literature, initial experiments were necessary to determine its methylation efficiency. A summary of the methylation efficiency for four FA is presented in Appendix B. Methylation efficiency of hydroxy FA had the lowest recovery (~50%), but it remained relatively consistent for all methylation reaction times. Methylation efficiency of cyclopropane FA was greatly influenced by reaction time; extended methylation periods caused degradation of the cyclopropane FA, with efficiency dropping to 10% at 2 h and random small peaks appearing on the chromatogram in tests over 40 min (data not shown). The original MIDI protocol suggests a 10 min methylation period, which does provide the optimal yield for all the FA tested in our result. Therefore, extraction of bacterial FA using the MIDI method in this study adhered strictly to a standardized 10 min methylation reaction time.

Because results of the methylation efficiency test clearly indicated variable methylation efficiency for different FA, it was decided to correct the GC raw data from all MIDI FA analysis results based on the methylation recovery percentages of the different FA classes: 50.9%, for hydroxy FA, 73.5% for *cis*-unsaturated FA; 83.5% for SAFA, branched-FA and *trans*-unsaturated FA, and 67.9% for cyclopropane FA. Considering that most lipid studies in literature usually report a single methylation efficiency value for all FA, this variable methylation efficiency would provide for a much more accurate assessment of FA concentration and relative compositions.

4.2 FA profile of different bacteria

The FA profiles of five different bacterial species were compared to indentify possible species-specific patterns or unique FA biomarkers (Appendix C, Table C1). Gram negative species included *P. aeruginosa* and *E. coli* whereas *S. aureus*, *B. subtilis* and *B. megaterium* represented the Gram positive species. Table 4.1 summarizes the concentrations and proportions of major FA classes for the five bacterial species. Gram negative species can be distinguished by the presence of hydroxyl FA, cyclopropane FA and MUFAs, whereas the majority of the FA in Gram positive species largely consists of either terminal branched FA or SAFAs (Oliver and Colwell, 1973; Gillan, 1986; Zhang and Rock, 2008;). Total FA in Gram negative species are higher than those of Gram positive, likely derived from excess of PLFA due to the presence of double cell membranes in Gram negative bacteria. No PUFAs were found in any of the species tested.

Table 4.1: Summary of major FA classes in Gram negative *P. aeruginosa*, *E. coli*, and Gram positive *S. aureus*, *B. subtilis* and *B. megaterium* grown in planktonic batch cultures at 37°C for 2 d. The amounts are presented as µg/mg dried weight tissue. Percentage shown in brackets is the proportion for the particular FA class to the total FA. N.D. = Not detected.

Major FA class	Gram negative		Gram Positive		
	P. aeruginosa (n=8)	*E. coli* (n=1)	*S. aureus* (n=4)	*B. subtilis* (n=2)	*B. megaterium* (n=1)
∑Hydroxy FA	12.17 (12.8%)	5.79 (9.9%)	N.D.	N.D.	N.D.
∑Branched FA	0.06	N.D	27.54 (71.42%)	33.79 (94.2%)	19.64 (85.6%)
∑iso-	*0.06*	-	*5.57*	*9.60*	*10.35*
∑anteiso-	*N.D.*	-	*21.98*	*24.19*	*9.29*
∑Cyc FA	10.14 (10.7%)	18.90 (32.4%)	N.D.	N.D.	N.D.
∑SAFA	29.12 (30.6%)	28.85 (49.4%)	10.41 (27.0%)	1.96 (5.5%)	3.17 (13.8%)
∑MUFA	43.63 (45.9%)	4.84 (8.3%)	0.61	0.12	0.13
∑Cis-	*40.75*	*4.84*	*0.61*	*0.12*	*0.13*
∑Trans-	*2.87*	*N.D.*	*N.D.*	*N.D.*	*N.D.*
∑PUFA	N.D.	N.D.	N.D.	N.D.	N.D.
Total	95.12	58.39	38.56	35.88	22.94

The relative proportion of individual FA for each bacterial strain can be found in Appendix C. *P. aeruginosa* had 14 major FA, which is in agreement with previously reported

findings (Dubois-Brissonnet *et al.*, 2000; Mrozik *et al.*, 2004). For this strain, 16:0, 16:1 and 18:1 were the most abundant, followed by 12:0, 3-OH 10:0, 2-OH 12:0, 3-OH 12:0 and the cyclopropane FA. When comparing the FA profile of *E. coli*, *P. aeruginosa* contains a greater proportion of hydroxy FA but composed of shorter chain lengths hydroxy FA (2-OH 14:0 and 3-OH 14:0). *P. aeruginosa* are known for their ability to produce rhamnolipid (Figure 4.1) biosurfactant that assist its motility (Inoue *et al.*, 2008). Excess rhamnolipid could be an alternative source of hydroxy FA aside from lipid-A of LPS in the Gram negative bacterial cell wall. Rhamnolipid production is characteristic of *Pseudomonas* spp. and is often associated with their ability to form biofilms (Soberón-Chávez *et al.*, 2005) therefore 3-OH 10:0 is a plausible candidate as a unique FA marker for *Pseudomonas* spp. Furthermore, the proportion of the different hydroxy FA had also been proposed as a comparable criterion to distinguish different *Pseudomonas* spp. (Wayne Moss and Dees, 1975).

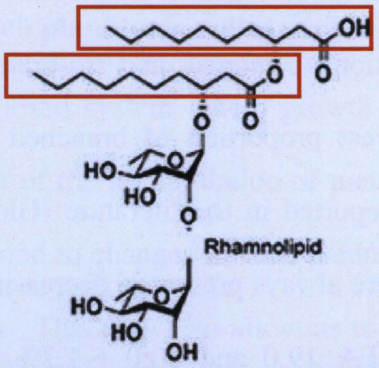


Figure 4.1: Chemical structure of R2-rahamnolipid. The red box indicates the possible source of 3-OH 10:0 fatty acid detected in *P. aeruginosa*.

A higher proportion of MUFA was found in *P. aeruginosa* (45.9%) compared to *E. coli* (8.3%). Conversely, *E. coli* had much higher proportions of cyclopropane FA (32.4% versus 10.7%) and SAFA (49.4% versus 30.6%) than *P. aeruginosa*. Furthermore, the majority of the cyclopropane FA and MUFA in *E. coli* were the shorter 17:0cyc (9, 10) and 16:1n7c, as opposed

to 19:0 (11, 12) and 18:1n7c in *P. aeruginosa*. Changes in the proportion of SAFA in relation to temperature fluctuations are well documented. For example, SAFA in *P. aeruginosa* have been shown to increase from 25% to 39% when the growth temperature increased from 15°C to 40°C (Dubois-Brissonnet *et al.*, 2000); closely resembling the 30.6% SAFA content in our *P. aeruginosa* cultures when they were held at 37°C. With respect to FA in membranes, a more saturated FA profile for *E. coli* would suggest that this species has relatively more rigid cell membranes. Given that the optimal growth temperature for *E. coli* is ~40.6°C (American Type Culture Collection; www.atcc.org); greater proportion of SAFA observed in *E. coli* may be the outcome of its homeoviscous adaptation to accommodate growing at a temperature (i.e. 37°C) lower than its optimal growth temperature.

Terminally branched FA (94.2%) was the major FA observed in the Gram positive *B. subtilis*, among which *anteiso*-branched FA were most prevalent (~61.6%, about 2:1 ratio to *iso*-branched FA). Another species from this genus, *B. megaterium*, had a lower proportion of branched FA (85.6%) and proportion of *anteiso*- and *iso*-branched FA was about equal. The bacterium *S. aureus* had the lowest proportion of branched FA (71.42%) compared to the *Bacillus* spp. we measured. As reported in the literature (Giotis *et al.*, 2007), the amount of branched chain FA in *S. aureus* were always present in decreasing order from; *a*-15:0 > *a*-17:0 > *i*-15:0 > *i*-17:0. Long-chain SAFA 19:0 and 20:0 (~1.2% and ~14.8%, respectively) was observed in *S. aureus*, but not in the other species tested. C19 branched-FA were also more prevalent in *S. aureus* at ~4.5%. These features can perhaps be considered as additional distinguishing features of *S. aureus*. Medium chain-length SAFA (13:0 and 15:0) were not found in any of *S. aureus* in this study, but Sado-Kamdem *et al.* (2009) reported them to be in trace amounts and noted further that their relative abundance increases with decreasing pH.

It is difficult to distinguish bacterial species solely based on unique FA markers, especially between the ubiquitous species compared in our study. Proportions of specific FA or FA classes can provide distinguishing features, but the ability of bacteria to efficiently modify their PLFA composition in response to changing environmental conditions (e.g. temperature and pH) complicate application of such an approach. Therefore special care should be taken in selecting FA as markers. Among the bacteria examined in this study (Appendix C, Table C2); gram negative *P. aeruginosa* can be distinguished by the abundance of 18:1n7c MUFA, presence of cyclopropane FA and its hydroxy FA pattern. The bacterium *S. aureus* is unique because of the presence of 19:0 and 20:0 long chain SAFA, presence of C19 branched-chain FA, also the relative amounts of the branched chain FA. Therefore, these FA were selected as distinguishing indicators for this study in the preceding experiments.

4.3 FA profiles of bacterial batch planktonic cultures over time

The batch culture is a closed system where growth typically ceases when a limiting nutrient is depleted or as a result of the accumulation of metabolic waste products. Observing how the planktonic bacteria respond to changes in such parameter can help us to delineate what controls microbial growth cycles. This may also allow us to determine the appropriate age for the planktonic batch cultures for comparisons with biofilm cultures. Figure 4.2 shows the trend of major FA classes in batch planktonic *P. aeruginosa* cultures from Day 1 to Day 4. Concentrations of hydroxy FA and SAFA were not significantly different amongst the four days and while MUFAs appeared to be decreasing with time, the trend was bordering statistical significance (ANOVA, $p = 0.05$). When the MUFA were separated into the different isomers, *cis*-MUFA were found to be decreasing with the age of the culture (ANOVA, $p = 0.03$). While

trans-MUFAs were showing increasing trend (~2.61 µg/mg at 1 d to ~6.92 µg/mg at 4 d), but the increase was not statistically significant (ANOVA, p = 0.22). The amount of cyclopropane FA was also increased (but not statistically significant) as the culture aged, increasing from 7.21 µg/mg on Day 1 to 18.38 µg/mg on Day 4.

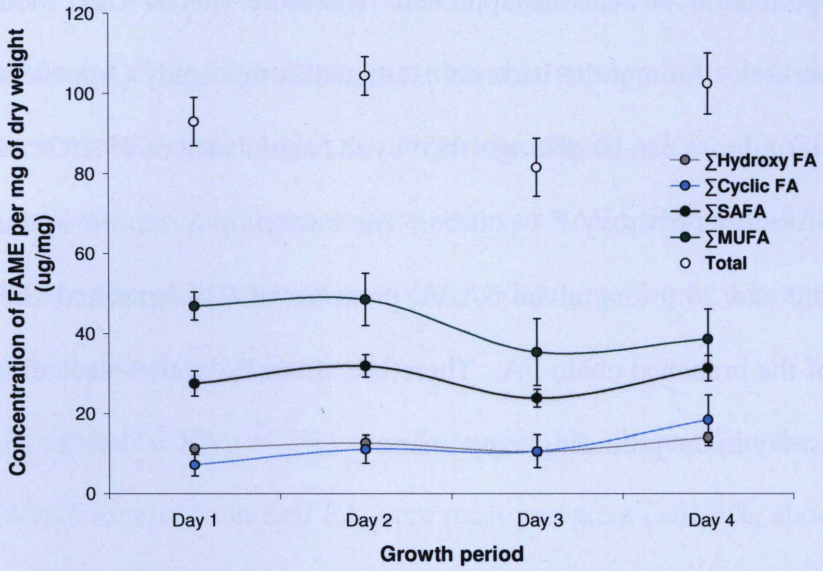


Figure 4.2: Trend over four days for the sum of major FA classes in batch planktonic *P. aeruginosa* grown in 10% TSB in 250 rpm shaker at room temperature (~25 to 27°C). The amounts presented as µg per mg of dried sample tissue weight.

Table 4.2: Ratio of FA derived from post-synthesis modification (cyclopropane FA and *trans*-MUFA) to *cis*-MUFA in *P. aeruginosa* batch planktonic cultures over 4 d. N = 6.

