FATE OF PHYTOSTEROLS IN PULP AND PAPER WASTEWATER TREATED IN A SIMULATED AERATED STABILIZATION BASIN

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SUMMARY

Phytosterols are steroid chemicals produced by plants for the purposes of membrane function and hormone production. Phytosterols can cause endocrine disruption in aquatic species at very low concentrations and are suspected of contributing to endocrine disruption linked to pulp and paper effluent. Wastewater from the pulp and paper industry is often treated biologically in aerated stabilization basins (ASBs) that expose phytosterols to a range of redox zones. Phytosterol removal in ASBs varies and stigmasterol has even been shown to increase across the treatment system. Little is known about the microbial processes that occur within ASBs and their effect on phytosterol removal. The objective of this research was to assess the biotransformation potential of phytosterols in a simulated ASB treatment system and to improve understanding of the processes that occur within the various redox zones and their impact on the removal of phytosterols. The specific objectives of this research were:

1. Investigate the biotransformation of phytosterols under aerobic conditions and the factors that influence phytosterol biodegradation.

2. Investigate the potential for phytosterols to be removed under anoxic and anaerobic conditions.

3. Assess the performance and phytosterol removal of a simulated ASB treatment system and investigate the processes occurring in the aerobic and anoxic/anaerobic zones.

To assess the biotransformation of phytosterols under aerobic conditions, three assays were conducted using a stock aerobic culture fed with pulp and paper wastewater.

In the first assay, phytosterols were tested as the sole carbon source added at a high concentration but no significant heterotrophic activity or phytosterol removal was observed. Nitrification was not inhibited, suggesting that heterotrophic activity was likely limited by substrate availability. Phytosterols were observed to aggregate within the liquid phase, reducing bioavailability. To test whether low heterotrophic activity or low solubility was responsible for the lack of phytosterol removal, a second assay was conducted with phytosterols and dextrin solution to act as an additional carbon source. Significant microbial activity was observed; however, no significant phytosterol removal occurred. A phytosterol-free control performed similarly to the phytosterol-amended series, confirming lack of inhibition. Therefore, the low availability of phytosterols as a substrate likely limited their biotransformation. A third aerobic assay was conducted using ethanol as both an additional carbon source and a phytosterol solubilizing agent. Phytosterol removal was nearly linear over a period of 6 days, a pattern that was repeated when the culture was re-spiked. On the 7^{th} day following the re-spiking, as the culture approached a stationary phase, phytosterol removal significantly accelerated, presumably due to an accelerated release of cholesterol oxidase.

The anoxic and anaerobic biotransformation of phytosterols was examined through a series of three semi-batch cultures maintained under nitrate-reducing, sulfate-reducing and fermentative/methanogenic conditions. A mixture of ethanol and phytosterols were fed to each culture and electron acceptors (i.e., nitrate, sulfate) were provided to the nitrate-reducing and sulfate-reducing cultures. All cultures experienced significant microbial activity over 16 feeding cycles (112 d). The nitrate-reducing culture removed significant amounts of phytosterols during the initial feeding cycles but in the

latter stages, microbial activity and phytosterol removal declined. It is likely that either inhibitors were present in the culture or that prior pH instability negatively impacted the culture. Phytosterol removal did not occur in the sulfate-reducing culture until the onset of the 8th feeding cycle, after which significant phytosterol removal was observed. No phytosterol removal was observed in the fermentative/methanogenic culture over the course of the assay, despite consistently robust microbial activity, suggesting phytosterols are recalcitrant under these conditions. Stigmast-4-en-3-one was identified as a potential intermediate in the biotransformation of phytosterols under sulfate-reducing conditions, which may assist in future elucidation of the microbial pathway for phytosterol biotransformation under sulfate-reducing conditions.

Phytosterol biotransformation was also examined in a lab-scale ASB fed continuously with pulp and paper wastewater. The ASB activity was monitored and the effluent and sediment characteristics were analyzed at steady-state for three different hydraulic retention times (HRT): 22.2, 11.1 and 5.6 days. The effluent characteristics remained relatively the same at each HRT but the sediment characteristics changed significantly. As the HRT was decreased, the steady-state concentration of phytosterols in the sediment increased significantly and the solids layer increased in depth. On average, 51% of phytosterols entering the ASB were removed via biotransformation, 40% were removed by settling and the remaining 9% exited in the effluent. At the shortest HRT, samples from the water column and sediment layer were taken and used as inoculum for an aerobic and an anoxic wastewater bioassay, respectively. Over a period of 28 days, the phytosterol concentration in the aerobic assay increased, primarily due to an increase in stigmasterol, and then decreased to below the initial concentration. The phytosterol

concentrations in the anaerobic assay increased across the 96 hour incubation period due to a release of phytosterols during solids degradation.

This research improves the understanding of biological processes within ASBs and their effect on phytosterol removal. The finding that phytosterols may be removed under sulfate-reducing conditions is of particular significance to the pulp and paper industry because of the presence of sulfate in pulp and paper wastewater. Additionally, the identification of a potential β-sitosterol biotransformation product under sulfate-reducing conditions may assist in the elucidation of an anaerobic metabolic pathway. This study also demonstrates both phytosterol release and removal within ASBs, which may assist in identifying better operating conditions to improve phytosterol removal. Overall, this research contributes to the understanding of the fate of phytosterols within ASBs for the purposes of designing wastewater treatment systems with improved phytosterol removal.

CHAPTER 1

INTRODUCTION

Steroid alcohols, or sterols, are produced by eukaryotic organisms including plants and animals. Phytosterols are sterols that are found in plant material and include both free phytosterols and bonded phytosterols (esterified steryls and steryl glycosides). Plants produce phytosterols for a number of important biological functions. Free phytosterols are incorporated into cell membranes where they help regulate membrane fluidity, assist in the control of membrane-associated metabolic processes and play a role in cell differentiation. Phytosterols also act as precursors to plant hormones and growth factors. Steryl esters are sterols in which the 3-hydroxyl group has been esterified by a fatty acid or phenolic acid, while steryl glycosides are sterols that are linked to a carbohydrate. Steryl esters, which are located intracellularly, act as a form of phytosterol storage and also play a role in the transport of phytosterols within the cell. Of the 250 different types of phytosterols known, β-sitosterol, stigmasterol and campesterol are the most prevalent (Piironen et al., 2000). Similar in structure to cholesterol, phytosterols differ by the chain length or degree of branching on the aliphatic side chain. Structures of cholesterol and the common phytosterols are shown in Figure 1.1.

Although phytosterols are produced naturally, they may have endocrine disrupting effects on marine species and small mammals, even at concentrations as low as 10 μg/L. Fish exposed to phytosterols display altered sexual development, changes in hormone production, decreased egg production and decreased spawning frequency (Mahmood-Khan and Hall, 2003; Orrego et al., 2010; Miskelly, 2009). Phytosterols may also act as

endocrine disruptors in small mammals. One study showed that mink exposed to β-sitosterol experienced changes in weight regulation and reproductive organ development (Ryokkynen et al., 2005). Another study showed that phytosterols impacted thyroid function and affected estradiol and testosterone levels in European polecats (Nieminen, 2002). Some evidence suggests that phytosterol biotransformation products may also contribute to endocrine disruption (Denton et al., 1985; Howell and Denton, 1989; Christianson-Heiska et al., 2007).

Phytosterols may be present at elevated concentrations in pulp and paper mill wastewater and are suspected of contributing to the endocrine disrupting effects observed in fish and aquatic species downstream of pulp and paper mills. The pulp and paper industry produces around 334 million tons of pulp each year, which consists of a mixture of wood or plant fibers (Ashtari, 2006). During the pulping and bleaching processes, phytosterols are released into wastewater, where concentrations of total phytosterols can range from 0.3 to 3.0 mg/L (Mahmood-Khan and Hall, 2003; Cook et al., 1997).

Wastewater from the pulp and paper industry is commonly treated in activated sludge or aerated stabilization basin (ASB) systems. Phytosterol removal is highly variable among treatment systems and also experiences large fluctuations over time, although activated sludge systems tend to have a higher phytosterol removal efficiency (Cook et al., 1997; Mahmood-Kahn and Hall, 2003). In a survey of U.S. pulp and paper mills, activated sludge systems typically removed 70-95% of total phytosterols, while ASBs typically removed 56-78%. Despite overall removal of phytosterols in ASBs, stigmasterol was found to increase across the treatment systems by an unknown mechanism (Cook et al., 1997). Phytosterols are hydrophobic molecules with a tendency

to associate with solids; thus, it is unclear how much of the phytosterol removal in treatment systems is due to biodegradation and how much is due to adsorption to solids. In ASB treatment systems, settling of solids may carry phytosterols into anoxic or anaerobic sediments. Although phytosterols are known to biodegrade under aerobic conditions, less is known of their fate under anoxic or anaerobic conditions.

The objective of this research was to assess the biotransformation potential of phytosterols in a simulated ASB treatment system and to improve understanding of the processes that occur within the various redox zones and their impact on the removal of phytosterols. The specific objectives of this research were:

1. Investigate the biotransformation of phytosterols under aerobic conditions and the factors that influence phytosterol biodegradation.

2. Investigate the potential for phytosterols to be removed under anoxic and anaerobic conditions.

3. Assess the performance and phytosterol removal of a simulated ASB treatment system and investigate the processes occurring in the aerobic and anoxic/anaerobic zones.

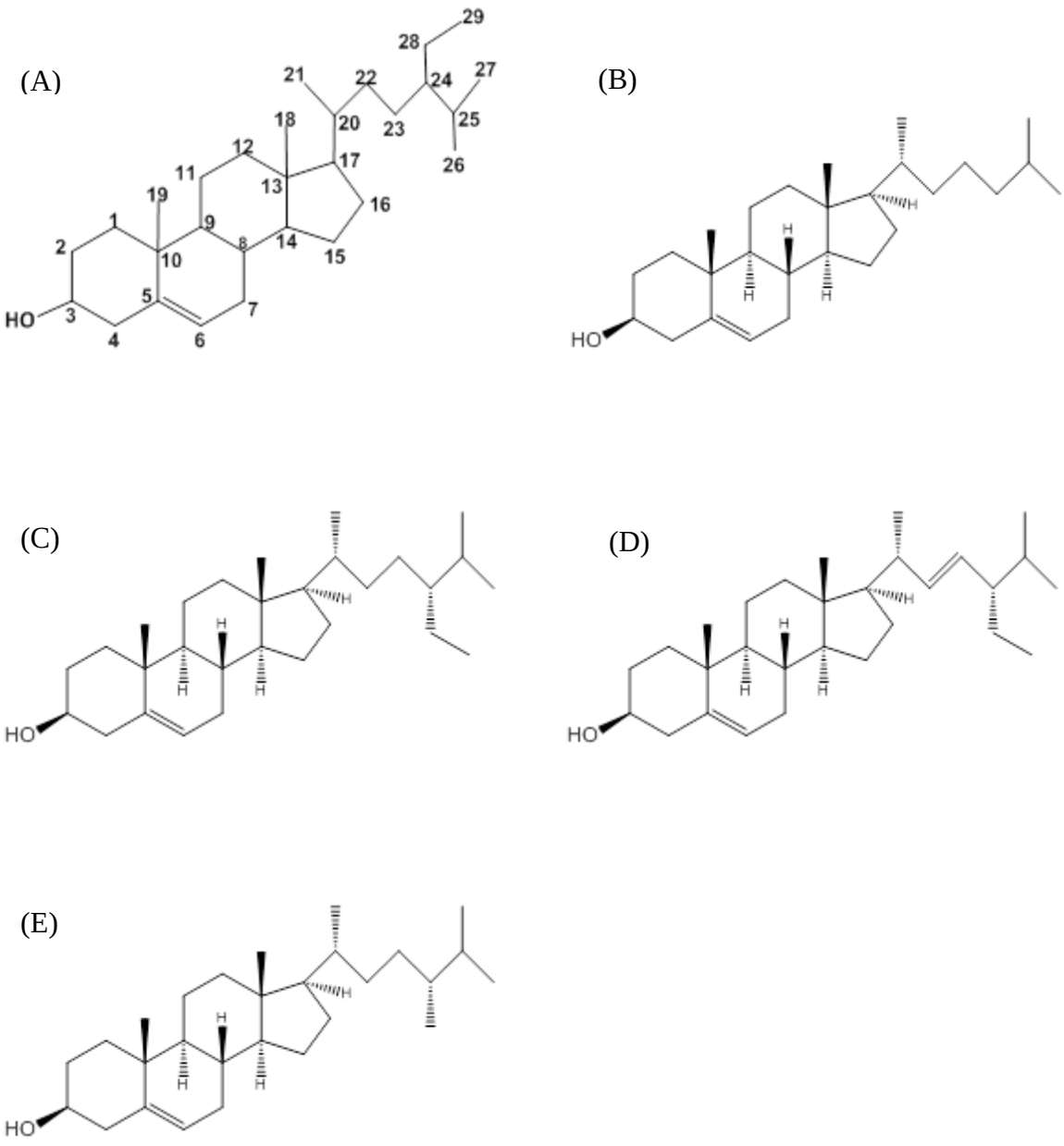


Figure 1.1. Molecular structure of the sterol skeleton (A), cholesterol (B), β-sitosterol (C), stigmasterol (D), and campesterol (E).

CHAPTER 2

BACKGROUND

2.1. Sources of Phytosterols

Sterols are found in eukaryotic cells of all kinds: animal cells primarily produce cholesterol, fungi produce mainly ergosterol, and plants produce a suite of sterols termed "phytosterols". The most common phytosterols are β-sitosterol, stigmasterol and campesterol, although hundreds of different plant phytosterols are known. Phytosterols may exist as free phytosterols (Figure 1.1C,D,E) or as conjugated phytosterols including steryl esters and steryl glycosides (Piironen et al., 2000). As a membrane component of plant cells, phytosterols are found in many different plant products. Canola oil contains 4.6-8.1 mg/g of total phytosterols, soybean oil contains 2.4-4.0 mg/g and sunflower oil contains 2.1-4.5 mg/g (Vlahakis and Hazebroek, 2000). Corn oil contains even higher amounts of phytosterols with 8 to 15 mg/g (Piironen et al., 2000). Dietary phytosterols are thought to have a number of health benefits in humans. Phytosterols, which are more hydrophobic than cholesterol, have a higher affinity for incorporating into intestinal micelles, reducing the solubility, and hence the uptake, of cholesterol. Replacing cholesterol in the diet with phytosterols results in a reduction of plasma LDL cholesterol, an increase in HDL cholesterol and an overall reduction in coronary heart disease (Alhazzaa et al., 2013). Increased phytosterol intake is also linked to anticancer effects (Llaverias et al., 2013). As a result of their perceived health benefits, phytosterols have been increasingly added to foods such as margarine, fats and mayonnaise to act as cholesterol-lowering and health-promoting ingredients (Alhazzaa et al., 2013). Unlike

cholesterol, however, phytosterols are poorly absorbed within the gastrointestinal system. While cholesterol absorption is around 56% in healthy subjects, only 1.9% of campesterol is absorbed and only 0.5% of sitosterol (Ostlund et al., 2002). The increasing prevalence of phytosterols in food products and the poor gastrointestinal absoroption of phytosterols indicates that phytosterols or their biotransformation products may be increasingly present in municipal wastewater. Indeed, phytosterols have been observed in municipal wastewater, with stigmasterol as the most abundant (Ikonomou et al., 2008).

Phytosterols are also of interest to the pharmaceutical industry as starting material for the microbial production of steroid hormones. Recent research has focused on mutant and specialty microbes that are able to selectively degrade the side chain of the phytosterol structure, producing key steroid intermediates (Bragin et al., 2013; Zhang et al., 2013a; Zhang et al., 2013b). Two of the most common steroid intermediates produced are 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD), which may in turn be used to produce pharmaceuticals such as testosterone, estradiol, progesterone, cortisol and prednisone (Malaviya and Gomes, 2008). While it is difficult to control the process of selective side chain degradation without allowing oxidation of the ring structure, the use of inexpensive, structurally-similar phytosterols as a starting material is attractive (Huang et al., 2006).

Although phytosterols are desirable in food products and useful as pharmaceutical precursors, they can be problematic when released in relatively large quantities into aquatic environments. Small concentrations of phytosterols are naturally present in water bodies. In a survey of 16 sites along 2 rivers in Canada, stigmasterol was most abundant with an average concentration of 0.15 μg/L and a maximum of 0.34 μg/L. β-sitosterol

concentrations averaged 0.10 μg/L, with a maximum of 0.15 μg/L, while campesterol averaged 0.02 μg/L with a maximum of 0.03 μg/L. Phytosterol concentrations were observed to increase downstream in both rivers, possibly due to increased influx of agricultural runoff (Jeffries et al., 2010).

One of the largest anthropogenic sources of phytosterols is the pulp and paper industry. Each year, the pulp and paper industry produces around 334 million tons of pulp, which consists of a mixture of wood or plant fibers (Ashtari, 2006). During the pulping and bleaching processes, phytosterols are released into wastewater, where concentrations of total phytosterols can range from 0.3 to 3.0 mg/L (Xavier et al, 2009). In one study, the total phytosterol concentration in primary effluent from a pulp and paper mill ranged from 0.25 to 1.2 mg/L. In the same study, the biologically treated secondary effluent contained 0.08 to 0.25 mg/L, showing overall removal across the system. However, the secondary sludge contained 10-32 mg/L total phytosterols, indicating that sorption or association with solids was a significant phytosterol removal mechanism (Mahmood-Khan and Hall, 2003).

2.2. Chemical and Physical Properties

Phytosterols are nonpolar molecules with a molecular weight around 400-415 g/ mol (Table 2.1). They are highly hydrophobic and have very low solubility in water. Experimental values for most properties are unavailable in the literature and due to their hydrophobic nature, laboratory measurement of phytosterol water solubility is difficult. Estimates of phytosterol water solubility, vapor pressure, Henry's law constant, K_{ow} and pK_a are shown in Table 2.1. The low Henry's law constant indicates that phytosterols are unlikely to escape to the atmosphere. Phytosterols also have a high octanol-water

partition coefficient, which is often used to determine the risk of bioaccumulation in fatty tissues. The high pK_a (>15) indicates that ionization of phytosterols will not occur under environmental conditions.

Table 2.1 – Select properties of phytosterols and cholesterol.

Sterol	Molecular Weight	Aqueous Solubility at 25°C, pH=7 (µg/L)	Vapor Pressure at 25°C[a] (Torr)	Henry's Law Constant at 25°C[b] (atm-m^3/mol)	log K_{ow}[c]	pK_a[a]
β-sitosterol	414.71	0.9[a], 0.5[b]	3.53E-12	2.95E-4	9.65	15.03 ± 0.7
Stigmasterol	412.69	1.6, 1.2	3.84E-12	2.59E-4	9.43	15.03 ± 0.7
Campesterol	400.68	2.0, 1.8	1.23E-11	2.22E-4	9.16	15.14 ± 0.7
Cholesterol	386.65	3.3, 3.6	2.95E-11	1.67E-4	8.74	15.03 ± 0.7

Table compiled by Giles (2012). [a]SciFinder (ACD Labs software estimation); [b]EPI Suite WATERNT estimate; [c]EPI Suite KOWWIN estimate

2.3. Biotransformation

2.3.1. Aerobic Biotransformation

Phytosterols are known to biodegrade under aerobic conditions. Indeed, the pharmaceutical industry utilizes aerobic biodegradation processes to produce steroid precursors. Because of their similarity in structure to cholesterol, phytosterols are thought to be degraded similarly to cholesterol, which has been studied in greater detail. The first step in cholesterol oxidation is the oxidation of the 3β-hydroxyl group to form cholest-5-en-3-one, followed by isomerization to cholest-4-en-3-one. Cholesterol oxidase, which uses oxygen as an electron acceptor is a bi-functional enzyme that is capable of

catalyzing both of these first steps. Cholesterol dehydrogenase/isomerase is an oxygen-independent enzyme that is capable of catalyzing the first two steps using NAD^+ or $NADP^+$ as cofactors (Li et al., 1993; Uhia et al., 2011). Following the activation of the molecule by the oxidation of the hydroxyl group, side chain oxidation and ring cleavage occur.

Cholesterol oxidase is a flavin adenine dinucleotide (FAD)-dependent enzyme that catalyzes the oxidation of the 3-β-hydroxyl group of cholesterol to form the intermediate, cholest-5-en-3-one. FAD acts as a coenzyme that is reduced to $FADH_2$ by the oxidase activity and then reoxidized with O_2 to form a flavin hydroperoxide ($FADH_2O_2$), which in turn decomposes to FAD and H_2O_2. Cholesterol oxidase also catalyzes the subsequent step of cholest-5-en-3-one isomerization to cholest-4-en-3-one. However, some species such as *Burkholderia cepacia* strain ST-200, *Pseudomonas* spp., and *Chromobacterium* sp. strain DS-1 produce cholesterol oxidases that generate 6β-hydroperoxycholest-4-en-3-one by peroxidation of the cholest-5-en-3-one intermediate (Doukyu, 2009). It has been shown that the oxidation of the 3-β-hydroxyl group is slower than the isomerization step (Brooks and Smith, 1975).

Depending on where they are found, cholesterol oxidases are classified as either membrane-bound or extracellular. Membrane-bound cholesterol oxidase is produced intracellularly and is anchored to the cell with an active domain near the membrane surface. Extracellular cholesterol oxidase is excreted into the surrounding medium. Both types may be produced by the same microorganism in varying amounts depending on the incubation conditions (Kreit et al., 1992; Kreit et al., 1994; Sojo et al., 1997). Although the substrate specificity of membrane-bound and extracellular cholesterol oxidases are

similar, the membrane-bound enzyme is more hydrophobic, has a broader optimal pH range and has a higher molecular mass than the extracellular enzyme (Kreit et al., 1994). Sojo et al. (1997) found that *Rhodococcus erythropolis* growing in a medium without cholesterol exhibited very low production of intracellular cholesterol oxidase. Addition of cholesterol induced the production of both membrane-bound and extracellular cholesterol oxidase (Sojo et al., 1997). Induction of the membrane-bound enzyme is also affected by the amount and age of the inocula, as well as the agitation speed (Kreit et al., 1994).

Cholesterol oxidase is capable of catalyzing the oxidation and isomerization of phytosterols. However, enzymatic activity is lower with phytosterols as a substrate than it is with cholesterol, and the structure of the C17 side chain affects the oxidation rate (Doukyu, 2009; MacLachlan et al., 2000). When β-sitosterol is used as a substrate instead of cholesterol, the relative enzyme activity can range from 20-84%, depending on the substrate specificity of the cholesterol oxidase produced. Stigmasterol is a less suitable substrate, incurring 12-59% of the relative enzyme activity, as compared with cholesterol (Doukyu, 2009; MacLachlan et al., 2000).

Cholesterol dehydrogenase (CDH) also catalyzes the oxidation of cholesterol to cholest-5-en-3-one followed by isomerization to cholest-4-en-3one. Unlike cholesterol oxidase, CDH simultaneously reduces NAD^+ or $NADP^+$ to NADH or NADPH. CDH is only known to be expressed intracellularly in *Nocardia* sp. and *Mycobacterium tuberculosis*. Enzyme production is transcriptionally activated when cholesterol is present in the medium (Kishi et al., 2000; Yang et al., 2007). The cholesterol dehydrogenase expressed by *M. tuberculosis* does not play a role in energy metabolism and *M. tuberculosis* is not able to grow on cholesterol as a sole carbon source. The optimal pH

range for cholesterol dehydrogenase activity is higher than that of cholesterol oxidases, with little or no catalytic activity at a neutral pH (Yang et al., 2007). The intracellular expression of cholesterol dehydrogenase and its higher active pH range likely limit its catalytic activity in wastewater treatment systems.

After the initial oxidation of the cholesterol 3β-hydroxyl group, side chain oxidation and ring cleavage occur. The two reactions may occur in any order or simultaneously. The side chain oxidation of cholest-4-en-3-one is initiated by the hydroxylation of C27 or C25 with molecular oxygen, which triggers a series of reactions that successively degrade the side chain, producing androst-4-en-3,17-dione (Chiang et al., 2008; Malaviya and Gomes, 2008). The cleavage of the ring structure is initiated by the hydroxylation of C9 and dehydrogenation at the C1-C2 bond. The reactions result in an unstable compound that spontaneously cleaves the B ring at C9 and the products undergo further degradation (Dutta et al., 1992).

2.3.2. Anoxic/Anaerobic Biotransformation

Little is known about anoxic/anaerobic biotransformation of cholesterol outside of mammalian gastric systems. One study enriched denitrifying bacteria capable of mineralizing 2-5% of the added cholesterol and transforming 20-30% into biotransformation products including 5α- and 5β-cholestan-3β-ol, cholest-4-en-3-one, 5α-androstan-3,17-dione and androst-4-en-3-17-dione (Taylor et al., 1981). Other studies have reported similar mineralization of cholesterol by denitrifying bacteria (Tarlera and Denner, 2003; Harder and Probian, 1997). In another study, microalga lipids containing phytosterols were assayed for biodegradation under anoxic conditions with a sediment sample containing nitrate-reducing, sulfate-reducing and methanogenic microorganisms.

At the end of 442 days of incubation, 27% of the total sterols had been removed (Grossi et al., 2001). *Sterolibacterium denitrificans* has been studied for its ability to degrade cholesterol under denitrifying conditions. A pathway has been proposed in which the first step is the anoxic oxidation of cholesterol to cholest-5-en-3-one, followed by isomerization to cholest-4-en-3-one (Chiang et al., 2008). These steps are the same as those found in the aerobic cholesterol oxidation pathway and therefore *S. denitrificans* utilizes a similar strategy to activate the ring structure for further degradation. However, the source of oxygen in the initial oxidation differs between the two pathways. In the aerobic pathway, molecular oxygen is the source of oxygen, whereas water is the source of oxygen in the anoxic pathway.

2.4. Endocrine Disruption

Phytosterols are capable of causing endocrine disruption in a number of aquatic species and some small mammals. Fish exposed to phytosterols have displayed reduced sex sterol levels, induced vitellogenin (egg yolk protein precursor) production in male fish, reduced gonad size, altered development of secondary sexual characteristics, decreased egg production and decreased spawning frequency (Orrego et al., 2009; Miskelly, 2009). A study exposing zebrafish to β-sitosterol found induced vitellogenin production in males, increased testosterone and accelerated spermatogenesis, with some effects found at concentrations as low as 10 μg/L (Christianson-Heiska et al., 2007). Male brook trout and goldfish exposed to β-sitosterol had reduced low-density liprotein cholesterol and triglyceride levels (Gilman et al., 2002). Goldfish exposure to 75 μg/L

phytosterols resulted in a reduction of reproductive steroid levels and changes in gonadal steroidogenesis (MacLatchy et al., 1997). Phytosterols are also capable of endocrine disruption in some small mammals such as mink and European polecat (Ryokkynen et al., 2006; Ryokkynen et al., 2005; Nieminen et al., 2002). Along with other components of pulp and paper wastewater, phytosterols are suspected of contributing to the endocrine-disrupting effects observed in receiving bodies of water (Orrego et al., 2011; Ellis et al., 2003). Additionally, there is evidence that phytosterol transformation products may also have endocrine-disrupting effects (Christianson-Heiska et al., 2007; Denton et al., 1985).

2.5. Pulp Mill Treatment Systems

Wastewater from the pulp and paper industry is usually treated biologically. Two of the most common treatment systems are activated sludge units and ASBs. Activated sludge systems can achieve 70- 90% COD removal and 40-60% removal of adsorbable organic halides (AOX) (Pokhrel and Viraraghavan, 2004). However, activated sludge systems may produce sludge with poor settling properties and may also be sensitive to shock loading or toxicity. Activated sludge units are also limited in their ability to remove recalcitrant substances from pulp and paper wastewater (Thompson et al., 2001).