Cyclopropane FA /MUFA	1 d	2 d	3 d	4 d
Cyc FA + *trans*-MUFA	9.81 ± 2.37	14.31 ± 1.89	16.46 ± 2.78	25.30 ± 3.37
Cyc FA + *trans*-MUFA /*cis*-MUFA	0.23 ± 0.06	0.36 ± 0.08	0.61 ± 0.14	0.84 ± 0.10
C19:0cyc (11, 12) /C18:1n7c	0.16 ± 0.07	0.28 ± 0.10	0.46 ± 0.18	0.63 ± 0.20
C17:0cyc (9, 10) /C16:1n7c	0.25 ± 0.10	0.44 ±0.16	0.58 ± 0.22	0.94 ± 0.33

When comparing specific FA type, combination of cyclopropane FA and *trans*-MUFA increased over the 4 d period, as well as its proportion to *cis*-MUFA (Table 4.2). Both cyclopropane FA and *trans*-MUFA in bacteria derive from post-synthesis modification of existing *cis*-MUFA, and it usually occurs during stationary phase upon resource depletion

(Denich *et al.*, 2003). It has been shown that Gram negative bacteria respond to acid stress or limiting nutrients by converting existing MUFA to cyclopropane FA in the membrane phospholipids (Piotrowskia-Segret and Mrozik, 2003; Kim *et al.*, 2005, Härtig *et al.*, 2005). This conversion was also observed in the present study by an increase in the ratio of the cyclopropane FA in *P. aeruginosa* and their respective MUFA precursors; 19:0cyc (11, 12)/18:1n7c and 17:0cyc (9, 1)/16:1n7c as the batch culture aged (Table 4.2).

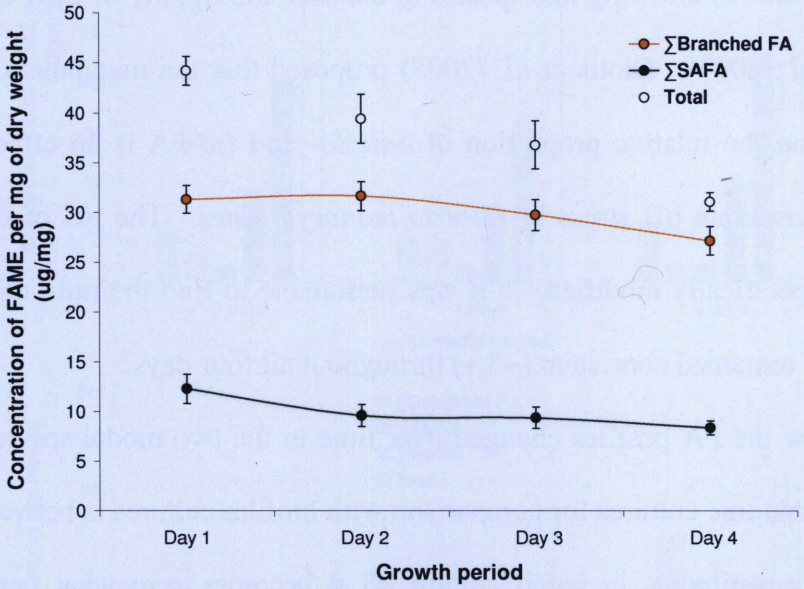


Figure 4.3: Trend over four days for the sum of major FA classes in batch planktonic *S. aureus* grown in 10% TSB under in 250 rpm shaker at room temperature (~25 to 27°C). The amounts presented as µg per mg of dried sample tissue weight.

Major FA classes were also compared for the Gram positive *S. aureus* (Figure 4.3). Although total FA appears to be decreasing with time, the differences among the days were not considered statistically significant ($p = 0.07$). The changes in both SAFA and branched chain FA over time were also insignificant. It is possible that *S. aureus* have already reached stable stationary phase within the first 24 h, and the slight downward trend in total FA may be due to gradual decline in viable biomass and rapid degradation of PLFA upon cell death (White *et al.*, 1988). In addition, since the unit of our quantitative measurement was concentration normalized

to the dry weight of sample tissue, accumulation of other biochemical compounds (i.e. protein, carbohydrate or other non-lipid products) would contribute to the weight of the sample, thus reducing the relative contribution of FA to the total extracted mass of material.

Gram positive bacteria exhibit a tremendous ability to modulate their FA composition in response to environmental conditions. Modification of phospholipids branched-chain FA in response to pH have been reported in the past. For example, the amount of *i*-15:0 increases with acidic pH (~5) in *S. aureus* allowing this species to enhance the rigidity of their cell membranes (Sado-Kamdem *et al.*, 2009). Giotis *et al.* (2007) proposed that the manipulation of branched chain FA content and the relative proportion of *anteiso-* and *iso*-FA is an effective means of adaptation to mild/moderate pH stress in *Listeria monocytogenes*. The pH of our experiment condition was not specifically modified, so it was reasonable to find the ratio of *anteiso*-FA to *iso*-FA for *S. aureus* remained consistent (~3:1) throughout all four days.

Based on how the FA profiles changed over time in the two model species, the optimal age for the batch planktonic cultures for comparison with biofilm cultures is between 1 to 2 d old. The individual FA constituents in batch culture ≥3 d becomes somewhat compromised, as indicated by the increasing cyclopropane FA to MUFA ratio in *P. aeruginosa* and gradual decline in the total FA in *S. aureus* over time.

4.4 Bacterial biofilm FA profile over time

The FA profiles of *P. aeruginosa* biofilms cultured in the continuous flow system were compared after 2, 4 and 6 d (Figure 4.4A). There was no significant difference for any of the major FA classes between the different groups (ANOVA, $p > 0.05$). The FA composition of Gram positive *S. aureus* also remained consistent with different culture age (Figure 4.4B). There

were no discernable differences in the individual FA, the proportion of *cis*- to *trans*-MUFA in *P. aeruginosa* and the *iso*- to *anteiso*-branched FA ratio in *S. aureus* did not change either. These results indicate the FA composition in biofilm culture remain stable for cultures up to 6 d.

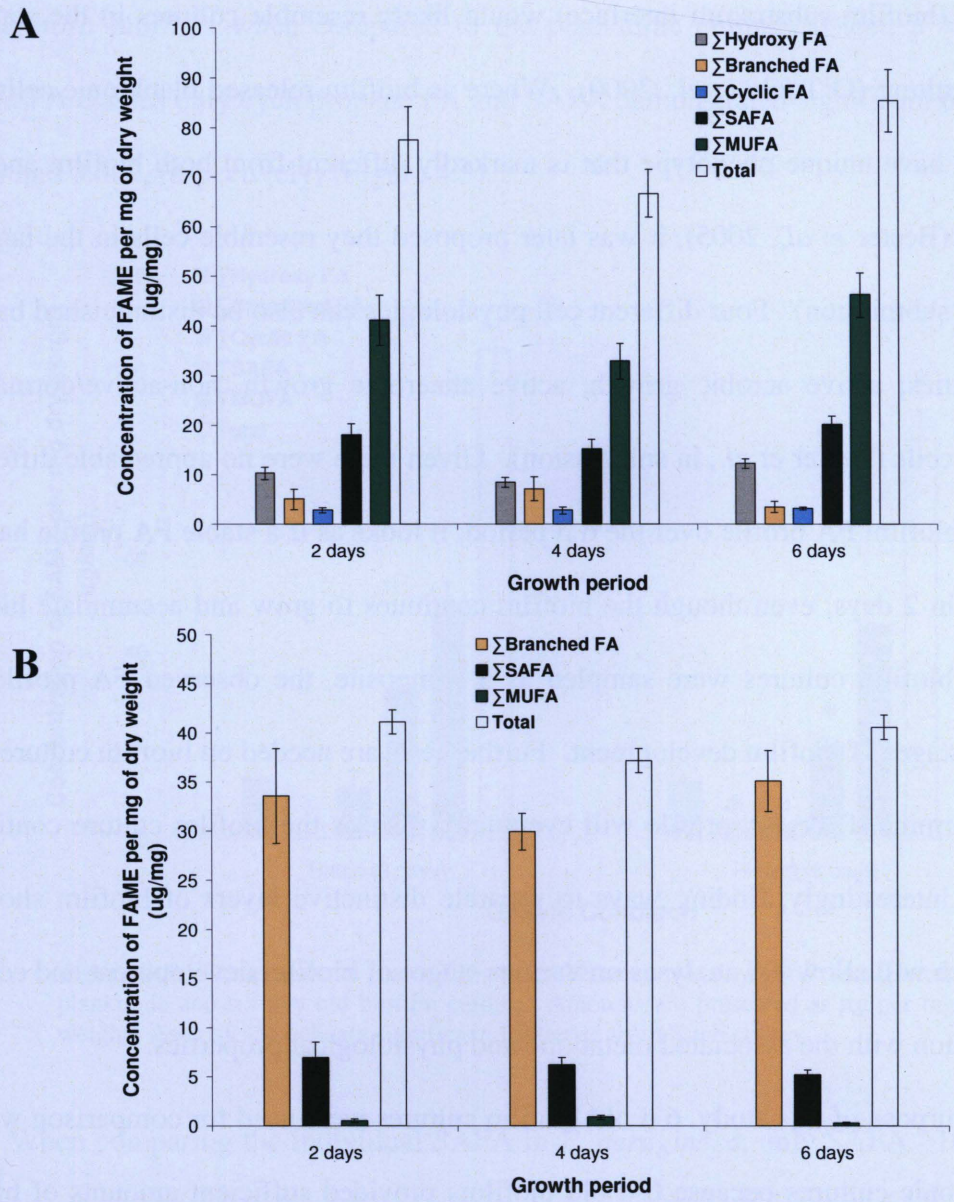


Figure 4.4: Comparison of major FA classes in 2, 4 and 6 d old A) *P. aeruginosa* and B) *S. aureus* biofilm cultures. Amounts are presented as µg per mg dried weight. No significant differences were found between the age groups for all FA classes.

Biofilms demonstrate unique phenotypes at different stages of development and posses properties that markedly differ from planktonic cells (Davies and Marques, 2009); it was

therefore expected that there should also be apparent differences in FA composition for cells at different stages of biofilm development. The active biofilm layers – those closest to the biofilm-liquid interface resemble cells in the growth phase of batch cultures. The deeper, surface associated zone (biofilm-substratum interface) would likely resemble cultures in the stationary phase of batch culture (O'Toole *et al.*, 2000). Where as biofilm-released planktonic cells in the bulk liquid may have unique phenotype that is markedly different from both biofilm and batch planktonic cells (Bester *et al.*, 2005), it was later proposed they resemble cells in the lag phase (Bester *et al.*, in submission). Four different cell physiologies can also be distinguished based on metabolic activities; active aerobic growth, active anaerobic growth, non-active/dormant but viable and dead cells (Bester *et al.*, in submission). Given there were no appreciable differences in the observed biofilm FA profile over the 6 d period, it looks as if a stable FA profile had been established within 2 days, even though the biofilm continues to grow and accumulate biomass. Also, since the biofilm cultures were sampled as a composite, the observed FA profile is an 'average' of all stages of biofilm development. Further tests are needed on biofilm cultures older than 6 d to determine if the FA profile will eventually shift as the biofilm culture continue to develop. More interestingly, finding ways to separate distinctive layers of biofilm should be considered, which will allow FA analyses on various stages of biofilm development and correlate the FA composition with the associated metabolic and physiological properties.

For the purpose of this study, 6 d old biofilm cultures were used for comparison with 2 d old batch planktonic cultures because 6 d old biofilms provided sufficient amounts of biomass and as this experiment have indicated the FA profiles were not influenced by biofilm age.

4.5 Comparing FA profile of bacterial biofilm and planktonic cells

Batch planktonic cultures were compared with biofilm cultures to determine how the FA profile differs between the two forms of microbial existence. There was a decrease in total FA in *P. aeruginosa* biofilms when compared to the planktonic cultures (t-test, p = 0.04). Of the different FA classes only cyclopropane FA and SAFA demonstrated significant declines (t-test, p = 0.014 and 0.002, respectively) (Figure 4.5).

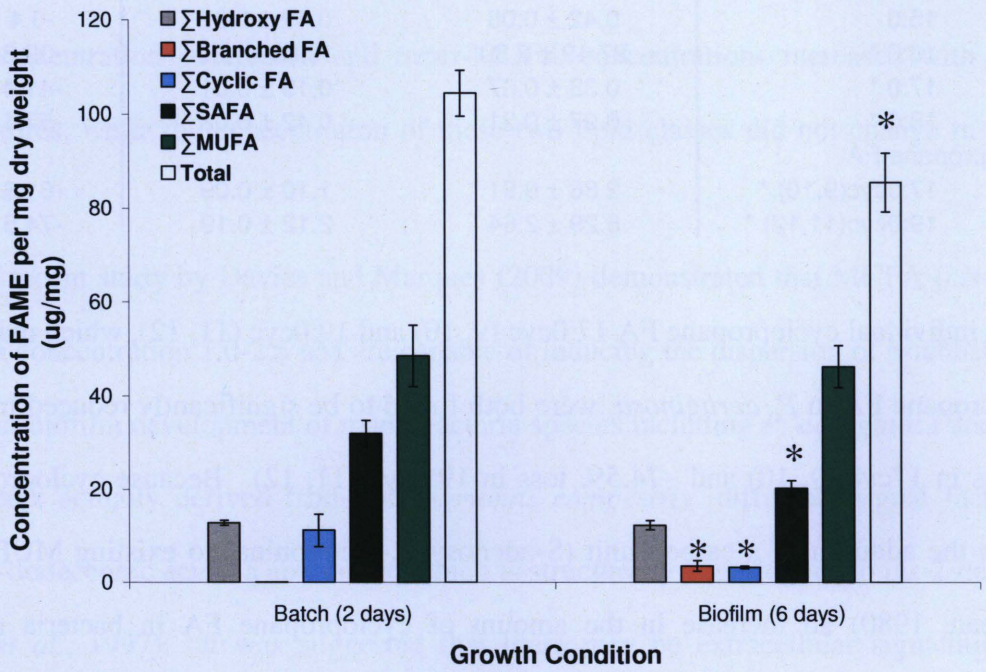


Figure 4.5: Comparison of major FA classes between two day old *P. aeruginosa* batch planktonic and six day old biofilm culture. Amounts are presented as μg per mg dried weight. Asterisk (*) indicate significant difference from batch culture.

When comparing the individual SAFA in *P. aeruginosa*, only SAFA ≥16C long showed significant decrease with the most abundant SAFA (palmitic acid; 16:0) showing a ~39.3% loss in biofilm compared with planktonic cells (Table 4.3). Although 18:0 is found only in low concentrations, there was more than 50% loss in the biofilm. The SAFA in biofilm conformed to a FA profile consisting of shorter FA chains and possibly less rigid membrane structure; contrary

to previous findings in surfaced-associated population on glass surface (Gianotti *et al.*, 2008) or deep subsurface sediment population (Tunlid *et al.*, 1989).

Table 4.3: Concentration (± standard error) and percent change of individual SAFA and cyclopropane FA in *P. aeruginosa* batch planktonic and biofilm cultures. Asterisk (*) indicate statistically significantly differences in the amount detected ($p < 0.05$).

	Planktonic (µg/mg) (n=6)	Biofilm (µg/mg) (n=9)	% change
SAFA			
12:0	2.17 ± 0.18	1.98 ± 0.36	-8.6
14:0	0.59 ± 0.03	0.56 ± 0.02	-4.7
15:0	0.42 ± 0.08	0.42 ± 0.02	-0.4
16:0 *	27.49 ± 2.30	16.69 ±1.38	-39.3
17:0 *	0.33 ± 0.07	0.19 ± 0.01	-41.4
18:0 *	0.97 ± 0.21	0.42 ± 0.08	-57.1
Cyclopropane FA			
17:0cyc(9,10) *	2.86 ± 0.91	1.10 ± 0.09	-61.6
19:0cyc(11,12) *	8.29 ± 2.64	2.12 ± 0.19	-74.5

The individual cyclopropane FA 17:0cyc (9, 10) and 19:0cyc (11, 12), which make up the total cyclopropane FA in *P. aeruginosa*, were both found to be significantly reduced in biofilm; ~61.6% loss in 17cyc (9, 10) and ~74.5% loss in 19:0cyc (11, 12). Because cyclopropane FA derive from the addition of a carbon unit (S-adenosyl-L-methionine) to existing MUFA (Buist and MacLean, 1980) an increase in the amount of cyclopropane FA in bacteria relates to decrease in MUFA (Cronan *et al.*, 1974; Smith *et al.*, 2000; Zhang and Rock, 2008). However, we found no differences in the concentration of MUFA between planktonic and biofilm cultures of *P. aeruginosa*. Cyclization of FA acyl chain is regarded as a mechanism for the bacteria to maintain membrane flexibility while controlling penetration of undesirable molecules under adverse conditions (e.g. nutrient depletion, pH increase) and as a way to improve membrane PLFA stability in the stationary phase of batch cultures (Kim *et al.*, 2005). Lower cyclopropane FA could indicate that the majority of the population within the biofilm cultures was relatively active and have not reached the stationary phase.

Trans-isomerization of MUFA is an alternative post-synthesis modification mechanism to modify membrane properties. This mechanism is highly active when bacteria are in a nutrient deprived state. Therefore, the *cis*:*trans* ratio of MUFA had been suggested as potential index for nutritional status in bacteria (Denich *et al.*, 2003). We did not find significant changes in the *cis*:*trans* ratio of MUFA between biofilm and batch planktonic cultures. This further confirms the validity of using 2 d old batch cultures for the comparison to biofilm cultures. In the previous section (FA profile of planktonic cultures over time) it was demonstrated that *cis*-MUFA concentrations decreased and *trans*-MUFA concentrations increased with age in the batch cultures, while the concentration of these two lipid classes did not change in the biofilm cultures.

A recent study by Davies and Marques (2009) demonstrated that MUFA (*cis*-2-decenoic acid), at a concentration 1.0-2.5 nM are capable of inducing the dispersion of established biofilm and inhibit biofilm development of many bacteria species including *P. aeruginosa* and *S. aureus*. The concept actually derived from *Xanthomonas campestris*' diffusible signal factor (*cis*-11-methyl-2-dodecenoic acid); a compound which is structurally homologous to *cis*-2-decenoic acid (Barber *et al.*, 1997). It was suggested that there may be extracellular signaling molecules homologous to *cis*-MUFA in a wide a range of bacteria that participate in the transition from biofilm to planktonic state. Although neither *cis*-2-decenoic acid nor *cis*-2-dodecenoic acid were available in the identification table of our analytical method, there were no apparent unidentified peaks observed in our planktonic or biofilm samples. However, our results cannot rule out their presence as signal molecules can be in small quantities that falls below the detection limit.

Another interesting observation was the greater concentration of branched-FA in the Gram negative *P. aeruginosa*. Branched chain FA in biofilm increased from ~0.1% in the batch

planktonic cultures to ~3.6% in the biofilm; an amount that was comparable to the cyclopropane FA (~3.3%). Among the total branched-FA found in *P. aeruginosa* biofilm, *i*-15:0 accounted for more than 50%; others include *a*-15:0, *i*-16:0, *i*-17:0 and *a*-17:0. Wayne-Moss and Dees (1975) looked at the FA composition of various *Pseudomonas* spp, and found that *iso*-branched FA, specifically *i*-15:0, accounted for 30-36% of total FA in *Pseudomonas maltophilia*. Mrozik *et al.* (2004) also found branched FA in *Pseudomonas stutzer* (0.8%) and *Pseudomonas vescularis* (7.5%). The proportion of branched, and especially *iso*-branched, FA increased when the cultures were exposed to naphthalene. It was suggested that the presence of branched chain FA is consistent with an adaptive mechanism to increase membrane rigidity and exclude aromatic compounds. *Anteiso*-branched *a*-15:0 were actually found to repress the flagella-driven motility of *P. aeruginosa* resulting in a 31% repression of biofilm formation (Inoue *et al.*, 2008). Perhaps branched FA have other prominent roles in biofilm physiology that have yet to be discovered.

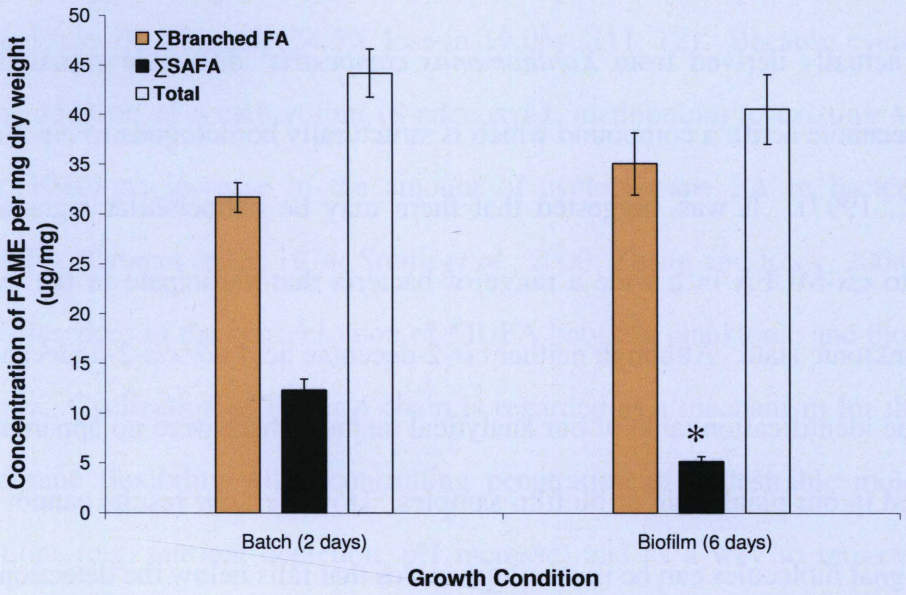


Figure 4.6: Comparison of major FA classes between two day old batch *S. aureus* planktonic and six day old biofilm culture. Amounts are presented as µg per mg dried weight. Asterisk (*) indicate significant difference from batch culture.

The concentration of total FA in *S. aureus* was relatively the same between biofilm and planktonic cells (Figure 4.6). The increase in concentration of branched-FA was not significant (t-test, $p = 0.43$). Trace amounts of MUFAs were found in biofilm cultures just as in planktonic cultures (~0.2 μg/mg, which accounted for <0.5% of the total FA). SAFA was much lower in biofilm (t-test, $p < 0.001$), representing 5.3% of the total FA in biofilm compared to 12.7% in planktonic cultures.

The SAFAs 18:0 and 20:0 showed significant decrease between biofilm and planktonic cultures; both of which were the more abundant SAFAs found in *S. aureus* (Table 4.4). Decreases in the proportion of 20:0 SAFA have been observed in *Staphylococcus haemolyticus* under organic solvent stress (i.e. toluene and benzene), but this change was accompanied by increases in the proportion of *anteiso*-FA so that the bacterium could increase its membrane fluidity (Nielsen *et al.* 2005). Although changes in the concentration of 14:0, 16:0 and 19:0 between planktonic and biofilm cultures were not statistically significant, it was intriguing to find that there were decreases in the even-number SAFA and increases in the odd-number SAFA in biofilms. Perhaps the physiological condition in biofilms may have some influence on the mechanism of odd-chain FA synthesis, but the high variability and low sample size in the results preclude us from drawing any formal conclusions.

Table 4.4: Concentration (±SE) and percent change of individual SAFA in *S. aureus* batch planktonic and biofilm cultures. Asterisk (*) indicate statistically significantly differences in concentration ($p < 0.05$).

	Planktonic (μg/mg) (n=5)	Biofilm (μg/mg) (n=8)	% change
Even chain SAFA			
14:0	0.25 ± 0.08	0.10 ± 0.04	-57.7
16:0	1.44 ± 0.37	1.02 ± 0.12	-29.0
18:0 *	4.63 ± 0.76	2.25 ± 0.20	-51.4
20:0 *	5.77 ± 0.05	1.16 ± 0.22	-79.8
Odd chain SAFA			
17:0 *	0.08 ± 0.01	0.22 ± 0.02	+64.7
19:0	0.20 ± 0.07	0.35 ± 0.09	+43.9

4.6 Fatty acids in extracellular polymeric substance (EPS)

The challenge in EPS analysis had always been finding an extraction method which retains the molecule(s) of interest without simultaneously disrupting the cells. Extractions using cation exchange resins have been shown to be effective in analyzing protein and DNA in EPS (Frolund *et al.*, 1996). However, the resin may retain the FA as trial FA analysis on activated-sludge EPS extract that used cation exchange resin found no trace of FA signals (results not shown). Chemical extraction methods are also available using EDTA, formaldehyde and/or sodium hydroxide (Liu and Fang, 2002), but they were not used in this study because of their potential to oxidize and otherwise modify FA. Physical separation by centrifugation was successful in separating the polysaccharide component of EPS and is, by far, the simplest method (Evans and Linker, 1973), but our initial trials found immense amounts of FA in the extract (results not shown). We suggest that centrifugation at 4000 rpm may not be sufficient to completely pellet all the cells; therefore an addition filtration step was applied in our study to remove all traces of cells in the supernatant and obtain the EPS extract in the eluate.