A typical ASB consists of a large lagoon with tapered aeration and is often baffled to create a long retention time (Sackellares et al., 1987). Wastewater that enters the ASB is aerated in a semi-mixed zone before moving to an increasingly quiescent zone where solids are removed through settling. Therefore, ASBs treat wastewater through a combination of biological treatment and settling. The biological activity within ASBs is

more complex than in activated sludge due to the various redox zones that exist throughout the ASB sediment layers and water column. ASBs are capable of removing 50-75% of BOD_7 and 70% AOX, making them less effective than activated sludge in the removal of organic matter but more effective in the removal of AOX (Pokhrel and Viraraghavan, 2004). ASBs are attractive treatment systems due to their ability to accept large variations in flow and wastewater composition. However, ASBs require large areas of land near the pulp and paper mill.

Both activated sludge systems and ASBs are capable of removing phytosterols, although removal is highly variable (Mahmood-Khan and Hall, 2003; Cook et al., 1997). In a survey of U.S. pulp and paper mills, activated sludge systems removed 70-95% of total phytosterols, while ASBs removed 56-78%. Unlike the other phytosterols measured, stigmasterol was found to increase across the treatment systems by an unknown mechanism (Cook et al., 1997). It is likely that phytosterol removal is due to both biotransformation and sorption to solids. As seen in one study, biological treatment by activated sludge reduced the sterol concentration from 0.25-1.2 mg/L to 0.80-.25 mg/L but the sludge solids contained 10-32 mg/L, indicating sorption as a significant removal mechanism (Mahmood-Khan and Hall, 2003). It is unclear how much removal in ASB systems is due to biotransformation and how much removal is due to sorption to solids. Moreover, little is known about the microbial processes that occur within ASB systems and how the aerobic and anoxic/anaerobic zones interact. Studying the microbial activity and phytosterol biotransformation within ASB systems is vital to developing systems with more efficient phytosterol removal.

CHAPTER 3

MATERIALS AND ANALYTICAL METHODS

3.1. General Analytical Methods

3.1.1. pH

All pH measurements were performed using the potentiometric method with a ATI Orion Model 370 digital pH meter (Orion Research Inc., Boston, MA) and a gel-filled combination pH electrode (VWR International, West Chester, PA). The meter was calibrated weekly with pH 4.0, 7.0, and 10.0 standard buffer solutions (Fisher Scientific, Pittsburg, PA).

3.1.2. Dissolved Oxygen (DO)

Dissolved oxygen in this study was measured using the luminescent dissolved oxygen method with a Hach HQ40d digital multimeter in conjunction with a LDO101 oxygen probe (Hach Company, Loveland, CO). The instrument was calibrated to water-saturated air at 22°C before each use.

3.1.3. Chemical Oxygen Demand (COD)

COD was measured using the closed reflux, colorimetric method as described in *Standard Methods* (Eaton et al., 2005). An aliquot of 3 mL digestion solution composed of 4.9 g $K_2Cr_2O_7$, 6 g $HgSO_4$, 6 g Ag_2SO_4 and 500 mL H_2SO_4 was transferred to HACH COD digestion vials (HACH Company, Loveland, CO) and then 2 mL of sample was added to the vial. After tumbling the vial for 4-8 times, the content in the vials was

digested at 150^{o}C for 2 hours and then cooled down to room temperature. The absorbance was measured at 620 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. Samples were centrifuged and filtered through a 0.45 μm polypropylene membrane filter if the soluble COD was measured, otherwise well-mixed samples were used after appropriate dilution for total COD measurements. All samples were prepared in triplicates and a calibration curve was prepared using 1 g/L standard solution of potassium hydrogen phthalate (KHP).

3.1.4. Ammonia

Ammonia concentration was measured using the distillation method described in *Standard Methods* (Eaton et al., 2005). The samples were centrifuged at 12,000 rpm for 15 minutes and filtered through a 0.2 μm nitrocellulose membrane filter (Fisher Scientific, Pittsburgh, PA). The ammonia distillation was performed using a Labconco distillation apparatus (Labconco Corp., Kansas City, MO) and the distillate was received in a mixed indicator solution of 20 g H_3BO_3/L, methyl red 13.3 mg/L and methylene blue 6.67 mg/L. The distillate then was titrated with 0.2 N H_2SO_4 and the ammonia was quantified.

3.1.5. Anions

Chloride (Cl^-), nitrite (NO_2^-), bromide (Br^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) anion concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Corporation, Sunnyvale, CA) equipped with a suppressed conductivity detector, a Dionex IonPac AG14A (4x50mm) precolumn, and a Dionex

IonPac AS14A (4x250 mm) analytical column. The unit was operated in autosupression mode with 1 mM $NaHCO_3$/8 mM Na_2CO_3 eluent and a flow rate of 1 mL/min. All samples were filtered through 0.2 μm membrane filters prior to injection. The minimum detection limit for each anion listed above was 0.03, 0.02, 0.03, 0.04, 0.02 and 0.05 mM, respectively.

3.1.6. Total and Volatile Solids (TS and VS)

Total solids content of samples were determined according to procedures outlined in *Standard Methods* (Eaton et al., 2005). Samples were weighed in pre-ignited (550^{o}C) and cooled ceramic crucibles using an Ohaus AP250D Analytical Balance (precise to ±0.02 mg up to 52 g, and to ±0.1 mg between 52 and 210 g). The samples were then dried at 105^{o}C for 24 hours in a Fisher Isotemp Model 750G oven. After drying, the crucibles were transferred to a desiccator until cooled, and then the dry weight was measured. If VS were to be determined, the crucibles were transferred to a Fisher Isotemp Model 550-126 muffle furnace and ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled in a desiccator and the remaining solids weight was measured. TS and VS were then calculated. Total solids were calculated as the difference between the weight of the crucible after the sample was dried at 105°C and the tare weight of the crucible divided by the sample volume. Volatile solids were calculated as the difference between the weight of the crucible after the sample was dried at 105°C and the weight of the crucible after the sample was burned at 550°C divided by the sample volume.

3.1.7. Total and Volatile Suspended Solids (TSS and VSS)

TSS and VSS were determined according to procedures described in *Standard Methods* (Eaton et al., 2005). Whatman GF/C glass fiber filters (47 mm diameter and 1.2 μm nominal pore size; Whatman, Florham Park, NJ) were washed with deionized (DI) water and ignited at 550^{o}C for 20 minutes in a Fisher Isotemp Model 550-126 muffle furnace before use. The filters were than cooled in a desiccator and weighed. Samples of known volume were filtered through the glass fiber filters. The filters were then rinsed with 10 mL DI water to remove dissolved organics and inorganic salts. The filters containing the samples were dried at 105^{o}C for 90 minutes. After cooling in a desiccator, the dry weight was recorded and the filters containing the dry samples were ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled down in a desiccator and the weight was measured. TSS and VSS concentrations were then calculated. Total suspended solids were calculated as the difference between the weight of the filter after the sample was dried at 105°C and the tare weight of the filter divided by the sample volume. Volatile suspended solids were calculated as the difference between the weight of the filter after the sample was dried at 105°C and the weight of the filter after the sample was burned at 550°C divided by the sample volume.

3.1.8. Total Gas Production

Total gas production in closed assay bottles and large volume reactors was measured by either the gas-water displacement method or with a VWR Pressure/Vacuum transducer (resolution –1 atm to 1.974 atm with an accuracy of 0.002 atm).

3.1.9. Gas Composition

The gas composition was determined by a gas chromatography (GC) unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane (CH_4) and dinitrogen (N_2) were separated with a 15 m HP-Molesieve fused silica, 0.53 mm i.d. column (Agilent Technologies, Inc.). Carbon dioxide (CO_2), hydrogen sulfide (H_2S), nitric oxide (NO) and nitrous oxide (N_2O) were separated with a 25 m Chrompac PoraPLOT Q fused silica, 0.53 mm i.d. column (Varian, Inc., Palo Alto, CA). Helium was used as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150^{o}C, the oven was set at 40^{o}C and the detector temperature was set at 150^{o}C. All gas analyses were performed by injecting a 100 μL gas sample. The minimum detection limits for CH_4, CO_2, H_2S, NO, N_2O and N_2 were 500, 800, 100, 500, 7 and 50 ppmv, respectively.

3.1.10. Volatile Fatty Acids (VFAs)

VFAs (C_2 to C_7; i.e., acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, iso-caproic, n-caproic and heptanoic acids) were measured after acidification of filtered samples with a 2.5% H_3PO_4 solution containing 1.5 g/L acetoin as the internal standard (sample:acid, 2:1 volume ratio) using an Agilent 6890 Series GC unit equipped with a flame ionization detector and a 35-m Stabilwax-DA, 0.53-mm I.D. column (Restek, Bellefonte, PA). Injections were performed with a 1:1 split ratio. Samples used for the measurement of VFAs were prepared by centrifugation at 10,000 rpm for 30 minutes and filtration through 0.22-μm PVDF membrane filters before acidification. The

minimum detection limit for each acid mentioned above was 0.25, 0.10, 0.03, 0.02, 0.10, 0.08, 0.02, 0.02, 0.05 mM, respectively.

3.2. Analysis of Phytosterols and Cholesterol

3.2.1. Chemicals

Cholesterol (≥ 99%) was obtained from Sigma Aldrich (St. Louis, MO). A phytosterol mixture derived from soybeans and containing 41.49% β-sitosterol, 26.83% stigmasterol and 23.04% campesterol, was purchased from Purebulk.com (Roseburg, OR). Iso-octane (> 99.5%) was purchased from Sigma-Aldrich. Stock solutions were prepared in ethanol (> 99%) at 8 and 4 g/L for cholesterol and the phytosterol mixture, respectively.

3.2.2. Separatory Funnel Extraction

For samples expected to have a moderate concentration of phytosterols, a separatory funnel extraction method was used. A sample (100 mL) was added to a 250 mL glass separatory funnel with glass stopper and Teflon stopcock. A cholesterol standard solution was added (50 μL) as a surrogate standard because cholesterol has similar properties to phytosterols but is not typically present in pulp and paper wastewater. Isooctane (50 mL) was added and the separatory funnel was shaken vigorously by hand for a total of 5 min. The emulsion was allowed to separate in the funnel and the isooctane layer was separated. The isooctane layer was removed and

evaporated under He or N_2 gas and the residue was reconstituted with 1 mL isooctane prior to analysis with GC-MS/FID. This method of extraction achieved ≥ 87% recovery.

3.2.3. Stir Plate Extraction

A stir plate extraction method was used for assays conducted in sacrificial flasks with high phytosterol concentrations. A Teflon-coated stir bar was added to the flask and used to mix the culture while a cholesterol solution was added as a surrogate standard. Under high mixing, isooctane (1:10 v/v isooctane to sample ratio) was added and the flasks were mixed for 3 hours. The resulting solutions was rinsed into glass separatory funnels and allowed to separate. Further recovery of the isooctane was achieved by centrifuging the emulsion in glass centrifuge tubes at 3000 rpm for 10 min and recovering the isooctane layer with a glass pipet. The isooctane phase was then evaporated under N_2 gas and the resulting residue was reconstituted with isooctane prior to GC analysis. Preliminary tests showed that this extraction method achieved ≥91% recovery of all three phytosterols.

3.2.4. Vortex Extraction

A vortex extraction method was used for rapid phytosterol measurement when phytosterol concentrations were low enough so that a representative sample could be obtained from a well-mixed reactor. Using a glass syringe, triplicate 20 mL samples were placed in 25 mL glass serum culture tubes. For total phytosterol concentrations anticipated to be greater than 1 mg/L, 2 mL isooctane was added to each culture tube, resulting in a 1:20 v/v isooctane to sample ratio. For total phytosterol concentrations expected to be less than 1 mg/L, triplicate 20 mL samples were taken and 1 mL isooctane

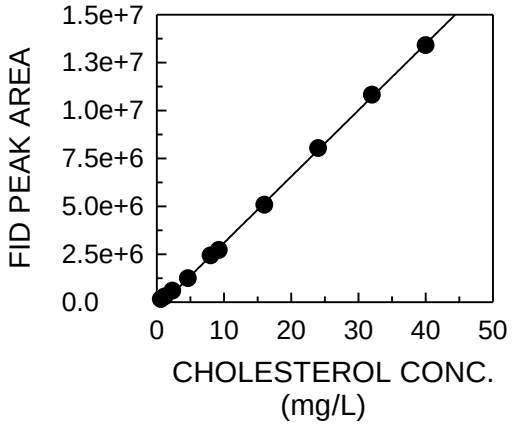
was added to each serum culture tube, resulting in a 1:40 v/v isooctane to sample ratio. Each tube was closed with a Teflon-lined cap and vortexed for 5 minutes. The resulting emulsion was then centrifuged for 10 min at 3000 rpm in glass centrifuge tubes with Teflon caps. The isooctane layer was drawn off with a glass pipet and directly used for GC analysis. Preliminary tests showed that this extraction method achieved ≥80% recovery of all three phytosterols.

3.2.5. Identification and Quantification of Phytosterols

Phytosterol concentrations were measured by liquid-liquid extraction followed by gas chromatography/mass spectroscopy and flame ionization detection (GC/MS-FID). Analysis of phytosterols and cholesterol was performed using an Agilent 7890 GC unit with a Zebron ZB-5HT column, which terminated in a Dean's switch for simultaneous collection of MS data for identification and FID data for quantification. The GC had a splitless liner in the inlet, which was maintained at 325°C and 23.4 psi. The injection volume was 1 µL and helium was used as a carrier gas with a flow rate of 2.4 mL/min. The oven temperature was held at 90°C for 1 min, then ramped up to 250°C at a rate of 20°C/min, held for 1 min, ramped up to 310°C at a rate of 6°C/min and then held at 310°C for 2 min until termination of the run. MS analysis was conducted by electrospray ionization with positive ion polarity at 70 eV fragmentation voltage and a mass scan range of m/z 50-500. MS spectra were compared to standards and those in the NIST library for identification of sterols. FID peak areas were used to quantify sterols using a standard curve prepared with phytosterol and cholesterol solutions in ethanol. Cholesterol was used as the surrogate and/or internal standard in all liquid-liquid extraction and GC

analyses. The method's minimum detection limit was 0.5 µg/mL in the injected solvent sample.