Since biofilm cultures have different FA composition than the planktonic cultures (see above), the next step was to determine how the EPS contributes to the observed differences. FA analyses on the EPS were able to detect presence of FA, but only trace concentrations were detected (~1 to 2 µg/mg dry weight) (Table 4.5). Results from the previous section demonstrated that *P. aeruginosa* biofilms have greater amounts of branched FA, less SAFA and cyclopropane FA compared to planktonic cultures, but only trace quantities of branched-FA (~0.07 µg/mg) found in the EPS. This suggests that the difference in FA profile between biofilm and planktonic *P. aeruginosa* was likely due to the cellular physiology, rather than accumulation of lipid material in the EPS. Hydroxy FA represents the largest proportion EPS FA composed of 3-OH

10:0, 2-OH 12:0 and 3-OH 12:0. These FA are most likely derived from the rhamnolipid secreted by *P. aeruginosa*.

SAFA found in *S. aureus* EPS consisted of the ubiquitous 16:0 and 18:0 and none of the shorter (14:0) or odd-number SAFAs (19:0 and 20:0). The largest proportion of FA found in *S. aureus* EPS were the branched-chain FA (65.0%). The proportion of *iso*-branched and *anteiso*-branched chain FA were similar (~30.0% each), whereas in the cells there was a larger proportion *anteiso*-FA (*a*-15:0 and *a*-17:0). The *i*-19:0 and *a*-19:0 FA present in the cells were not found in the EPS.

Table 4.5: Comparison of major FA classes in the filtered supernatant (EPS) and pelleted cells of six day old *P. aeruginosa* and *S. aureus* biofilm cultures. Amounts are presented as μg per mg dried weight. Percent show in brackets ( ) are the proportion of the amount in total FA detected. (-) = not detected.

	P. aeruginosa (n=3)		*S. aureus* (n=1)	
	Supernatant (EPS)	Cells	Supernatant (EPS)	Cells
∑Hydroxy FA	0.41 (47.9%)	10.75 (12.9%)	-	0.14 (0.4%)
∑Branched FA	0.07 (8.6%)	3.68 (4.4%)	1.19 (65.0%)	30.41 (86.2%)
∑*iso*	0.07 (8.6%)	2.94 (3.5%)	0.56 (31.0%)	9.00 (25.5%)
∑*anteiso*	-	0.74 (0.9%)	0.62 (34.0%)	21.41 (60.7%)
∑Cyclic FA	-	3.30 (4.0%)	-	-
∑SAFA	0.31 (36.4%)	20.24 (24.3%)	0.57 (31.4%)	4.41 (12.5%)
∑MUFA	0.25 (28.9%)	45.22 (54.4%)	0.05 (2.5%)	0.34 (1.0%)
∑*Cis*	0.25 (28.9%)	43.24 (52.0%)	0.05 (2.5%)	-
∑*Trans*	-	1.98 (2.4%)	-	0.34 (1.0%)
Total	0.85	83.19	1.83	35.30

4.7 Biofilm grazing by *Hyalella azteca*

In order to determine whether *H. azteca* grazes and consume the biofilm, a feeding trial with GFP tagged *P. aeruginosa* (CTO7::*gfp-2*) biofilm on cotton gauze was performed. Fluorescent signal from CTO7::*gfp-2* on the cotton gauze and the transfer of fluorescent signal to the *H. azteca* would provide an indication of biofilm grazing. Figure 4.7 shows two threads pulled from the cotton gauze. The thread on the left (A) was not exposed to *H. azteca* grazing

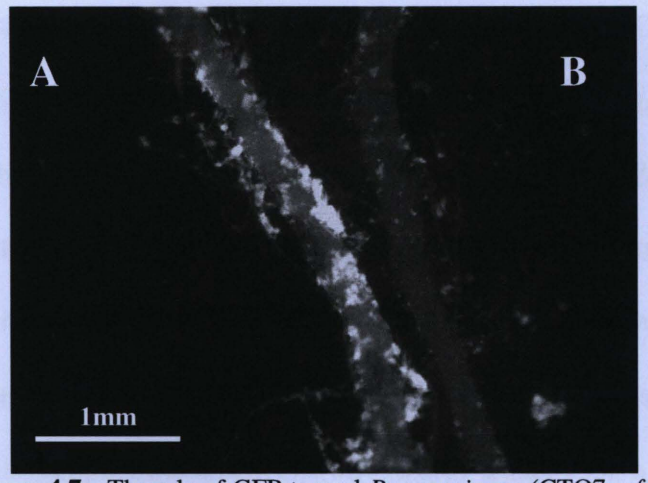


Figure 4.7: Threads of GFP tagged *P. aeruginosa* (CTO7::*gfp-2*) biofilm covered cotton gauze viewed under 10 x magnifications using a dissection microscope equipped with UV light. The bright white color indicates green fluorescent signal. A) Not exposed to *H. azteca* grazing. B) Exposed to *H. azteca* grazing for 1 day.

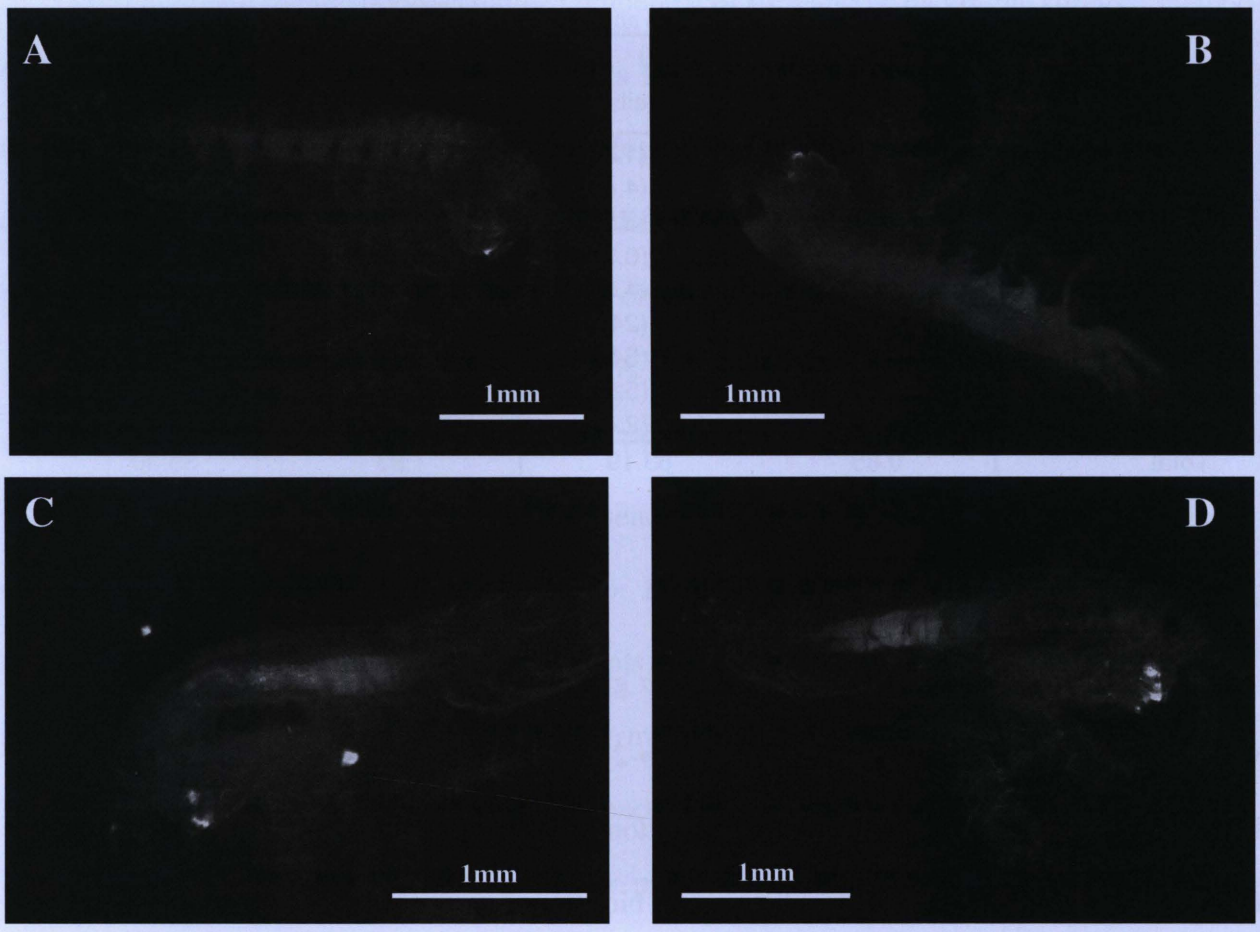


Figure 4.8: *H. azteca* from the stock culture tank without exposure to CTO7::*gfp-2* biofilm (A and B), and *H. azteca* exposed to CTO7::*gfp-2* biofilm for one day (C and D). The images were taken at 10 x magnifications with UV light and green fluorescent filter. White color indicates the green fluorescent signal.

and the fluorescent signal of CTO7::*gfp-2* biofilm can be seen in patches. The thread on the right (B) was exposed to *H. azteca* grazing and the CTO7::*gfp-2* biofilm accumulation was absent; likely consumed by the *H. azteca*.

Figure 4.8 shows images of *H. azteca* without (Figure 4.8A & B) and with (Figure 4.8C & D) exposure to CTO7::*gfp-2* covered cotton gauze for 1 d. Fluorescent signal can be seen between the foregut and mid-gut of the *H. azteca* in Figure 4.8C and near the hind-gut in figure 4.8D. Fluorescent signal can also be seen on the mandibles of both *H. azteca* specimens. A total of 4 amphipods were tested in this experiment, the other two expressed similar fluorescent pattern in the mid-gut (not shown). These results confirm that *H. azteca* actually consumed the biofilm growing on the cotton gauze.

The fact that the fluorescent signals are only seen in one section of the intestinal tract suggests the *H. azteca* may go through alternate periods of feeding and digestion to allow food to pass through its gut. This observation also demonstrates the importance of depuration time before collecting the *H. azteca* in a feeding trial to prevent batches of food remaining in the intestinal tract that can skew the FA signals in our investigation.

A concern that arose from the results is that the GFP signal was still observed in the hind-gut (Figure 4.8D). Hepatopancreatic caeca in the mid-gut are the sole source of digestive enzymes; it is the main site for nutrient absorption and storage in amphipods (Schmitz and Scherrey, 1983). Content that passed into the hind-gut should be fully digested and ready to be excreted. The presence of fluorescent signal would suggest the GFP, or perhaps the whole cells, may have only been ingested, and not fully digested. This would also mean bacteria specific diet may not provide the necessary nutritional requirement for *H. azteca* since they simply pass through the digestive system. Alternatively, the GFP may have passed through the intestinal

tract without modification. Investigating *H. azteca*'s FA profile should provide some insight into whether the ingested bacteria were digested and metabolized. Further experiments may be necessary through a more direct approach by dissection to extract and analyze the *H. azteca* gut content, which would be able to provide more solid evidence to the status of the ingested bacterial cells.

4.8 Starvation of *H. azteca* to determine basal FA profile

Amphipods without feed were analyzed to determine their basal FA profile, at the same time, to assess how *H. azteca* FA composition would respond to extended periods of fasting. Examples of *H. azteca* FA profiles can be seen in Appendix D. Similar to literature reported values for gammarids, the 18:1n9 MUFA and 16:0 SAFA are the most prominent FA; levels of ω-3 PUFA ranged between 11-31% of total FA, among which proportion of LIN is the highest (5-18%), followed by EPA (5-16%). Only trace quantity of DPA (0.5-2.4%) and DHA (1.5-4.2%) have been observed in gammarids (Kolanowski *et al.*, 2007). As with the European fresh water amphipod *Dikerogammarus villosus* (Maazouzi *et al.*, 2007) we found greater proportion of C18-PUFA (LIN and ALA) and less C20-PUFA (ARA, EPA, DPA and DHA) in *H. azteca*.

Figure 4.9 shows the proportion of the three major classes of FA found in the *H. azteca* under various fasting periods. Proportion of SAFA fluctuated between different fasting periods, but differences were not significant (ANOVA, $p = 0.40$). The proportion of MUFA remained relatively constant (~19.8 to 25.3%) (ANOVA, $p = 0.89$) and there was no significant difference in the proportion of PUFA (KW test, $p = 0.10$) between all fasting periods. ω-6 PUFAs accounted for 23.2% of total FA in the control and 13.6% for ω-3; the ratio of ω-6 to ω-3 FA (~1.6:1) was consistent across all feeding periods. Bacteria-specific hydroxyl FA and

cyclopropane FA were not detected in the *H. azteca* specimens, and only trace amounts (< 1% of total FA) of C15 and/or C17 branched-chain FA were present.

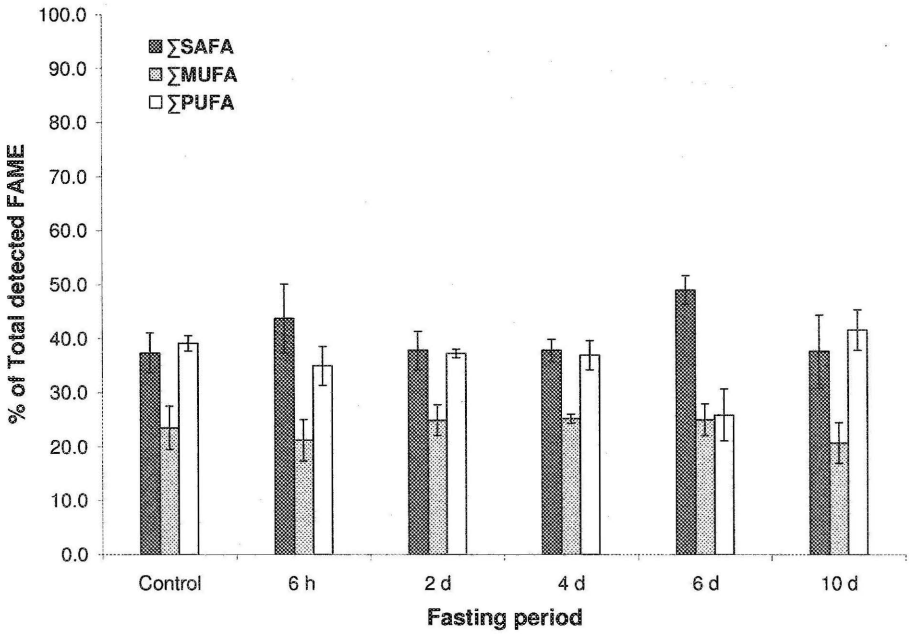


Figure 4.9: Proportion of major FA classes found in *H. azteca* under different starvation periods. Control group consisted of *H. azteca* directly from the stock culture tank without going through any period of starvation. h = hour, d = day. No significant differences were found for all FA classes between groups.

Although the essential fatty acid ALA (18:3n3), precursor to the ω-3 FA EPA (20:5n3) and DHA (22:6n3) appear to have decreased with increasing fasting period (Figure 4.10), the difference between the groups were not statistically significant (ANOVA, $p = 0.08$). Drastic fluctuations can be seen in the proportion of EPA between the fasting periods but there was no significant difference (ANOVA; $p = 0.06$); this is caused by the high variability within the each fasting period. Proportion of DPA ranged between 0.4-1.2% (not included in the figure) and DHA between 1.5-2.2%, changes in both FA were insignificant as well (DPA, ANOVA, $p = 0.28$; DHA, KW test, $p = 0.47$).

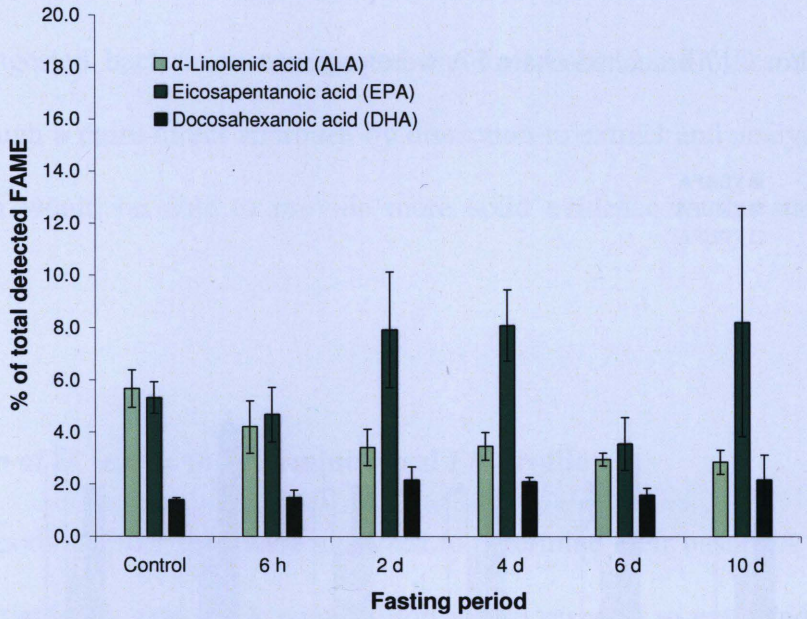


Figure 4.10: Changes in proportion of the three ω-3 PUFAs found in *H. azteca* with increasing period of fasting. The control group consisted of *H. azteca* directly from the stock culture tank. h = hour, d = day. DPA was not included in the figure because it represented <1% of total FA. No significant differences were found for all ω-3 PUFAs.

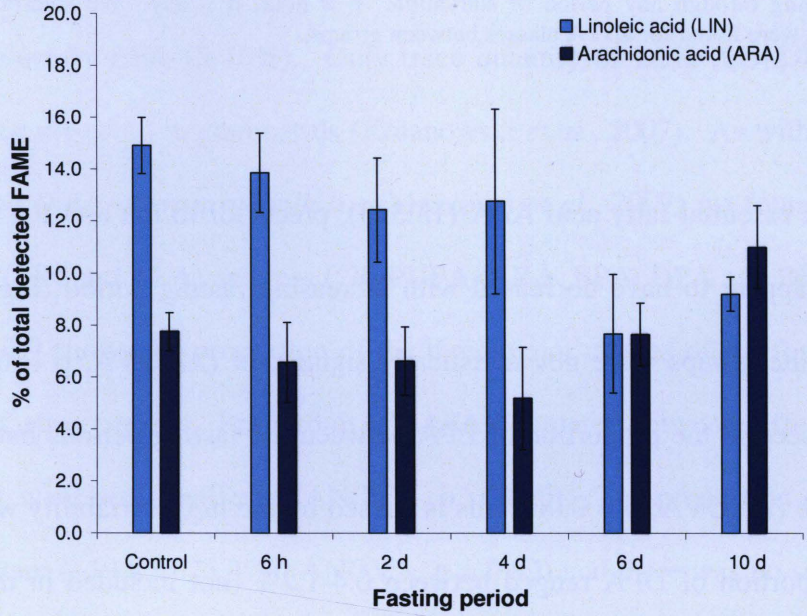


Figure 4.11: Changes in proportion of the two ω-6 PUFAs found in *H. azteca* with increasing starvation period. Control group consisted of *H. azteca* directly from the stock culture tank without going through any period of starvation. h = hour, d = day. No significant differences were found for all ω-6 PUFAs.

Although the differences in the proportion of both LIN (18:2n6) and ARA (20:4n6) were not statistically significant (ANOVA, $p \geq 0.20$), the proportion of LIN did appear to have decreased from 4 d to 6 d of fasting (Figure 4.11).

The difference in the concentration of total FA among all groups; ranging from 36.8-67.9 µg/mg, were not significant. It was somewhat unexpected to find *H. azteca* FA to remain so consistent during all stages of the starvation period. Large marine amphipods fed on detrital microbiota have been found to exhibit TAG-phospholipid ratio ~ 0.7 and starvation for a week depresses that ratio to ~0.13 due to loss of TAG (Budge *et al.*, 2006). This loss should translate to decrease in total FA or shift in the FA classes. Reproduction had been found to be a major drain on EPA from female *Daphnia magna* as EPA are selectively stored in eggs (Park *et al.*, 2002). The highly fluctuating proportions of EPA in our experimental fasting regime may be caused by presence of females at different stages of egg development.

Overall, despite the lack of statistical significance, the proportion of PUFA precursors (i.e. ALA and LIN) did show a decreasing trend with starvation. The effect may not have been very pronounced because of the high variability in our results. In addition, the fasting period may not be long enough for the changes to manifest as essential fatty acids are typically the last to be catabolized. The estuarine amphipod *Melita plumulosa* demonstrate an ability to retain ω-3 PUFA as evident by having higher levels of ω-3 PUFA than what was available for them in their food source (Hyne *et al.*, 2009). Our fasting experiment would also suggest *H. azteca* has the capacity to retain PUFA up to 10 d.

4.9 *Hyalella azteca* fed with biofilm

The statistical powers for all the comparisons in the *H. azteca* feeding trials fell below the desire value (i.e. power < 0.800), which means it was less likely to detect a difference when one actually exists. A way to improve this is to increase the sample size for each treatment group. Power analysis using data from the *P. aeruginosa* biofilm feeding experiments indicate upward of 150 samples per group are required in order to obtain desirable statistical power, which was outside the realm of possibility in the current study. Therefore, current results should be considered as preliminary assessments, further studies with larger sample size are necessary to achieve better resolution in our comparisons.

4.9.1 Transfer of bacterial FA signatures

One of the unique features of bacteria is their ability to synthesize odd number SAFA and terminal branched *iso* or *anteiso*-FA. When accumulated, these FA can potentially be used as indicator of bacterial contribution (Maazouzi *et al.*, 2007). Figure 4.12 show the proportion of odd number SAFA + branched-FA in *H. azteca* fed with *P. aeruginosa* and *S. aureus*. In the control group, *H. azteca* from the stock culture tank showed greater proportion than the *H. azteca* that underwent 10 d fasting. The stock culture tank contained complex populations of microorganisms, it is reasonable to expect some level of bacterial input. The 10 d fasted *H. azteca* showed a wide error bar because two out of the three samples actually had no odd-number SAFA or branched-chain FA.

H. azteca fed with either *P. aeruginosa* or *S. aureus* showed greater proportion of odd number SAFA and branched-FA than the control groups, with the exception of when the feeding period reaches 8 d. A factor that was missing from our experimental design that may explain this unexpected shift in the FA pattern was the duration of instars and molting throughout *H. azteca*'s

development. One of the physiological and behavioral alterations that come with molting is an accompanying fasting period. For example, Pacific white shrimp (*Litopenaeus vannamei*) exhibit a fasting period up to 5 d during molting that leads to decreases in protein, glycogen and lipid (Sánchez-Paz *et al.*, 2007). Morris *et al.* (1987) also found that the lipid composition of the crustacean *Gammarus duebeni*'s FA in gill membrane was influenced by the onset of molting. The *H. azteca* in this study was selected based on a size that roughly correlated to the adolescent stage of *H. azteca* development and the duration of the instars during adolescent stage had been shown to be ~8 to 10 d (Geisler, 1944).

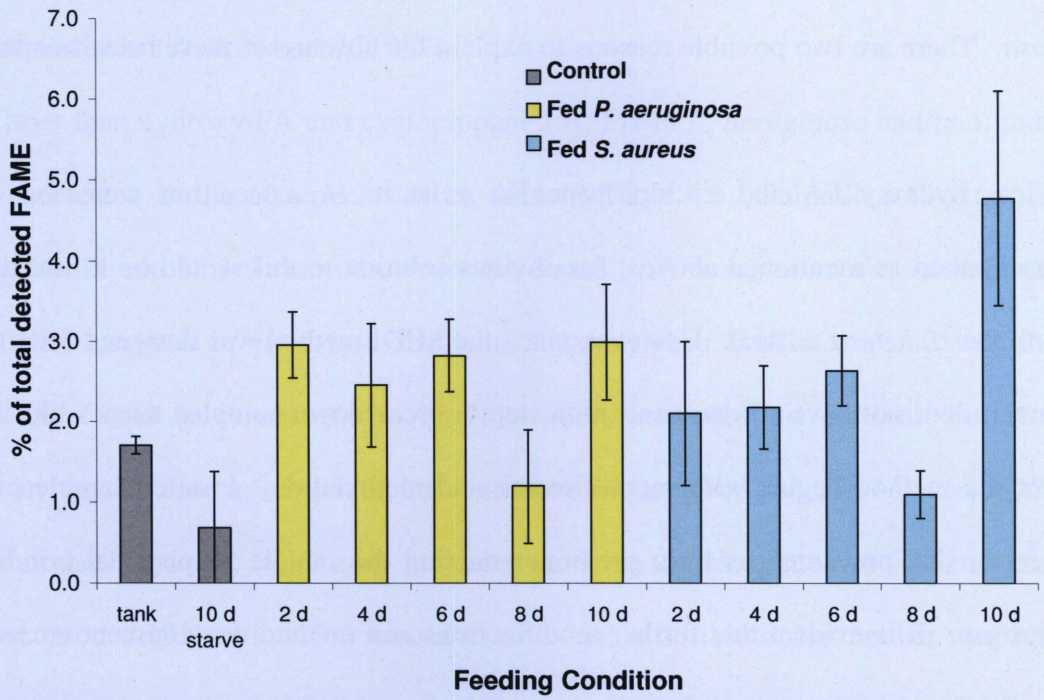


Figure 4.12: Comparison of the sum of the amount of odd-chain SAFA and branched FA detected in *H. azteca* fed with *P. aeruginosa* or *S. aureus* biofilm. Amounts are presented as µg per mg sample dry weight. Two control groups consisted of *H. azteca* from the stock culture tank and *H. azteca* starved for 10 d as negative control.