Calibration curves for the quantification of cholesterol and phytosterols were constructed by analyzing serial dilutions of a cholesterol or phytosterol mixture stock solution. Using a glass syringe, 50 µL of each serial dilution was transferred to a glass GC injection vial. A light stream of N_2 gas was directed into the vial to evaporate the ethanol in the sample. After evaporation, 1 mL of isooctane was added to the vial to reconstitute the residue, the vial was vortexed and then analyzed by GC-MS/FID. Quantification of phytosterols by FID was more accurate than quantification by MS, thus the MS data was used for identification and the FID data was used for quantification. The calibration curve for cholesterol is shown in Figure 3.1. The calibration curves for β-sitosterol, stigmasterol and campesterol are shown in Figure 3.2.



$y = 667{,}313\ x$ $R^2 = 0.998$

Chol (mg/L) = FID Area / 667,313

Figure 3.1. FID calibration curve for cholesterol. Chol = cholesterol.

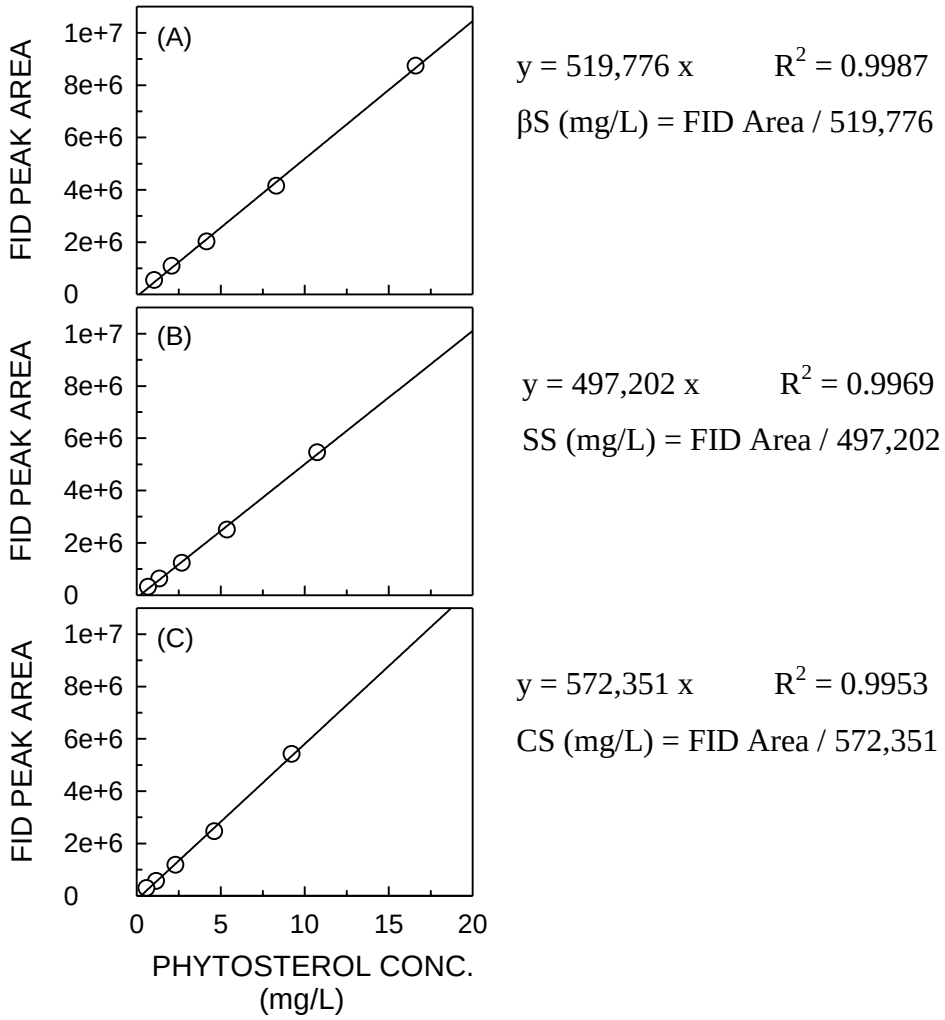


Figure 3.2. FID calibration curves for β-sitosterol (A), stigmasterol (B), and campesterol (C). βS = β-sitosterol; SS = stigmasterol; CS = campesterol.

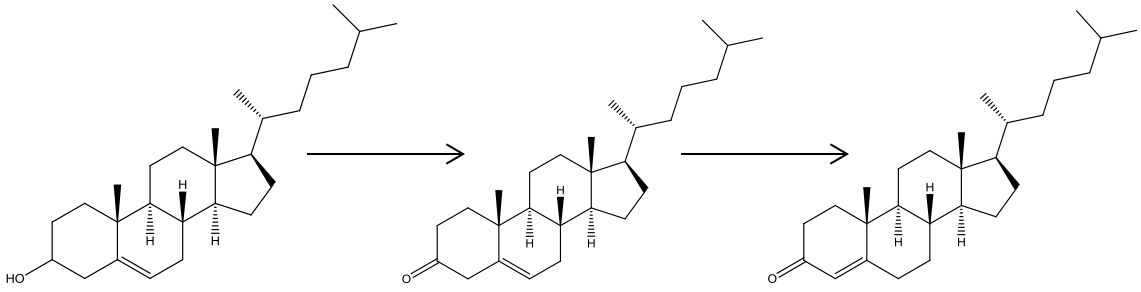
CHAPTER 4

AEROBIC BIOTRANSFORMATION OF PHYTOSTEROLS

4.1. Introduction

Phytosterols are known to biodegrade under aerobic conditions but little is known about how they are biotransformed. Because of their structural similarity, phytosterols are expected to follow a pathway similar to cholesterol, which has been studied in more detail. In the aerobic pathway, cholesterol is first oxidized to cholest-5-en-3-one, followed by isomerization to cholest-4-en-3-one (Figure 4.1). The initial reactions can be catalyzed by two types of enzymes: cholesterol oxidases, which use oxygen as an electron acceptor, and cholesterol dehydrogenases/isomerases, which require NAD^+ or $NADP^+$ as cofactors (Li et al., 1993; Uhia et al., 2011).

It is not well known what factors may limit the aerobic biotransformation of phytosterols and contribute to the observed variability in the removal efficiency of treatment systems. In order to optimize phytosterol removal, it is necessary to understand the role of the physical characteristics of phytosterols (i.e., hydrophobicity and solubility) as well as the role of an additional carbon source in phytosterol biotransformation. The objective of this portion of the study was to assess the biotransformation potential of three common phytosterols -- β-sitosterol, stigmasterol and campesterol -- by a mixed aerobic culture when phytosterols were the sole added carbon source, together with dextrin, or as an ethanol solution or suspension.



cholesterol cholest-5-en-3-one cholest-4-en-3-one

Figure 4.1. Initial steps in the biodegradation of cholesterol. Cholesterol is oxidized to cholest-5-en-3-one, followed by isomerization to cholest-4-en-3-one.

4.2. Materials and Methods

4.2.1. Phytosterols

A mixture of three phytosterols (β-sitosterol, stigmasterol and campesterol) derived from soybeans was purchased from Purebulk.com (Roseburg, OR). The mixture contained 41.5% β-sitosterol, 26.8% stigmasterol and 23.0% campesterol. A stock solution was prepared in ethanol (>99%) with a total phytosterol concentration of 3.6 g/L and a well-mixed suspension was prepared in ethanol with a total phytosterol concentration of 36.3 g/L.

4.2.2. Stock culture development and maintenance

A stock mixed culture was developed with mixed liquor collected from a pulp and paper mill ASB located in the southeastern United States. The culture was maintained at room temperature (20-22°C) under alternating aerobic/anoxic conditions. Aeration was provide via pre-humidified, compressed air for six days of each weekly feeding cycle and stopped for one day to accommodate an anoxic period to remove the nitrate produced by

ammonia oxidation during the oxic period. The culture was fed the supernatant of settled, untreated pulp and paper mill wastewater, amended with $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ (50 mg/L each), NH_4Cl (60 mg N/L), phosphate buffer (700 mg P/L; pH 7.2) and trace metals (Table 4.1). Feed characteristics are shown in Table 4.2. The reactor was operated with a HRT of 4.2 d and SRT of 21 d. Phytosterols were measured in the feed, mixed liquor and effluent by liquid-liquid extraction with isooctane. Prior to use in the biotransformation assays, mixed culture that was wasted at the end of a feeding cycle was aerated overnight to reduce the residual degradable organic matter.

Table 4.1. Composition of trace metals stock solution

Chemical	**Concentration (g/L)**
$ZnCl_2$	0.5
$MnCl_2.4H2O$	0.3
H_3BO_3	3.0
$CoCl_2.6H_2O$	2.0
$CuCl_2.2H_2O$	0.1
$NiSO_4.6H_2O$	0.2
$Na_2MoO_4.2H_2O$	0.3

Table 4.2. Characteristics of the stock aerobic culture feed

Parameter	Feed
pH	7.30 ± 0.02[a]
TSS (mg/L)	3.6 ± 1.0
VSS (mg/L)	2.7 ± 0.5
Total COD (mg/L)	267 ± 3
Soluble COD (mg/L)	266 ± 7
Nitrite (mg N/L)	ND[b]
Nitrate (mg N/L)	ND
Ammonia (mg N/L)	150 ± 5
Chloride (mg Cl/L)	407 ± 12
Phosphate (mg P/L)	1877 ± 19
Sulfate (mg S/L)	37.7 ± 0.1
Total phytosterols (µg/L)	180.3 ± 6.7
β-sitosterol (µg/L)	146.3 ± 6.7
Stigmasterol (µg/L)	19.3 ± 0.7
Campesterol (µg/L)	14.7 ± 0.3

[a]Mean ± standard deviation (n = 3)

[b]ND, not detected

[c]Culture mixed liquor sampled at the end of the feeding cycle (day 7).

[d]Settled supernatant collected at the end of the feeding cycle after the culture was allowed to settle for 2 hours.

4.2.3. Phytosterol biotransformation assays

4.2.3.1. Phytosterols as the sole added carbon source

To investigate whether aerobic biotransformation of β-sitosterol, stigmasterol and campesterol would occur when phytosterols were the sole exogenous carbon source, a biologically active series was prepared. An abiotic control series was prepared identically

and then sodium azide was added to a concentration of 200 mg/L to inhibit microbial growth. Each series consisted of seven 250 mL Erlenmeyer flasks with a total liquid volume of 200 mL. Within each flask, aerobic culture, phytosterol powder and ammonium chloride were combined to reach initial concentrations of total phytosterols and ammonium chloride of 46 mg/L and 60 mg N/L, respectively. The initial pH of the series was 6.9. The flasks were incubated at room temperature (20-22°C) on an orbital shaker at 190 rpm and aerated with pre-humidifided, compressed air delivered with glass pipets. At times throughout the incubation period, a flask from each series was removed and analyzed for pH, COD, nitrate and nitrite. Phytosterols were measured following a liquid-liquid extraction of the remaining volume (190 mL) using the stir-plate extraction method (Section 3.2.3).

4.2.3.2. Dextrin as an additionalcarbon source

Phytosterols are associated with other sources of organic matter in pulp and paper wastewater streams. To investigate whether aerobic phytosterol biotransformation would be enhanced by the presence of an additional degradable carbon source, dextrin was added as a proxy for the more complex carbon sources in pulp and paper wastewater. A series of ten 250 mL Erlenmeyer flasks were prepared with aerobic culture, phytosterol mixture (46 mg total phytosterols/L) and ammonium chloride (60 mg N/L) to a total liquid volume of 200 mL. The initial pH was 7.0. The flasks were incubated at room temperature (20-22°C) on an orbital shaker at 190 rpm and aerated with pre-humidified, compressed air delivered with glass pipets. At various times throughout the incubation, a flask was removed and analyzed for pH, COD, nitrate and nitrite. The remaining volume (190 mL) was used to measure phytosterols following liquid-liquid extraction using the

stir-plate method (Section 3.2.3). A phytosterol-free control was prepared in a 2 L glass reactor with aerobic culture, ammonium chloride (60 mg N/L) and an initial pH of 7.0. The control was maintained at room temperature (20-22°C) under mixing and aerated with pre-humidified, compressed. Samples were taken periodically to measure pH, COD, nitrate and nitrite. The control was maintained in parallel with the above-described phytosterol-containing culture series; thus, dextrin solution (1,150 mg COD/L) was added on the third day to reach a total COD of 1,580 mg/L.

4.2.3.3. Ethanol as an additional carbon source and solubilizing agent

Phytosterols have low solubility in water, limiting their accessibility by microorganisms. To overcome this issue, ethanol was used as both an additional external carbon source and a solubilizing agent. Phytosterols were added either as part of a well-mixed suspension (36.3 g/L total phytosterols) or as part of a completely solubilized solution (3.6 g/L total phytosterols). The assay using the phytosterol/ethanol suspension was conducted in a series of seven sets of triplicate 250 mL Erlenmeyer flasks. Aerobic culture and ammonium chloride (60 mg N/L) were added for a total liquid volume of 200 mL. To add the phytosterol suspension to each of the flasks, 125 µL of the phytosterol suspension was first pipetted into the flask and then 125 µL of pure ethanol (>99%) was rinsed through the pipet tip into the flask. Each flask initially contained 22.6 ± 2.1 mg/L total phytosterols with an added COD of 2,063 mg/L and an initial pH of 7.0. The flasks were incubated on an orbital shaker at 190 rpm at room temperature (20-22°C) and aerated with pre-humidified, compressed air delivered with glass pipets. At various times throughout the incubation, sets of three flasks were removed and extracted, without prior sampling or transfer, using the stir plate method (Section 3.2.3). A preliminary test was

conducted under the same conditions, which showed that DO levels remained above 5 mg/L throughout the assay.

The assay conducted using the phytosterol/ethanol solution delivered the phytosterols to the system in a solubilized form; thus, a representative sample could be collected from a single reactor and sacrificial flasks were not necessary. For this assay, a single, 4L glass reactor with a bottom spigot and Teflon stopcock was set up with aerobic culture and ammonium chloride (60 mg N/L) for a total liquid volume of 3 L. A glass syringe was used to transfer the phytosterol stock solution into the reactor to achieve a total phytosterol concentration of 4.49 ±0.09 mg/L. The initial pH was 7.0 and the initial soluble COD was 2,542 mg/L. The culture was incubated at room temperature (20-22°C) and aerated with pre-humidified, compressed air delivered from a glass pipet. The reactor was continuously mixed with a Teflon coated stir bar and preliminary tests under the same conditions showed that DO remained above 7 mg/L throughout the incubation. Periodically, samples were taken in triplicate using a glass syringe and extracted using the vortex extraction method to determine phytosterol concentrations (Section 3.2.4). An additional 10 mL sample was taken for microbial activity measurements.

4.3. Results and Discussion

4.3.1. Stock Culture Performance

The pH of the stock culture remained within the range of 6.8-7.3 over a feeding cycle. The pH declined during the aerated portion of the feeding cycle due to nitrification and increased slightly during the anoxic phase due to denitrification (Figure 4.2A). The

COD concentration in the reactor over the course of a 7-day feeding cycle is shown in Figure 4.2B. The stock culture was fed twice over the course of a feeding cycle (on days 0 and 3) and the majority of the available soluble COD was removed within 1 day of each feeding. The total and soluble COD followed a similar pattern. Nitrification took place during the feeding cycle as shown by falling ammonia concentrations and rising nitrate concentrations (Figure 4.2C). The ammonia concentration declined from 50 to 5 mg N/L over one day following each feeding and was undetectable afterwards. Transient nitrite was detected at low levels on the days following each feeding. Nitrification occurred from day 1-3 and 4-7, with nitrate levels rising to a maximum of 53 mg N/L on days 3 and 7. On the 3rd day of the feeding cycle, the nitrate concentration declined from 53 to 7 mg N/L because of the replacement of the culture supernatant with fresh feed. Between days 3 and 4, the culture underwent a 24-hour period of denitrification in which the remaining nitrate was completely removed.

The stock culture was continuously maintained for over three years. The performance characteristics of the stock culture at steady state are given in Table 4.3. The culture was capable of reducing soluble COD by 67%. After settling the culture for 2 hours, the supernatant carried only16.5% of the soluble COD present in the feed. The stock culture was fed with the supernatant of settled pulp and paper wastewater; thus, phytosterols were continuously carried into the culture. With the feed considered as the influent and the culture's settled supernatant as the effluent, the culture removed 90.9% of the total phytosterols. However, without settling, the phytosterol removal was 78.3%. The mixed culture prior to settling contained a higher total phytosterol concentration (39.2 μg/L) than the settled effluent (16.4 μg/L), indicating a tendency for the

phytosterols to be associated with solids. The stock culture was able to remove 163.9 μg/L total phytosterols, of which 13.9% was removed through settling and 86.1% presumably through biotransformation. The performance of the stock culture shows that both biotransformation and association with solids are important mechanisms controlling phytosterol removal.

Table 4.3. Characteristics of the stock aerobic culture feed, mixed liquor and effluent at steady-state.

Parameter	**Feed**	**Mixed Liquor[c]**	**Supernatant[d]**
pH	7.30 ± 0.02[a]	6.86 ± 0.02	6.86 ± 0.02
TSS (mg/L)	3.6 ± 1.0	737 ± 82	21.5 ± 0.8
VSS (mg/L)	2.7 ± 0.5	265 ± 16	7.14 ± 0.5
Total COD (mg/L)	267 ± 3	438 ± 27	60 ± 3
Soluble COD (mg/L)	266 ± 7	89 ± 8	44 ± 2
Nitrite (mg N/L)	ND[b]	ND	ND
Nitrate (mg N/L)	ND	50.9 ± 3.1	52.5 ± 2.4
Ammonia (mg N/L)	150 ± 5	ND	ND
Chloride (mg Cl/L)	407 ± 12	242.0 ± 15.4	256.2 ± 10.4
Phosphate (mg P/L)	1877 ± 19	117.0 ± 7.8	952.9 ± 36.0
Sulfate (mg S/L)	37.7 ± 0.1	302.3 ± 18.6	53.5 ± 1.4
Total phytosterols (μg/L)	180.3 ± 6.7	39.2 ± 9.4	16.4 ± 1.6
β-sitosterol (μg/L)	146.3 ± 6.7	32.4 ± 9.4	5.5 ± 1.6
Stigmasterol (μg/L)	19.3 ± 0.7	3.2 ± 0.1	9.8 ± 0.3
Campesterol (μg/L)	14.7 ± 0.3	3.6 ± 0.3	1.1 ± 0.1

[a]Mean ± standard deviation (n = 3)

[b]ND, not detected

[c]Culture mixed liquor sampled at the end of the feeding cycle (day 7).

[d]Settled supernatant collected at the end of the feeding cycle after the culture was allowed to settle for 2 hours.

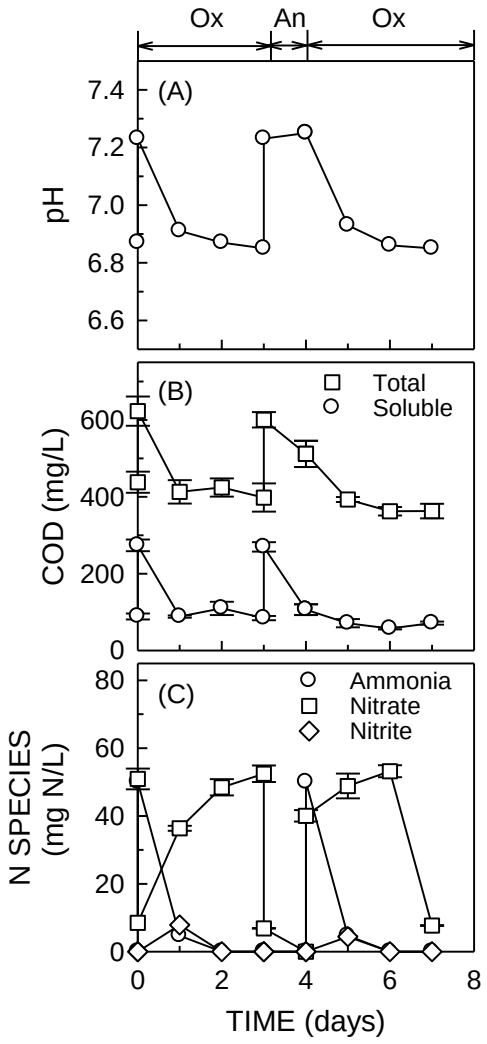


Figure 4.2. Aerobic stock culture pH (A), total and soluble COD (B), and nitrogen species (C) over the course of a 7-day feeding cycle. Error bars represent mean values ± one standard deviation, *n* = 3 (Ox = Oxic; An = Anoxic).

4.3.2. Phytosterol biotransformation assays

4.3.2.1. Phytosterols as the sole added carbon source

The culture series with phytosterols as the sole carbon source, added as a powder, showed only minor activity over a 26 day incubation period. During the first two weeks, the total COD decreased from 477 to 393 mg/L in the biotic assay, compared with the

control series average of 497 ± 31 mg COD/L. The soluble COD in the biotic series also decreased from 164 to 123 mg/L, compared with the control series average of 153 ± 7 mg COD/L. Nitrification took place as shown by an increase in the nitrate concentration from 51.5 to 122 mg N/L over 14 days; nitrite was only observed at the 12-hour measurement in trace amounts. The abiotic control maintained a steady nitrate concentration of 49 ± 4 mg N/L. The phytosterol concentrations in the biotic and abiotic flasks over the 26 day incubation period are shown in Figure 4.3. Phytosterol concentrations did not significantly change across the incubation period and were similar between the biotic assay (46 ± 6 mg/L total phytosterols) and abiotic control (47 ± 3 mg/L total phytosterols). The relative percentage of each phytosterol remained stable throughout the incubation period in both the abiotic and biotic series. The biotic series had a distribution of 45% β-sitosterol, 27% stigmasterol and 28% campesterol at the beginning of the incubation period, and at the end contained 44% β-sitosterol, 29% stigmasterol and 27% campesterol. For the abiotic series, β-sitosterol, stigmasterol and campesterol represented 45, 27 and 28%, respectively, at the beginning of the incubation period and 45, 28 and 27%, respectively, at the end of the incubation period.

Significant phytosterol biotransformation was not observed and heterotrophic activity was very low; thus, primary metabolism of phytosterols could not be confirmed. Lack of phytosterol removal could be due to the very low heterotrophic activity, especially if phytosterol degradation takes place via secondary metabolism, and/or the inability of microorganisms to access phytosterols. Inhibition of the mixed culture by the phytosterols was unlikely because nitrifiers, which are slower-growing and more prone to inhibition, were active throughout the incubation period. Instead, heterotrophic activity

was likely limited by low substrate concentrations. The bioavailability of phytosterols was limited by their low solubility and aggregation in the liquid phase. Phytosterols have an inherently low water solubility and were observed to form aggregates in the liquid phase due to the hydrophobic effect, in which nonpolar species aggregate in a polar solvent to decrease the interfacial surface area (Southall et al., 2002). The bioavailable phytosterol concentration in the solution was therefore limited by both the low solubility of the phytosterols and their tendency to form aggregates that resist dissolution. The results of this assay agree with other studies which found that cholesterol oxidase was not induced when cholesterol was the only carbon source present (Kreit et al., 1992; Sojo et al., 1997). In one study, the addition of cholesterol alone failed to induce the production of cholesterol oxidase by *R. erythropolis*; however, enzyme induction occurred when cholesterol was added as an emulsion in a detergent, suggesting that the low solubility of cholesterol limits the induction of cholesterol oxidase (Sojo et al., 1997). Other studies have found that the presence of a detergent or organic solvent greatly increases both the induction of cholesterol oxidase and the oxidation reaction rate (MacLachlan et al., 2000; Moradpour et al., 2013). Solubilizing agents are thought to act by dispersing cholesterol as well as enhancing the flux of compounds to and from the cell (Moradpour et al., 2013). Without a solubilizing agent present with the phytosterols, induction of cholesterol oxidase was likely minimal in the assay performed in the present study, which resulted in insignificant phytosterol removal.

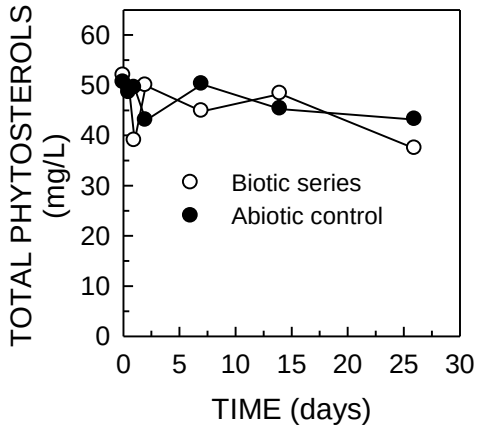


Figure 4.3. Total phytosterol concentration during the 26-day aerobic bioassay with phytosterols added as a powder and no additional carbon source.

4.3.2.2. Dextrin as an additional carbon source

A series of flasks were set up with phytosterols, monitored for 3 days and then spiked with dextrin to stimulate microbial heterotrophic growth. After spiking, the culture series had a total and soluble COD of 1,560 mg/L and 1,136 mg/L, respectively. The readily-degradable COD was consumed within 5 days. At the end of incubation (7 days after spiking), the total COD was 908 mg/L and the soluble COD was 230 mg/L. The COD and nitrate profiles were similar in both the phytosterol-amended series and a phytosterol-free control (Figure 4.4A-C). Nitrification was observed throughout the assay; nitrate increased from 42.9 to 138.9 mg N/L over 10 days of incubation. The phytosterol concentration over the 13 day incubation is shown in Figure 4.4D. No appreciable change was observed in the total phytosterol concentration, which averaged 47 ± 6 mg/L across the series. The relative percentage of each phytosterol did not

significantly change during the incubation period. β-sitosterol represented 45% of the total phytosterols and both stigmasterol and campesterol were at 27%-28%.

Significant phytosterol removal was not observed despite the addition of another carbon source to achieve high heterotrophic microbial activity. The phytosterol concentration across the dextrin series was comparable to the average concentration across the above-discussed phytosterol-only series (46 ± 6 mg/L). Addition of dextrin and the resulting increase in heterotrophic microbial activity did not significantly enhance phytosterol biotransformation. The bioavailable phytosterol concentration was again limited by low solubility. It is also likely that dextrin was not a suitable carbon source to induce significant production of cholesterol oxidase. Phytosterols are able to induce cholesterol oxidase production when another carbon source is present, although the effect of different carbon sources can vary. Cholesterol oxidase produced by *Rhodococcus* sp. GK1 with hexanoic acid as a carbon source was reported to have a specific activity of 0.84 enzyme units/mg protein, whereas when D-ribose, D-glucose, cholesterol or soybean sterols were the carbon source, there was insignificant cholesterol oxidase activity (Kreit et al., 1992). Dextrin contains polymers of D-glucose units and therefore may not be capable of inducing significant levels of cholesterol oxidase.