Over the course of the study, it became clear that the chloroform-methanol FA extraction method for *H. azteca* can not retain the hydroxy FA and cyclopropane FA. Initial trial

experiments with the chloroform-methanol method on bacterial samples were unable to detect either hydroxy FA or cyclopropane FA (result not shown). This may be due to the extended methylation period (15 h) being too harsh and causing degradation of cyclopropane FA, or because the more volatile hydroxy FA are simply lost to the atmosphere. Therefore, it was necessary to adopt the MIDI method that was originally designed for microbial FA analysis to extract FA from bacterial samples in this study. When using the chloroform-methanol method for the *H. azteca*, neither hydroxy FA nor cyclopropane FA was observed in any sample. This was unfortunate as these FA can be considered reliable markers for bacteria and often in considerable quantities, especially cyclopropane FA for Gram-negative bacteria like *P. aeruginosa*. There are two possible reasons to explain the absence of these bacteria-specific FA markers:

First, hydroxy FA and cyclopropane FA exist in *H. azteca* but were lost due the extraction method as mentioned above. An obvious solution to this would be to use the MIDI method on the *H. azteca* as well. However, since the MIDI method was designed for single cell organisms, it does not have a homogenization step to break down complex tissues like an entire *H. azteca*; the method begins with saponification and methylation. Limited sample mass was another factor that prevented us from pre-homogenizing the sample by physical grinding with liquid nitrogen. It is evident that further modifications and method development are necessary (see 'Conclusion and future perspectives').

Second, both hydroxy FA and cyclopropane FA may not be retained or they may be rapidly modified by the *H. azteca*. Not all FA provide equal information about diet due to predator metabolism; short or medium chain FA (i.e. <C14) are often immediately oxidized when consumed (Iverson *et al.*, 2004). Hydroxy FA derives from Lipid-A of LPS from Gram

negative bacteria and rhamnolipid of *P. aeruginosa*, both are related to bacteria-specific structure and functions. There is no evidence to indicate hydroxy FA can participate as FA chain in membrane phospholipids or storage TAG. Although in Gram negative bacteria, cyclopropane FA has a similar function as MUFA to modify membrane integrity, to our knowledge, such mechanism have not been reported in amphipods. In fact, although the presence of hydroxy FA and cyclopropane FA is clearly associated with Gram negative bacteria, ω-7 MUFAs (and/or branched-FA) are generally considered as microbial markers, for example, in mesograzers' fecal pellets (Itoh *et al.*, 2007), intertidal sediment (Van Oevelen *et al.*, 2006), crustacean (Phleger *et al.*, 2005), gammarids (Makhutova *et al.*, 2003), copepods (Goedkoop *et al.*, 2000; Stevens *et al.*, 2004) and mollusks (Zhukova *et al.*, 1992).

Other than hydroxy FA and cyclopropane FA, FA in *P. aeruginosa* and/or *S. aureus* that were also detected in the *H. azteca* can be seen in Table 4.6. Palmitic acid (16:0) and ω-7 MUFA (16:1n7c and 18:1n7c) are the major FA found in *P. aeruginosa*, but neither were found in significant amounts in *H. azteca* fed with *P. aeruginosa* biofilm. *i*-15:0 was the major branched-FA found in *P. aeruginosa* biofilms (shown in previous section), but it was not detected in the *H. azteca*. Instead, trace amount of branched FA (*i*-17:0 and *a*-17:0) was observed in the *H. azteca*, which may have derived from the elongation of C15 branched-FA it consumed. Both *i*-15:0 and *a*-15:0 were also abundant in *S. aureus*, but neither was detected in *H. azteca* fed with *S. aureus*. C17 branched-FA were two of the more abundant FA found in *S. aureus*, especially *a*-17:0, but C17 branched-FA were only detected in *H. azteca* fed with *S. aureus* up to 6 d. Even then, the amounts were not significantly greater than *P. aeruginosa* fed *H. azteca*.

Therefore, regardless of diet, *H. azteca* demonstrated a lack of capacity to utilize or retain bacteria-specific branched-FA. Branched-chain FA in bivalve mollusks can contribute from 3.4 to 7.7% of total FA, but such high proportion originated from sulfide-oxidizing endosymbiotic bacteria (Zhukova *et al.*, 1992). Some bivalves showed the proportion of bacteria-derived 18:1n7 to be as high as 14%, so combination of 18:1n7 and branched-chain FA was appropriate as the biochemical indicator of bacteria symbionts in mollusks. But different groups of meiobenthic/macrobenthic organisms have variable capacity to incorporate bacterial PLFA through diet, both in amount and duration. Copepods for example had only a marginal increase in bacteria-derived PLFA signal, which the authors concluded that bacterial FA has limited contribution to benthic organisms' total carbon requirement (Van Oevelen *et al.*, 2006).

Overall, it was difficult to identify any obvious FA profile patterns in the *H. azteca* that coincide with a bacteria-based diet. Notable difference in the appearance of C17 branched-FA and slightly higher amount of SAFA, such as 16:0, 18:0 and 20:0, did not specifically coincide with FA profile of the test bacterial strains, nor did it follow an increasing trend over extended feeding period as one would normally expect.

Table 4.6: Concentration of specific FA found in the *H. azteca* using chloroform-methanol extraction method that were also found in the model bacterial species *P. aeruginosa* and *S. aureus*. The amounts are presented as µg per mg dry weight. Each FA is color coded according to which bacterial species it can be found in; (yellow) is *P. aeruginosa*, (blue) is *S. aureus*, and (green) can be found in both. Two control groups consist of *H. azteca* directly from stock culture tank (0 d) and amphipods without feed for 10 d.

	Controls (no feed)		Fed with *P. aeruginosa*					Fed with *S. aureus*				
	0 d	10 d	2 d	4 d	6 d	8 d	10 d	2 d	4 d	6 d	8 d	10 d
SAFA												
12:0	-	0.35	0.14	0.11	0.55	0.20	0.21	0.17	0.29	0.21	0.17	0.21
14:0	0.84	5.65	0.89	5.38	1.27	0.17	2.37	0.67	2.50	4.42	0.23	2.14
15:0	-	-	0.13	0.24	0.16	-	0.19	0.23	0.54	0.32	0.25	1.82
16:0 [P]	9.24	6.85	8.18	11.83	6.84	4.83	7.64	9.12	13.06	16.80	4.17	9.56
17:0	0.64	0.78	0.68	0.77	0.57	0.44	0.51	0.64	0.76	0.89	0.21	0.93
18:0	2.93	3.72	3.56	8.83	3.83	1.87	4.67	5.12	8.34	14.26	1.52	5.84
19:0	-	-	-	-	-	-	-	-	-	-	-	-
20:0	-	0.22	0.26	0.51	0.45	0.08	0.09	0.76	0.29	0.64	-	-
Branched FA												
i-15:0 [S]	-	-	-	-	-	-	-	-	-	-	-	-
a-15:0 [S]	-	-	-	-	-	-	-	-	-	-	-	-
i-16:0	-	-	-	-	-	-	-	-	-	-	-	-
i-17:0	-	-	0.17	0.17	-	0.10	0.14	0.30	0.18	0.29	-	-
a-17:0 [S]	-	-	0.41	0.58	0.46	0.21	0.44	0.63	0.23	0.53	-	-
i-19:0	-	-	-	-	-	-	-	-	-	-	-	-
a-19:0	-	-	-	-	-	-	-	-	-	-	-	-
MUFA												
16:1n7c [P]	1.19	0.47	0.47	0.58	0.29	0.18	0.26	0.39	0.49	0.18	-	0.64
16:1n7t	-	-	-	-	-	-	-	-	-	-	-	-
18:1n7c [P]	0.80	1.48	0.39	1.97	0.92	0.47	1.89	0.75	2.37	4.76	0.10	2.78
18:1n7t	-	-	-	-	-	-	-	-	-	-	-	-

[P] = FA that was found in greater amount in *P. aeruginosa* (>5.0µg/mg)
[S] = FA that was found in greater amount in *S. aureus* (>5.0µg/mg)
- = Not detected

4.9.2 Nutritional value of bacteria-specific diet - P. aeruginosa biofilms as food

The proportion of total PUFA and the ω-3 (ALA, EPA, and DHA); ω-6 (LIN and ARA) PUFAs were used to assess the nutritional condition of *H. azteca*. For *H. azteca* fed with *P. aeruginosa* biofilms (Figure 4.13), changes in the proportions of SAFA (ANOVA, $p = 0.10$) and MUFA (ANOVA, $p = 0.96$) were not significant.

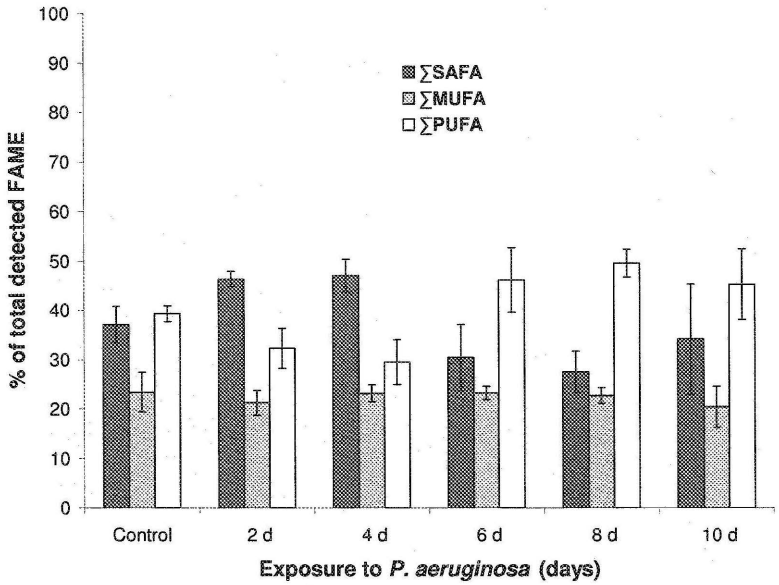


Figure 4.13: Proportion of the three major FA classes in *H. azteca* fed with *P. aeruginosa* biofilm over a 10 d period. Amounts shown are proportions to the total detected FA. Control group consist of *H. azteca* from the stock culture tank. No significant differences were found in each FA classes.

Proportion of PUFA was not significant either (KW test, $p = 0.07$). Comparison of individual PUFAs showed that the proportion of ω-3 ALA was significantly lowered on Day 4 compare to the control (ANOVA, $p = 0.01$; Tukey's test, $p = 0.01$). Proportions of EPA, DPA and DHA were consistent for all feeding conditions (Figure 4.14). Changes in the most abundant ω-6 LIN were not significant (ANOVA, $p = 0.26$). The proportion of ARA (ANOVA, $p = 0.02$) however, was significantly higher in *H. azteca* fed with *P. aeruginosa* for 6, 8 and 10 d when compared with other feeding conditions (Tukey's test, $p < 0.05$).

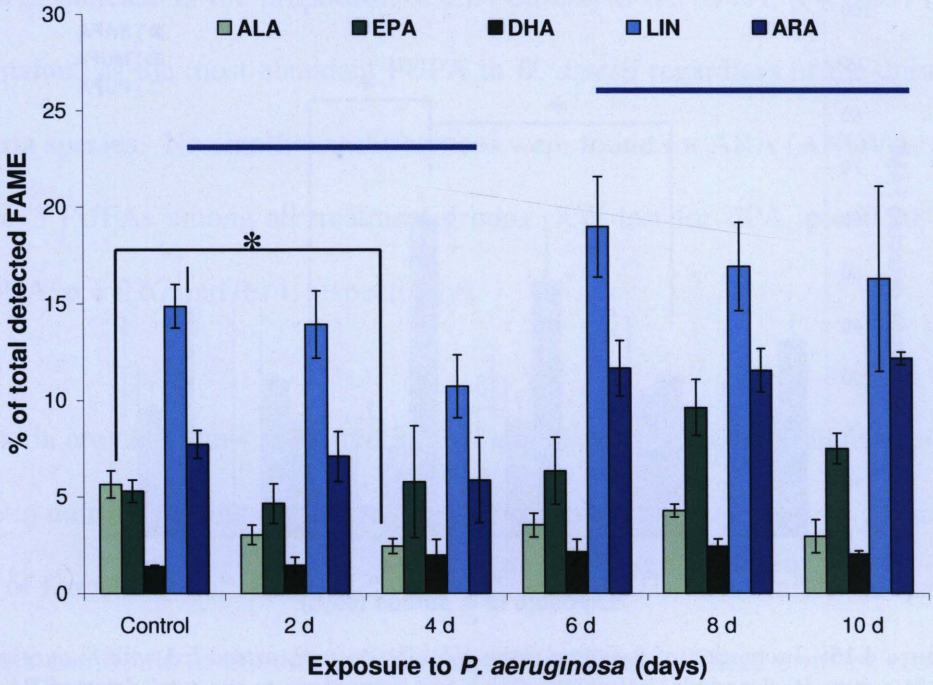


Figure 4.14: Proportion of essential ω-3 FA ALA, EPA, DHA and ω-6 FA LIN, ARA found in *H. azteca* fed with *P. aeruginosa* biofilm over 10 d period. DPA was not included in the figure because it represented <1% of total FA. Amounts shown are proportions to the total detected FA. Control group consist of *H. azteca* from the stock culture tank. For Tukey's test result, asterisk (*) indicate significant difference ($p < 0.05$), and lines connecting the treatment groups indicate no significance for FA of the corresponding color code in the legend.