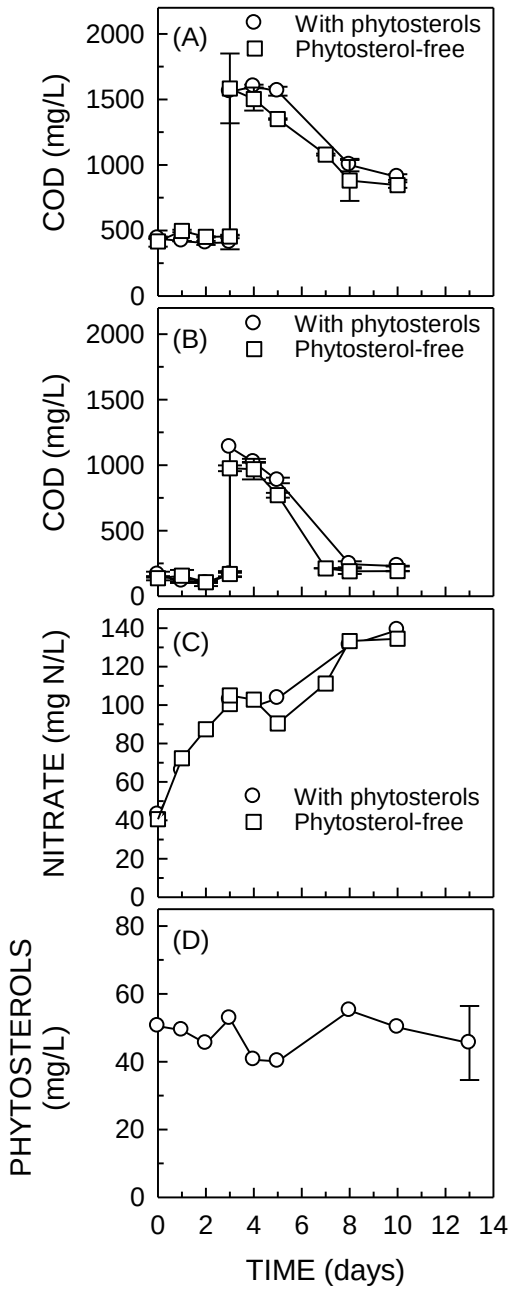


Figure 4.4. Total COD (A), soluble COD (B), nitrate (C), and total phytosterols (D) in the phytosterol-amended flasks and phytosterol-free control during the incubation of the aerobic bioassay conducted with dextrin added on day 3. Error bars represent mean values ± one standard deviation, $n = 3$.

4.3.2.3. Ethanol as an additional carbon source and solubilizing agent

Two assays were conducted with ethanol as both a carbon source and a solubilizing agent for phytosterols. In the first assay, a well-mixed suspension of phytosterols in ethanol was added to sacrificial Erlenmeyer flasks. Flasks were extracted without sampling to reduce measurement variability caused by sampling a non-homogeneous mixture. The measured phytosterol concentrations from triplicate flasks over the 6 day assay are shown in Figure 4.5. Phytosterol biotransformation could not be determined with certainty as any phytosterol removal that occurred fell within the measurement variability. One study with *Rhodococcus erythropolis* found that induction of intracellular cholesterol oxidase was not observed until after 36 h exposure to cholesterol. The intracellular enzyme peaked on day 6 and declined sharply afterward, followed by the appearance of extracellular cholesterol oxidase (Sojo et al., 1997). If extracellular cholesterol oxidase was not expressed until near the end of the assay in the present study, a variation in its emergence between flasks may have been responsible for the larger error bars on days 5 and 6 (Figure 4.5).

In the second assay, a single reactor was set up with an ethanol-dissolved phytosterol solution at a lower phytosterol concentration and re-spiked on the sixth day. The total and soluble COD are shown in Figure 4.6A. Within the first 3 d, 93% of the soluble COD and 50% of the total COD was removed. Until day 3, the soluble COD was consumed at an average rate of 800 mg COD/L-d. Following the re-spiking on day 6, the soluble COD was consumed at a faster average rate of 1,000 mg COD/L-d over days 6-8 and 90% of the soluble COD was consumed within 2 days. Periodic nitrate measurements corresponded closely with data from preliminary tests that showed robust nitrification

(data not shown). The measured total phytosterol concentrations over the 22-day incubation are shown in Figure 4.6B. The phytosterol concentration decreased steadily over a period of 6 days, at which time the reactor was re-spiked with the phytosterol and ethanol solution. The phytosterol concentration again decreased steadily over 7 days before phytosterol removal accelerated sharply and then slowed as the phytosterol concentration declined. Phytosterol concentrations were undetectable on day 24 (<18.75 μg/L) and day 26 (<12.5 μg/L). More accurate phytosterol measurements were not possible due to limited culture volume available for extraction. Phytosterol biotransformation intermediates were not detected during the assay, indicating that the initial phytosterol oxidation reaction was more likely the rate-limiting step of biotransformation and/or that intermediates were not sufficiently hydrophobic to be collected with the extraction procedure used in this study.

Phytosterols were added as part of an ethanol solution and entered the liquid phase as dissolved molecules, increasing their bioavailability and contact with microorganisms. When the phytosterol/ethanol solution was added to the reactor, the phytosterol concentration declined rapidly over the first 24 hours at a rate of 0.62 mg/L-d, a pattern that was repeated when spiked on the sixth day, achieving a removal rate of 0.74 mg/L-d over 24 hours. After the first addition of ethanol and phytosterols, the phytosterol concentrations declined in a linear fashion. Linear regression of the data points yielded an overall phytosterol removal rate of 0.47 mg/L-d ($R^2 = 0.998$) for the initial period through day 6. After the second spiking, two distinct stages of phytosterol removal were observed. The first stage lasted for the first 7 days following the spike. During this time, the phytosterol removal was nearly linear ($R^2 = 0.968$), with an overall phytosterol

removal rate of 0.32 mg/L-d. The second stage began on the 7th day following the spike (day 13). The phytosterol removal rate increased sharply, leading to a large drop in phytosterol concentration (Figure 4.6B). As the phytosterol concentrations declined, their rate of removal also declined and their concentrations almost leveled off before they fell below the detection limit.

The three phytosterols in the mixture used were not degraded at a similar rate as seen by the dissimilar slopes in Figure 4.6C showing that β-sitosterol was preferentially transformed. Initially, β-sitosterol made up 47% of the total phytosterols but by day 22 dropped to 42%. The 5% reduction was compensated by an increase in the stigmasterol and campesterol share of the total phytosterols by 2% and 3%, respectively. The greater biotransformation of β-sitosterol could be due to its relatively higher concentration and/or its side chain structure. Although cholesterol oxidase attacks the 3β-hydroxyl group on the sterol molecule, the side chain structure affects the positioning of the sterol within the enzyme's active site (Brooks and Smith, 1975; Doukyu, 2009; Sampson and Vrielinek, 2003). In a study of cholesterol oxidase of *Rhodococcus* sp. GK1, the enzyme was most active with cholesterol (100%) and less active with β-sitosterol (70%) and stigmasterol (40%) (MacLachlan et al., 2000). Therefore, the faster rate of β-sitosterol oxidation observed in the present study could be due in part to the enzyme specificity of cholesterol oxidase.

The first stage in the decline of phytosterol concentration could represent the relatively slower metabolism of phytosterols via membrane-bound cholesterol oxidase. A similar initial stage was reported in the batch cultivation of *R. erythropolis*, during which exponential growth occurred along with an increase in membrane-bound cholesterol

oxidase activity, while extracellular cholesterol oxidase activity was insignificant (Sojo et al., 1997; Sojo et al., 2002). In the present study, the initial stage of phytosterol removal (day 6-13) occurred during a period of cell growth, as shown by the increase in VSS in Figure 4.6D. Unlike extracellular cholesterol oxidase, the membrane-bound enzyme is involved in cell metabolism of cholesterol, which contributes to a small degree to cell growth (Fernandez de las Heras, 2011; Sojo et al., 1997). The second stage in the decline of phytosterol concentration could be due to an increase in the release of extracellular cholesterol oxidase, which has been reported to appear towards the end of the exponential growth phase (Sojo et al., 1997). In this stage, cell growth, measured in terms of VSS, did not appear to correlate with the removal of phytosterols. The period of fastest phytosterol removal, in which the total phytosterol concentration declined by 78% in 2 d (days 13-15), occurred while the biomass concentration was in decline (Figure 4.6D). A study on *Nocardia rhodocrous* found that the amount of extracellular cholesterol oxidase continued to increase after the end of the exponential growth phase but that the membrane-bound cholesterol oxidase decreased proportionally; thus, the total amount of enzyme remained constant (Buckland et al., 1976). Sojo et al. (1997) also showed a decline in the intracellular cholesterol oxidase of *R. erythropolis* coupled with an increase in extracellular cholesterol oxidase, with kinetics that suggested the excretion of extracellular enzyme was due to the partial solubilization of membrane-bound cholesterol oxidase. Therefore, the results of the present study are in agreement with reports that intracellular cholesterol oxidase contributes slightly to cell growth, while extracellular cholesterol oxidase does not (Fernandez de las Heras, 2011; Sojo et al., 1997).

The results of the above-discussed batch assays conducted in the present study may be used to interpret the observed phytosterol removal by the stock culture. The stock culture undergoes exponential growth within the first day following feeding, after which the majority of the available COD is depleted and the microbial activity slows down (Figure 4.6). Therefore, the aerobic culture spends approximately 70% of the feeding cycle under low growth conditions conducive to the release of extracellular cholesterol oxidase. Thus, relatively long residence times, which correspond to low growth rates likely lead to conditions under which cholesterol oxidase is expressed leading to enhanced biotransformation of phytosterols.

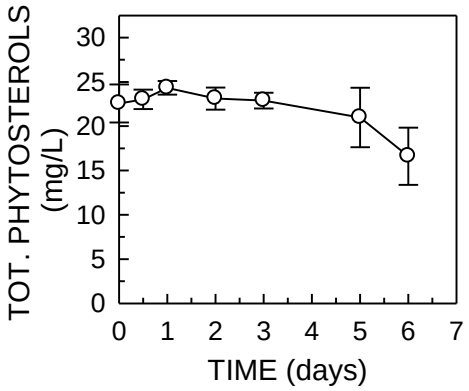


Figure 4.5. Total phytosterol concentration during the 6-day aerobic bioassay with phytosterols added as a high concentration suspension in ethanol. Error bars represent mean values ± one standard deviation, $n = 3$.

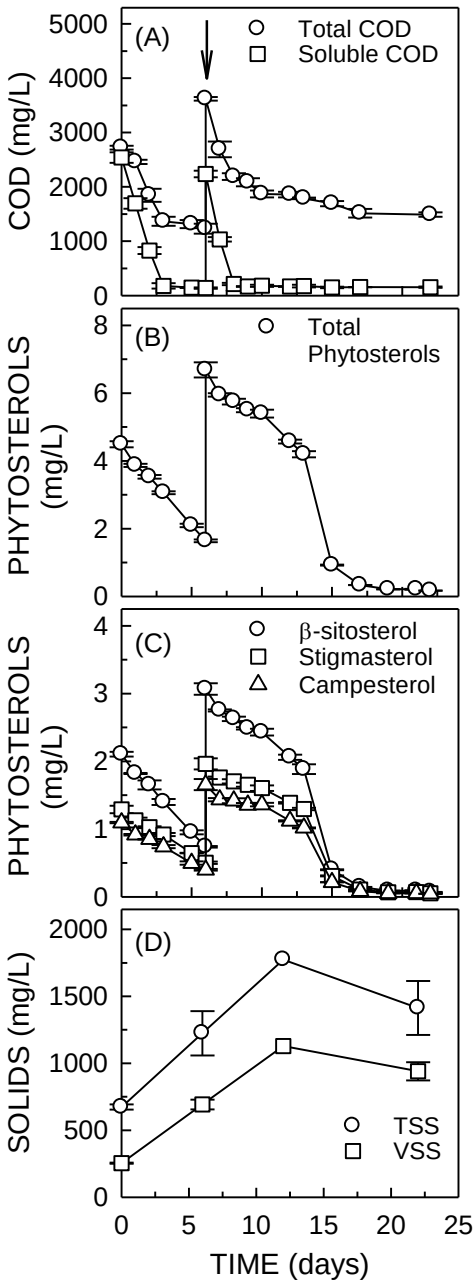


Figure 4.6. Total and soluble COD (A), total phytosterols (B), β-sitosterol, stigmasterol and campesterol (C) and TSS and VSS (D) during the 22-day aerobic bioassay with phytosterols added as a solution in ethanol. Arrow in panel A indicates re-spiking with phytosterols ethanol solution. Error bars represent mean values ± one standard deviation, $n = 3$.

4.4. Summary

Phytosterols were aerobically degraded when dissolved in ethanol. After spiking with a phytosterol/ethanol solution, a two-stage phytosterol removal was observed, with an initial linear degradation period followed by a period of accelerated removal. β-sitosterol was preferentially degraded, as compared to stigmasterol and campesterol, although all three phytosterols fell below detection limits by the 24th day of incubation. On the other hand, no significant phytosterol removal was observed in assays conducted with phytosterol powder, either with or without an additional carbon source. This study shows that phytosterol biotransformation under aerobic conditions is limited by their low solubility. When phytosterol solubility is enhanced, as was the case with ethanol in this study, a period of steady phytosterol removal, followed by an accelerated removal phase was observed as the culture approached the stationary phase. The appearance of a second phase of phytosterol removal with significantly different kinetics suggests two different mechanisms are involved in phytosterol biotransformation. Membrane-bound and extracellular cholesterol oxidase are thought to be involved in the two mechanisms responsible for phytosterol oxidation. The late appearance of a period of accelerated phytosterol removal suggests that longer residence times may be necessary to achieve greater phytosterol biotransformation. Further study is needed to better understand the fate of phytosterols in complex systems like ASBs in which oxic and anoxic/anaerobic zones are part of a dynamic system.

CHAPTER 5

ANOXIC AND ANAEROBIC BIOTRANSFORMATION POTENTIAL OF PHYTOSTEROLS

5.1. Introduction

Phytosterols are hydrophobic molecules with low water solubility and a tendency to adsorb to solids within wastewater treatment systems. ASBs treat pulp mill wastewater through a combination of biological treatment and settling, which carries solids-associated phytosterols into sediments where anoxic or anaerobic conditions prevail. Although phytosterols may be biodegraded under aerobic conditions, less is known about their potential for transformation under anoxic or anaerobic systems. Similar in structure to cholesterol, phytosterols are thought to follow similar biotransformation pathways. In the mammalian gut, cholesterol undergoes microbial conversion to coprostanol either by following a pathway with intermediate formation of 4-cholesten-3-one or by direct reduction of the 5-6 double bond (Gerard et al., 2007). The hydrogenation of cholesterol to cholestanol in oxygen-depleted sediments was observed at a rate of 0.5% conversion over a period of 3 months (Gaskell and Eglington, 1975).