4.9.3 Nutritional value of bacteria-specific diet S. aureus biofilms as food

The proportion of MUFA also remained consistent in *H. azteca* fed with *S. aureus* biofilm over time (ANOVA, $p = 0.35$) (Figure 4.15). Significant differences were found in the SAFA among the groups (ANOVA, $p = 0.02$), but this was largely due to the huge spike in SAFA on Day 6 (Tukey's test, $p < 0.05$ compared to Day 2 and Day 8). Conversely, the proportion of PUFA conversely was lowest on Day 6, but the difference was only significant when compared to Day 8 (ANOVA, $p = 0.03$; Tukey's test, $p = 0.04$). No significant differences were found between the control and other treatment groups.

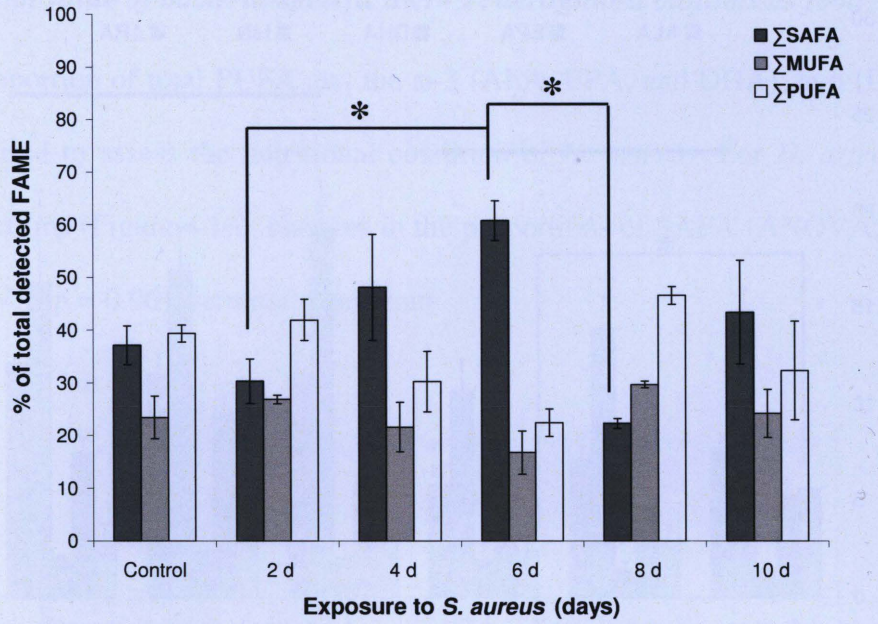


Figure 4.15: Proportion of the three major FA classes in *H. azteca* fed with *S. aureus* biofilm over 10 d periods. Amounts shown are proportions to the total detected FA. Control group consist of *H. azteca* from the stock culture tank directly. For Tukey's test result, asterisk (*) indicate significant difference ($p < 0.05$)

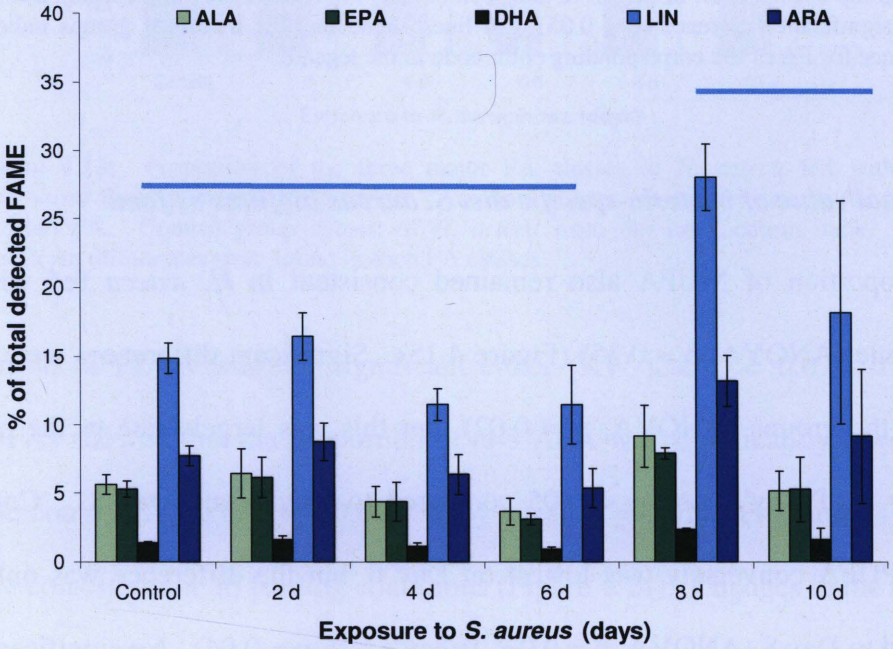


Figure 4.16: Proportion of essential ω-3 FA ALA, EPA, DHA and ω-6 FA LIN, ARA found in *H. azteca* fed with *S. aureus* biofilm over 10 d period. DPA was not included in the figure because it represented <1% of total FA. Amounts shown are proportions to the total detected FA. Control group consist of *H. azteca* from the stock culture tank directly. Lines connecting the treatment groups show Tukey's test result with no significance for FA of the corresponding color code in the legend.

A large increase in the proportion of LIN on Day 8 (ANOVA, $p = 0.03$) (Figure 4.16), but LIN remained as the most abundant PUFA in *H. azteca* regardless of the duration fed with either bacteria species. No significant differences were found for ARA (ANOVA, $p = 0.22$) and any of the ω-3 PUFAs among all treatment groups (KW test for EPA, $p = 0.20$; ANOVA for DPA and DHA, $p = 0.67$ and 0.14, respectively).

Bacteria are ubiquitous and often in high abundance in aquatic sediments, so, in addition to their role in nutrient cycling and degradation of organic matter they can be considered a major component of faunal diets of sediment-dwellers (Nichol, 2003). This raises the question of how much bacteria contribute to the diet and nutritional condition of these primary consumers. Van Oevelen *et al.* (2006) attempted to answer this question by using stable isotope-labeled glucose as carbon supply. It was found that bacterial contribution to the carbon intake was not considered significant; only 3% of ingested bacteria were actually assimilated during gut passage for the annelid *Heteromastus filiformis*. Much of the results presented in this study appear to support this finding.

Using PUFA to assess nutritional status, this study looked into the trophic contribution of bacteria to the freshwater amphipod *H. azteca*. Feeding trials with the model bacteria *P. aeruginosa* and *S. aureus* found insignificant changes in the *H. azteca* FA profile up to 10 d. Microscope images involving fluorescent labeled bacteria strain found strong fluorescent signal in the hind gut; combined with lack of bacteria-specific FA markers in *H. azteca* perhaps suggest that the majority of the bacteria could potentially being consumed but not digested.

A small amount of ω-3 PUFA was always present in the *H. azteca*, although the proportion of ALA, EPA, DPA and DHA were not significantly affected by the strict-bacteria

diet. ω-6 PUFAs represented a major portion of the total PUFA in the *H. azteca*. LIN specifically can reach as high as 25% of the total FA, comparable to the proportion of total MUFA. Proportions of LIN fluctuated quite drastically among the different feeding conditions, but it did not appear to have any correlation to the bacteria-specific diet or the duration of the feeding. Overall, our results did not show any clear-cut changes in *H. azteca*'s nutritional condition when fed strictly with *P. aeruginosa* or *S. aureus* biofilm. In some cases, the concentration of ω-6 LIN was higher while consuming bacteria without PUFA. These results may once again emphasize their ability to retain PUFA from previous diet as demonstrated by *H. azteca*'s ability to maintain consistent level of PUFA from our fasting experiments (see above). Alternatively, intestinal or previously ingested microbiota may also contribute to the PUFA detected in *H. azteca*; cyanobacteria and chlorphyta for example have been known to be able to synthesize LIN (Arts *et al.*, 2009). Gut content analysis or additional treatment (e.g. antibiotics) to remove all intestinal microbiota will be necessary to test this speculation.

Furthermore, EPS may be an alternative nutrient source for the *H. azteca*. Joubert *et al.*, (2006) have shown preferential grazing on non-cellular biofilm matrix by protists; it was proposed that the extracellular polymeric matrix may serve as source of nutrients and energy for protists. As pointed out in the earlier section that the amount of lipids in the EPS are in trace quantities, so perhaps the abundance of carbohydrate and/or protein in the EPS were sufficient to sustain the amphipods without the need for PUFA catabolism or dietary lipid intake within the duration of the feeding experiments.

Chapter 5: CONCLUSIONS AND FUTURE PERSPECTIVES

In the course of this study, bacterial biofilm FA were evaluated in two ways:

First, FA profiles of biofilm cultures were compared with batch planktonic bacterial cultures to evaluate FA physiology of these two distinct bacterial growth behaviors. In terms of FA, biofilm cultures of *P. aeruginosa* and *S. aureus* were able to reach stable state within 2 d and remained consistent up to 6 d. Biofilms showed reduced concentrations of SAFA for both of *P. aeruginosa* and *S. aureus* when compared to their respective batch planktonic cultures. Recent work by Bester *et al.* (in submission) was able to use carbon dioxide production rate as a measure of metabolic activity to differentiate the various biofilm phases. Their bubble perturbation technique can potentially be used to separate populations in various biofilm layers and correlate their FA composition to the overall biofilm profile and associate with their respective metabolic and physiological conditions. We did not find sufficient amount of FA in biofilm EPS to consider it having major contribution to the observed differences in the overall FA profile between biofilm and batch planktonic cultures. Therefore, the differences in FA that we observed between bacteria in the biofilm versus the planktonic phases most likely derived from the bacteria's intracellular physiology. However, extracellular signaling molecules are typically highly specific and can be functional at a concentration below the detection limits for these compounds. Therefore, our results do not unequivocally rule out the presence of extracellular lipidic molecules associated with cell-to-cell signaling in biofilm (Barber *et al.*, 1997; Davies and Marques, 2009).

Second, the FA profile of *H. azteca* grazing on a pure biofilm diet was compared to evaluate the nutritional contribution of biofilm to *H. azteca*. Feeding trials with a GFP-labeled bacterial strain provided a clear indication that *H. azteca* ingested biofilm. In a 10-day long fasting experiment it was observed that *H. azteca* did not exhibit any substantial changes in their

ω-3 and ω-6 PUFA proportion, perhaps indicative of their ability to retain PUFA. Further trials with longer fasting period may be necessary for such changes to manifest themselves. The ability of *H. azteca* to retain PUFA would also mean that biofilm diets are unlikely to have significant impacts on PUFA profiles of the *H. azteca* over the 10 d period. This was shown in our results; with the exception of increased proportion of ARA after 6 d with *P. aeruginosa* biofilm. In addition, much of the results in the *H. azteca* feeding trial may also be masked by the variability within each group, future comparisons with larger sample size is recommended.