Sterolibacterium denitrificans is capable of transforming phytosterols under denitrying conditions. The first step in the proposed pathway is the anoxic oxidation of the C3 hydroxyl group of cholesterol, forming cholest-5-en-3-one. Next, cholest-5-en-3-one undergoes isomerization to cholest-4-en-3-one. It is thought that these two steps are the result of a single bifunctional enzyme, as in the case of cholesterol oxidase under aerobic conditions. The third step is the dehydrogenation of the A ring to result in

cholesta-1,4-dien-3-one, or the hydroxylation of the side chain at the C25 position. Cholesta-1,4-dien-3-one may also undergo similar C25 hydroxylation of the side chain. The remaining steps in the anoxic cholesterol degradation pathway are unclear but a radiolabelling experiment showed *Sterolibacterium denitrificans* was capable of both cholesterol assimilation and mineralization to CO_2 (Chiang et al., 2007).

The fate of phytosterols under anoxic/anaerobic conditions is unclear and little is known about the quantity of phytosterols likely to enter ASB anoxic/anaerobic zones through settling. Therefore, the objective of this study was to i) determine the amount of phytosterols likely to enter anoxic/anaerobic zones by fractionating wastewater through settling; and ii) assess the biotransformation potential of three phytosterols (campesterol, stigmasterol and β-sitosterol) under denitrifying, sulfate-reducing and fermentative/methanogenic conditions.

5.2. Materials and Methods

5.2.1. Phytosterols

A soybean-derived phytosterol mixture containing β-sitosterol, stigmasterol and campesterol was purchased from Purebulk.com (Roseburg, OR). A stock solution was prepared in ethanol (>99%) with a total phytosterol concentration of 4.77 g/L. Analysis of the stock solution by GC-MS/FID determined it to contain 2.12 g/L β-sitosterol, 1.33 g/L stigmasterol and 1.32 g/L campesterol.

5.2.2. Stock Cultures and Inocula

The stock aerobic culture, described in Section 4.2.2, was capable of nitrate-reduction and was used as inocula for the denitrifying assay. A sulfate-reducing culture and a fermentative/methanogenic culture were developed with sediment from the ASB of a pulp and paper mill. Both sulfate-reducing and fermentative/methanogenic cultures were fed settled solids from untreated pulp and paper mill wastewater, amended with phosphate buffer (372 mg P/L, pH 7.2) and NH_4Cl (120 mg N/L). The resulting feed characteristics were: pH, 7.1±0.2; total COD, 2,090±28 mg/L; soluble COD, 300±3 mg/L; TSS, 3.2±0.05 g/L; VSS, 1.3±0.03 g/L (Giles, 2012). The sulfate-reducing culture was also amended with Na_2SO_4 (1.5 g S/L) and 50 mL of anaerobic culture medium (Table 5.1), which contained vitamin stock (Table 5.2). Some sulfate was present in the methanogenic culture feed (33±1 mg S/L) due to the presence of sulfate in the pulp and paper mill wastewater solids. Both cultures were maintained at room temperature (20-22^{o}C) with an HRT/SRT of 21 days.

Table 5.1. Composition of the anaerobic culture medium

Compound	**Solution Concentration**
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
NH_4Cl	0.5 g/L
$Na_2S.9H_2O$	0.5 g/L
$NaHCO_3$	3.5 g/L
1 g/L Resazurin Stock	1.0 mL/L
Vitamin Stock[a]	1.0 mL/L
Trace Metal Stock[b]	1.0 mL/L

[a]Vitamin stock solution composition is described in Table 5.2. [b]Trace metal stock solution composition is described in Table 4.1.

Table 5.2. Composition of vitamin stock solution

Vitamin Stock	Solution Concentration
Biotin	0.2 g/L
Folic Acid	0.2 g/L
Pyridoxine hydrochloride	1.0 g/L
Riboflavin	0.5 g/L
Thiamine	0.5 g/L
Nicotinic Acid	0.5 g/L
Pantothenic Acid	0.5 g/L
Vitamin B12	0.01 g/L
p-Aminobenzoic Acid	0.5 g/L
Thioctic Acid	0.5 g/L

5.2.3. Semi-continuous cultures

Three semi-continuously fed cultures were prepared to assess the biotransformation potential of the three phytosterols under nitrate-reducing, sulfate-reducing and fermentative/methanogenic conditions. The aerobic stock culture described above in Section 5.2.2. was used as inoculum for the nitrate-reducing culture. The stock methanogenic and sulfate-reducing cultures were fed with the settled solids of pulp and paper wastewater and thus contained a significant quantity of partially-degraded wastewater solids. To avoid introducing solids that could potentially release additional phytosterols over the course of incubation, wasted volumes from the methanogenic and sulfate-reducing stock cultures were allowed to settle for one hour and the low-solids supernatant was used as inoculum for the methanogenic and sulfate-reducing semi-continuous cultures. The three semi-continuous cultures were prepared in 2.2 L glass flasks pre-flushed with helium. Inoculum (500 mL) was combined with medium, (Table 5.2.1) for a total liquid volume of 1.5 L. The cultures were fed 1 mL of the stock phytosterol/ethanol solution, adding 1,100 mg/L COD and resulting in an initial

phytosterol concentration of 3.18 mg/L. After the initial feeding, the cultures were incubated for 14 days to allow time for acclimation. The cultures were maintained at room temperature (20-22^{o}C) and gently mixed by hand once per day. Following the second feeding on day 14, the cultures were fed every 7 days. Feedings were conducted by replacing 0.5 L of well-mixed culture with fresh medium (Table 5.3) and adding 1 mL of the stock phytosterol/ethanol solution. The resulting retention time of all three cultures was 21 days. The waste at each feeding was analyzed for pH, soluble COD, nitrate, nitrite, sulfate, VFAs and phytosterols. Gas production was measured daily and gas composition was measured at the end of each feeding cycle. After 16 feeding cycles (112 days), the three cultures were batch incubated for 7 months and then re-analyzed.

Table 5.3. Medium composition for semi-continuous cultures

Component	Denitrifying Medium Concentration	Sulfate-Reducing Medium Concentration	Methanogenic Medium Concentration
K_2HPO_4	0.9 g/L	0.9 g/L	0.9 g/L
KH_2PO_4	0.5 g/L	0.5 g/L	0.5 g/L
NH_4Cl	0.5 g/L	0.5 g/L	0.5 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L	0.2 g/L	0.2 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L	0.1 g/L	0.1 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L	0.1 g/L	0.1 g/L
$Na_2S \cdot 9H_2O$	-	-	0.5 g/L
$NaHCO_3$	-	-	3.5 g/L
Vitamin stock	1 mL/L	1 mL/L	1 mL/L
Trace metal stock	1 mL/L	1 mL/L	1 mL/L
Resazurin stock	-	-	2 mL/L

5.3. Results and Discussion

5.3.1. Biotransformation assays

The nitrate-reducing, sulfate-reducing and fermentative/methanogenic were monitored over 16 feeding cycles (112 days). All three cultures were active, as shown by the time course measurements of culture pH, soluble COD, electron acceptor (i.e., nitrate or sulfate) and gas production (i.e., headspace pressure) in Figure 5.1. Phytosterol removal was observed in the nitrate-reducing culture throughout the assay, although microbial activity and phytosterol removal declined following the 9^{th} feeding cycle. Phytosterol removal was also observedin the sulfate-reducing culture after the 8^{th} feeding cycle (Figure 5.1A5, B5). No significant phytosterol removal was observed in the methanogenic culture. The concentration of each of the three phytosterols (β-sitosterol, campesterol and stigmasterol) was measured at the end of each feeding cycle and is shown in Figure 5.2. Each phytosterol concentration decreased during phytosterol removal by the nitrate-reducing or sulfate-reducing cultures. Phytosterol biotransformation was shown to be highly dependent upon the redox condition, with removal possible under nitrate-reducing and sulfate-reducing conditions. Under fermentative/methanogenic/ conditions, no detectable removal was observed. The difference in phytosterol removal under the three redox conditions highlights the complexity of assessing the fate of solids-associated phytosterols within anoxic or anaerobic sediments.

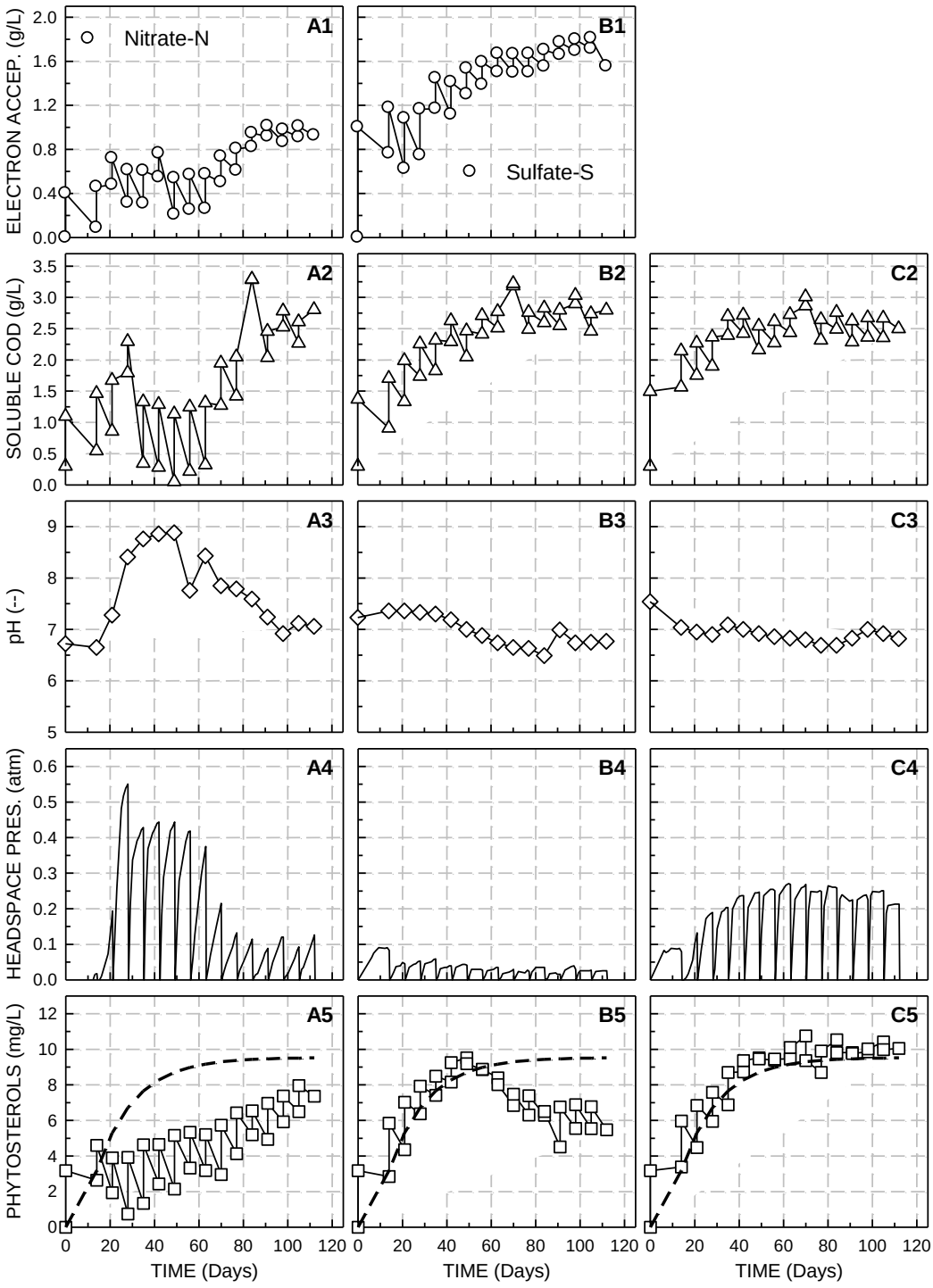


Figure 5.1. Time course of (1) electron acceptor (i.e., nitrate or sulfate), (2) soluble COD, (3) pH, (4) headspace pressure, and (5) total phytosterols in semi-continuously fed nitrate-reducing (A), sulfate-reducing (B), and methanogenic (C) cultures (Broken lines represent phytosterol concentrations calculated taking into account wasting and feeding of the cultures and assuming complete recalcitrance of the three phytosterols).

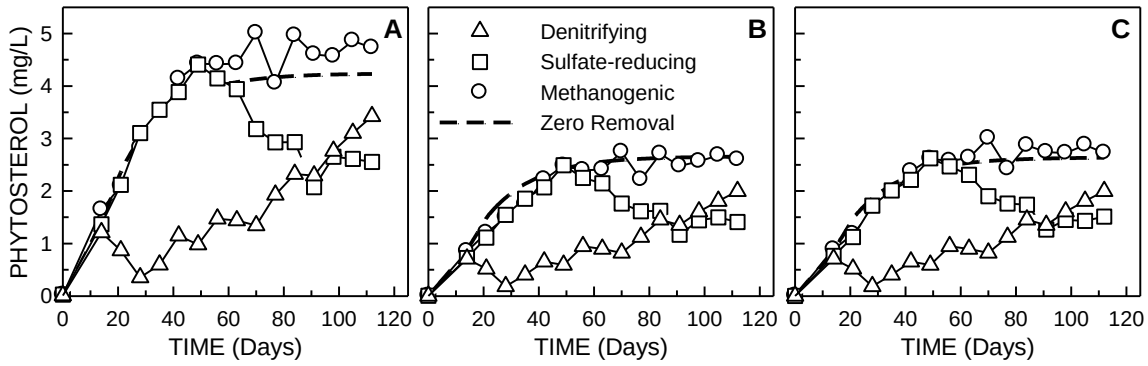


Figure 5.2. Phytosterol profiles in the three semi-continuously fed cultures. A, β-sitosterol; B, Stigmasterol; C, Campesterol (Broken lines represent phytosterol concentrations calculated taking into account wasting and feeding of the cultures and assuming complete recalcitrance of the three phytosterols).