Certain bacteria-specific FA markers in *H. azteca* were not found in this study, nor could we demonstrate a correlation to the dietary bacterial species and feeding duration. The lipid extraction and derivatization method will need to be reconsidered in order to detect alternative bacteria-specific FA markers (i.e. hydroxyl and cyclopropane FA). Nielson *et al.* (2005) were able to detect the hydroxy FA and cyclopropane FA in bacterial samples using a modified chloroform-methanol extraction method with the addition of amylene (as stabilizer) along with potassium hydroxide base-catalyzed methylation. This is an alternative method to consider and incorporate for future *H. azteca* FA analysis. Therefore, current results are insufficient to conclude the applicability of using *H. azteca* to monitor the biofilm community by evaluating the transfer of bacteria-specific FA markers. Nutritional contribution of biofilm in *H. azteca* cannot be determined based strictly on evaluating its ω-3 and ω-6 PUFAs because they appear to be well retained by *H. azteca* up to 10 d. However, FA analyses on *P. aeruginosa* and *S. aureus* confirm they are not a source of ω-3 and ω-6 essential FA. And while *P. aeruginosa* and *S. aureus* biofilm FA profiles differ from planktonic cultures, future work to correlate with populations at different developmental stages within a biofilm community and their respective FA composition should be of great interest.

APPENDIX A – GC parameters and temperature ramping

Agilent 6890GC with Programmable Temperature Vaporization (PTV) inlet

Column: Supelco SP-2560 fused silica capillary column (100 m x 0.25 mm i.d., 0.2 µm film)
Carrier Gas: Helium at 1.2 mL/min
Oven temperature ramping:
- Initial temp: 70°C hold for 1.0 min
- Increase to 140°C at 20°C/min, hold for 5.0 min
- Increase to 240°C at 4°C/min, hold for 15.5 min

Injection volume: 2.0 mL with Agilent 7683 auto sampler
Inlet (PTV):
- Initial temp: -10°C hold for 1 min
- Increase to 40°C at 360°C/min, hold for 0.1 min
- Increase to 720°C at 260°C/min, hold for 2.0 min
- Vent time: 0.75 min at 20.0 mL/min (4.0 psi)
- Purge time: 2.0 min at 50.2 mL/min
- Gas type: Helium

Detector (FID):
- Temp: 260°C
- Hydrogen flow at 40.0 mL/min
- Air flow at 450.0 mL/min
- Nitrogen (makeup gas) flow at 45.0 mL/min

Total run time: 50.0 min Detection Limit: 0.02µg/mL

Varian CP-3800GC

Column: Agilent HP-Ultra 2 capillary column (5% phenyl-methylpolysiloxane; 25 m x 0.20 mm i.d., 0.33 µm film)
Carrier Gas: Helium at 1.0 mL/min
Oven temperature ramping:
- Initial temp: 50°C hold for 0.5 min
- Increase to 170°C at 35°C/min
- Increase to 225°C at 4°C/min
- Increase to 290°C at 15°C/min, hold for 1.0 min
- Increase to 310°C at 60°C/min, hold for 1.15min

Injection volume: 0.5 µL with Varian CP-8400 auto sampler
Inlet temperature: 300°C
- Split ratio (split vent flow/column flow): 1.0

Detector (FID):
- Temp: 300°C
- Hydrogen flow at 30 mL/min
- Air flow at 300 mL/min
- Nitrogen (makeup gas) flow at 29 mL/min

Total Rune time: 24.5 min Detection Limit: 0.02ug/mL

APPENDIX B – Methylation efficiency

Methylation efficiency test with FA standard mix composed of four different FA using different methylation incubation time. (Green) highlight indicate the incubation time with optimal yield. Asterisk (*) indicate the methylation efficiency value used in quantitative analysis in MIDI FA extraction.

FA class	Actual Conc. (ug/mL)		Methylation Period	Conc. Detected by GC (ug/mL)		Methylation efficiency (%)		Avg. methylation efficiency (%)
	Trial 1	Trial 2		Trial 1	Trial 2	Trial 1	Trial 2	
Hydroxy FA (2-OH C10:0)	49.64	24.82	5 min	24.54	9.74	49.4	39.2	44.3
			10 min	24.08	13.22	48.5	53.3	50.9 *
			20 min	24.82	11.92	50.0	48.0	49.0
			40 min	27.12	11.88	54.6	47.9	51.3
			1h 20 min	22.82	12.06	46.0	48.6	47.3
			2 h	25.30	12.94	51.0	52.1	51.6
Cis-MUFA (C18:1n7c)	25.00	12.50	5 min	16.94	7.38	67.8	59.0	63.4
			10 min	18.46	9.14	73.8	73.1	73.5 *
			20 min	18.04	8.86	72.2	70.9	71.5
			40 min	20.06	8.34	80.2	66.7	73.5
			1h 20 min	16.84	8.58	67.4	68.6	68.0
			2 h	17.02	7.76	68.1	62.1	65.1
Trans-MUFA (C18:1n7t)	50.00	25.00	5 min	38.04	16.64	76.1	66.6	71.3
			10 min	42.16	20.66	84.3	82.6	83.5 *
			20 min	41.00	20.18	82.0	80.7	81.4
			40 min	45.90	19.02	91.8	76.1	83.9
			1h 20 min	38.40	19.68	76.8	78.7	77.8
			2 h	38.92	17.58	77.8	70.3	74.1
Cyclopropane FA (Cyc-C19:0 (9,10))	51.65	25.83	5 min	35.14	15.32	68.0	59.3	63.7
			10 min	35.90	17.14	69.5	66.4	67.9 *
			20 min	27.68	12.50	53.6	48.4	51.0
			40 min	21.54	7.52	41.7	29.1	35.4
			1h 20 min	8.44	4.08	16.3	15.8	16.1
			2 h	7.04	1.70	13.6	6.6	10.1

APPENDIX C – Bacterial FA

Table C1: Example of FA profiles showing proportion of individual FA to total FA in different bacteria species from a single sample. The bacteria samples were grown in batch planktonic culture for 2 d at 37°C. Asterisk (*) shows the model bacteria species selected for this study. (-) indicate not detected.

	Gram negative		Gram positive		
FA	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*	*B. megaterium*
11:0	-	-	-	-	-
2-OH 10:0	-	-	-	-	-
3-OH 10:0	3.2	-	-	-	-
12:0	2.6	4.0	-	-	-
13:0	-	0.1	-	-	-
2-OH 12:0	5.9	-	-	-	-
3-OH 12:0	2.6	-	-	-	-
14:0	0.6	6.8	0.6	0.3	0.9
15:0i	-	-	8.2	17.4	28.3
15:0a	-	-	38.5	42.0	32.1
15:0	0.5	0.5	-	-	0.4
2-OH 14:0	-	0.4	-	-	-
3-OH 14:0	-	9.5	-	-	-
16:0i	0.1	-	1.3	3.3	3.7
16:1n7c	4.5	2.9	-	-	-
16:1n7t	0.2	-	-	-	-
16:0	32.5	35.9	1.8	3.3	7.9
17:0i	0.1	-	5.1	11.0	11.3
17:0a	-	-	10.2	17.3	8.1
17:0cyc(9,10)	3.8	24.7	-	-	-
17:0	0.5	0.3	0.2	-	0.3
2-OH 16:0	-	-	-	-	-
3-OH 16:0	-	-	-	-	-
18:0i	-	-	1.2	-	-
18:2n6	-	-	-	-	-
18:1n9c	0.4	0.6	0.3	-	0.6
18:1n7c	26.9	4.8	0.2	-	-
18:1n9t	-	-	-	-	-
18:1n7t	-	-	-	-	-
18:0	1.0	1.8	7.4	2.3	4.3
19:0i	-	-	4.4	0.5	1.8
19:0a	-	-	4.7	2.3	0.4
19:0cyc(9,10)	-	-	-	-	-
19:0cyc(11,12)	14.6	7.6	-	-	-
19:0	-	-	1.2	-	-
20:0	-	-	14.8	0.3	-
∑Hydroxy FA	11.8	9.9	-	-	-
∑Branched FA	0.1	-	73.5	93.8	85.6
∑iso	*0.1*	-	*20.2*	*32.2*	*45.1*
∑anteiso	-	-	*53.4*	*61.6*	*40.5*
∑cyclic FA	18.3	32.4	-	-	-
∑SAFA	37.8	49.4	26.0	6.2	13.8
∑MUFA	32.0	8.3	0.5	-	0.6
∑Cis	*31.8*	*8.3*	*0.5*	-	*0.6*
∑Trans	*0.2*	-	-	-	-

Table C2: FA profiles of model bacterial species *P. aeruginosa* and *S. aureus* showing the average concentration for each individual FA ± standard error. (n) Indicate sample size. Cultures were grown in batch planktonic culture for 2 d at 37°C. (-) indicate not detected.

FA	*P. aeruginosa* (n=8)	*S. aureus* (n=4)
11:0	-	-
2-OH 10:0	-	-
3-OH 10:0	3.32 ± 0.25	-
12:0	2.08 ± 0.17	-
13:0	-	-
2-OH 12:0	5.72 ± 0.49	-
3-OH 12:0	3.13 ± 0.24	-
14:0	0.54 ± 0.04	0.34 ± 0.07
15:0i	-	2.60 ± 0.23
15:0a	-	16.37 ± 0.71
15:0	0.42 ± 0.06	-
2-OH 14:0	-	-
3-OH 14:0	-	-
16:0i	-	0.42 ± 0.04
16:1n7c	8.54 ± 1.54	-
16:1n7t	1.76 ± 0.93	-
16:0	24.92 ± 2.48	1.39 ± 0.39
17:0i	-	1.45 ± 0.24
17:0a	-	4.28 ± 0.60
17:0cyc(9,10)	2.75 ± 0.68	-
17:0	0.31 ± 0.05	0.06 ± 0.01
2-OH 16:0	-	-
3-OH 16:0	-	-
18:0i	-	0.25 ± 0.07
18:2n6	-	-
18:1n9c	0.18 ± 0.07	0.45 ± 0.26
18:1n7c	32.04 ± 3.45	0.13 ± 0.06
18:1n9t	-	-
18:1n7t	1.11 ± 0.65	-
18:0	0.86 ± 0.20	3.62 ± 0.28
19:0i	-	0.84 ± 0.33
19:0a	-	1.32 ± 0.25
19:0cyc(9,10)	-	-
19:0cyc(11,12)	7.40 ± 2.03	-
19:0	-	0.19 ± 0.09
20:0	-	4.80 ± 0.96
∑Hydroxy FA	12.17 ± 0.71	-
∑Branched FA	-	27.54 ± 1.76
∑*iso*	-	5.57 ± 0.87
∑*anteiso*	-	21.98 ± 1.40
∑yclic FA	10.14 ± 2.65	-
∑SAFA	29.12 ± 2.86	10.41 ± 0.75
∑MUFA	43.63 ± 5.87	0.58 ± 0.31
∑*Cis*	40.75 ± 4.91	0.58 ± 0.31
∑*Trans*	2.87 ± 1.57	-
Total	95.06 ± 7.57	38.53 ± 2.12

APPENDIX D – *Hyalella azteca* FA

Concentration of specific FA in *H. azteca* (µg/mg)		
FA	Sample 1	Sample 2
6:0	-	-
8:0	-	-
10:0	-	-
11:0	-	-
12:0	-	-
13:0	-	-
14:0	0.356	0.292
15:0i	-	-
15ai	-	-
14:1n5	-	-
15:0	0.150	0.130
16:0i	-	-
15:1	-	-
16:0	9.717	7.888
16:1n7	1.445	0.823
17:0	0.487	0.546
16:2n4	-	-
16:3n4	-	-
17:1	-	-
18:0	2.227	2.206
18:1n9t	-	-
18:1n9c	12.921	10.424
18:1n7	-	-
18:2n6t	-	0.325
18:2n6c (LIN)	5.817	5.636
20:0	-	-
18:3n6	0.220	0.195
20:1n9	0.730	0.708
20:1n7	-	-
18:3n3 (ALA)	3.108	2.511
21:0	-	-
20:2n6	0.857	0.927
22:0	-	-
20:3n9	-	-
20:3n6	-	-
22:1n9	-	-
20:3n3	0.198	0.307
20:4n6 (ARA)	2.228	2.765
23:0	-	-
22:2	-	-
24:0	-	-
20:5n3 (EPA)	2.049	2.191
24:1n9	0.116	-
22:4n6	-	-
22:5n6	-	-
22:5n3c (DPA)	0.184	0.264
22:6n3 (DHA)	0.511	0.510
∑SAFA	12.937	11.062
∑MUFA	15.213	11.955
∑PUFA	15.172	15.630
Total	43.322	38.647

Proportion to total FA in *H. azteca* (%)		
FA	Sample 1	Sample 2
∑SAFA	29.9	28.6
∑MUFA	35.1	30.9
∑PUFA	35.0	40.4
∑ω3	14.0	15.0
ALA	*7.2*	*6.5*
EPA	*4.7*	*5.7*
DPA	*0.4*	*0.7*
DHA	*1.2*	*1.3*
∑ω6	21.1	25.5
LIN	*13.4*	*14.6*
ARA	*5.1*	*7.2*

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