5.3.1.1. Nitrate-reducing conditions

The nitrate-reducing culture was highly active for the first 9 feeding cycles. During this period, the culture pH continually increased between 7.5 and 8.9, necessitating periodic adjustments with 0.1 N HCl. Both COD and nitrate removal were high and gas production was robust (Figure 5.1A1-A4). With the onset of the 10th feeding cycle, the gas production decreased and the residual soluble COD gradually increased, reaching 2,800 mg/L by the end of the last feeding cycle (Figure 5.1A). During the last 7 feeding cycles, the culture pH ranged from 7.0 to 7.8. Phytosterol removal was significant during the first 70 days (10 feeding cycles), with 69%-88% removal of all three phytosterols. However, the phytosterol removal began to decrease during the last 7 feeding cycles and was only 19% by the end of the 16th feeding cycle (Figure 5.1A5). The total phytosterol concentration at the end of the 16th feeding cycle was 7.36 µg/L,

consisting of 3.42 µg/L β-sitosterol, 2.00 µg/L campesterol and 1.94 µg/L stigmasterol. Phytosterol biotransformation products were not detected during the assay.

The results show that significant removal of phytosterols is possible under nitrate-reducing conditions. Indeed, biodegradation of cholesterol under nitrate-reducing conditions has been previously reported (Harder and Probian, 1997; Chiang et al., 2007; 2008). The reason for the observed decline in microbial activity and phytosterol removal following the 10th feeding cycle is unknown. It is possible that an inhibitor was present or that the pH instability experienced during the initial feeding cycles affected the microbial community. In ASB treatment systems, nitrate-reducing microenvironments may be present in anoxic zones, although activity is likely to be limited by the fact that low nitrate levels are found in pulp and paper wastewater.

5.3.1.2. Sulfate-reducing conditions

The sulfate-reducing culture was active throughout all of the 16 feeding cycles (Figure 5.1B). The pH ranged between 6.8 and 7.3 and the culture produced 20 to 42 mL of gas (at 25°C and 1 atm) per feeding cycle, as measured by the pressure in the headspace (Figure 5.1B3,B4). The soluble COD at the end of each feeding cycle increased to 2,400 mg/L by the end of the 16th feeding cycle due to an accumulation of VFAs, primarily acetate. The sulfate-reducing culture removed approximately 25% of the soluble COD during the last feeding cycle. The extent of sulfate removal (27%) was similar to the extent of soluble COD removal (22%) in the last feeding cycle. Sulfate gradually accumulated over the duration of the test and reached 1,500 mg S/L by the end. No significant phytosterol removal was observed during the first 7 feeding cycles (Figure

5.1B5). However, a significant decrease in phytosterol concentration was observed following the onset of the 8th feeding cycle. By the end of the test, the percent removal of β-sitosterol, stigmasterol and campesterol over one feeding cycle was 40%, 47% and 43%, respectively (Figure 5.2) The final β-sitosterol, stigmasterol and campesterol concentrations were 2.55, 1.41 and 1.51 µg/L, respectively (total phytosterol concentration of 5.57 µg/L). At the end of the 14th feeding cycle, stigmast-4-en-3-one was identified by GC-MS with 90% similarity to the stigmast-4-en-3-one in the NIST08.L Database (Figure 5.3). Stigmast-4-en-3-one is most likely to be a biotransformation product of β-sitosterol because of its similarity in structure (Figure 5.4). Stigmast-4-en-3-one has a saturated side chain, unlike stigmasterol, and a two carbon branch on its side chain, unlike campesterol. Recently, the production of stigmast-4-en-3-one from β-sitosterol by *Rhodococcus erythropolis* was reported under oxidative conditions (Grishko et al., 2012). It is possible that stigmast-4-en-3-one is an early intermediate in both the oxic and anoxic metabolic pathways. In studies with cholesterol, cholest-4-en-3-one is reported to be an intermediate of cholesterol biotransformation under both oxic and anoxic (denitrifying) conditions (Uhia et al., 2011; Chiang et al., 2007; 2008). Additionally, *Rhodococcus erythropolis* has been implicated in the reduction of sulfate to sulfite (Takarada et al., 2005). The onset of phytosterol removal (8th feeding cycle) occurred as the pH decreased below 7.0 and the sulfate levels increased above 1.2 g S/L. The relatively low pH and high sulfate levels could have contributed to the enrichment of particular sulfate-reducing bacteria and those bacteria may have also been capable of phytosterol biotransformation.

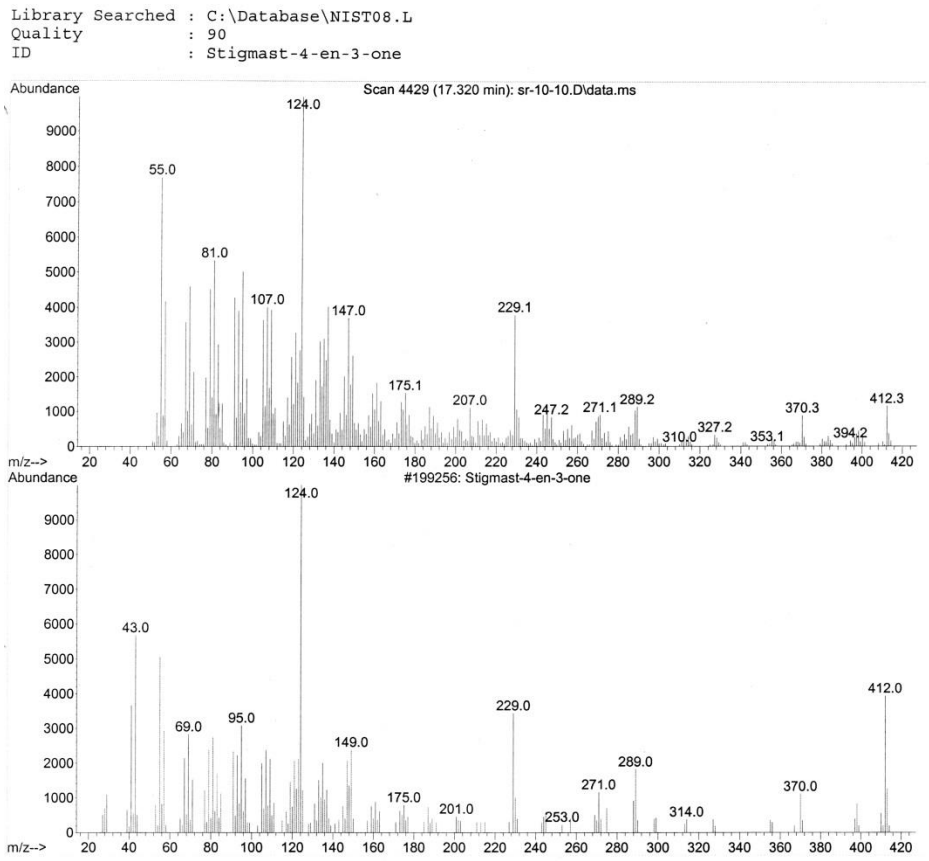


Figure 5.3. Comparison of the detected compound in the 14th feeding cycle of the sulfate-reducing culture with the NIST database for stigmast-4-en-3-one.

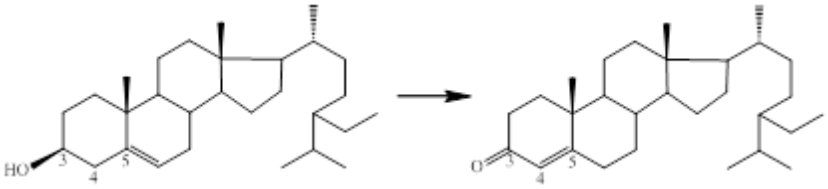


Figure 5.4. Production of stigmast-4-en-3-one from β-sitosterol.

5.3.1.3. Methanogenic conditions

The methanogenic culture was active throughout all of the 16 feeding cycles (Figure 5.1C). The pH ranged between 6.8 and 7.0 and the culture produced 130 to 190 mL of gas (at 25°C and 1 atm) per feeding cycle, as measured by the pressure in the headspace (Figure 5.1C3, C4). The gas produced was 66% methane, indicating active methanogenesis. The residual soluble COD increased over the first 4 feeding cycles and then remained nearly constant at approximately 2,500 mg/L through the end of the test. At the end of the 16^{th} feeding cycle, COD removal was about 24%. Residual COD was primarily due to an accumulation of VFAs, particularly acetate. No phytosterol removal was observed under methanogenic conditions and all three phytosterols were not degraded (Figures 5.1C and 5.2). The final β-sitosterol, stigmasterol and campesterol concentrations were 4.73, 2.60 and 2.73 µg/L, respectively (total phytosterol concentration of 10.06 µg/L). Although the hydrogenation of cholesterol to cholestanol by fermentative bacteria has been reported (Gaskell and Eglington, 1975), biotransformation of cholesterol or phytosterols by methanogens has not been reported.

5.3.2. Long-term batch incubation

Following 16 feeding cycles, the nitrate-reducing, sulfate-reducing and methanogenic cultures were incubated for 7 months without wasting or feeding. Samples were then collected under mixing and analyzed for pH, soluble COD, VFAs, electron acceptors (i.e., nitrate, sulfate), ammonia and phytosterols. The measurements obtained following batch incubation are shown in Table 5.2. In the nitrate-reducing culture, significant nitrate and soluble COD were present, indicating inhibition of the culture.

Nitrite was not detected. The ammonia concentration was similar to that in the other two cultures, indicating a lack of dissimilatory nitrate reduction to ammonia. VFAs, primarily in the form of acetate, were present in low levels in the nitrate-reducing culture. The reason for the inhibition observed in the nitrate-reducing culture is not clear. The phytosterol concentration in the nitrate-reducing culture was nearly unchanged from the beginning of the incubation period, also indicating little activity.

The soluble COD in the sulfate-reducing culture was high and consisted primarily of accumulated VFAs, particularly acetate. Sulfate was consistently removed by an average of 29% over each feeding cycle, thus the remaining sulfate gradually accumulated in the system. After 7 months incubation, the total phytosterol concentration in the sulfate-reducing culture decreased by 23% to 4.20 μg/L. β-sitosterol was preferentially removed (26%), while stigmasterol and campesterol were removed at a nearly equal extent (20% and 22%, respectively). The phytosterol removal indicates some activity occurred during batch incubation in the sulfate-reducing culture.

The fermentative/methanogenic culture had a low level of soluble COD and VFAs were not detected. The culture was active during incubation and showed no indication of inhibition. Despite evidence of microbial activity, the phytosterol concentrations remained relatively unchanged. These results show that phytosterols are recalcitrant under fermentative/methanogenic conditions even over long periods of time.

Table 5.4. Analyses of the nitrate-reducing, sulfate-reducing and fermentative/ methanogenic cultures following long-term incubation[a]

Parameter	Nitrate-reducing Culture	Sulfate-reducing Culture	Fermentative/ Methanogenic Culture
pH	8.23 ± 0.02[b]	6.90 ± 0.02	7.27 ± 0.02
Soluble COD (mg/L)	891 ± 88	2,460 ± 87	623 ± 92
VFAs (mg COD/L)	58.5 ± 3.7	2,140 ± 138	ND
Nitrate (mg N/L)	141.2 ± 3.8	ND	ND
Sulfate (mg S/L)	ND[c]	1,656 ± 78	ND
Ammonia (mg N/L)	109.2 ± 4	114.8 ± 6	114.8 ± 3
Phytosterols (mg/L)	7.31 ± 0.20	4.20 ± 0.11	10.61 ± 0.23
Campesterol	1.98 ± 0.06	1.21 ± 0.04	3.06 ± 0.04
Stigmasterol	2.00 ± 0.04	1.10 ± 0.03	2.77 ± 0.05
β-Sitosterol	3.33 ± 0.10	1.88 ± 0.03	4.79 ± 0.14

[a] After 7 months batch incubation; [b] Mean ± standard deviation (n = 3); [c] ND, not detected

5.4. Summary

Removal of phytosterols was observed under nitrate-reducing and sulfate-reducing conditions, but not under fermentative/methanogenic conditions. The nitrate-reducing culture was capable of significant phytosterol removal during the first 9 feeding cycles but phytosterol removal declined as the microbial activity decreased for unknown reasons. The sulfate-reducing culture did not remove phytosterols until the onset of the 8th feeding cycle, after which significant phytosterol removal was observed. It is unknown why the sulfate-reducing culture experienced a long lag time before phytosterol biotransformation occurred. It is possible that enrichment of certain sulfate-reducing

bacteria could have contributed to the significant phytosterol removal observed in the latter part of the assay. Stigmast-4-en-3-one was identified under sulfate-reducing conditions as a potential intermediate generated from the anaerobic oxidation of β-sitosterol as an initial step in biodegradation. Biotransformation of phytosterols, particularly under sulfate-reducing conditions is relevant to the treatment of pulp and paper wastewater where sulfate is often present. More research is needed to better understand the factors and conditions that affect phytosterol biodegradation, especially under sulfate-reducing conditions. Improving understanding may lead to the development of more efficient biological treatment systems for the removal of phytosterols.

CHAPTER 6

FATE OF PHYTOSTEROLS IN A SIMULATED AERATED STABILIZATION BASIN

6.1. Introduction

ASBs are non-homogeneous systems that consist of both spatial and temporal gradients of nutrients, oxygen and various wastewater components. In the direction of flow, oxygen gradients are controlled by tapered aeration that provides more dissolved oxygen near the entrance to the ASB where wastewater exerts a higher oxygen demand. Perpendicular to the direction of flow, gradients exist due to settling within the ASB. Wastewater solids are carried into sediment layers while soluble components remain in the water column. The various gradients within the system are a result of a multitude of interrelated, complex processes.

The simplest representation of an ASB may consider the system to consist of two distinct zones: an aerobic zone within the water column, and an anoxic/anaerobic zone within the sediment. Considering the aerobic zone, inputs include the influent wastewater (consisting of both solids and soluble components), oxygen from aeration, and benthal feedback of both soluble and gaseous products from the anoxic/anaerobic zone. Within the aerobic zone, oxidation of organics occurs along with production of biomass, which may settle into the sediment layer. Outputs from the aerobic zone include the settling of solids and the release of soluble components and non-settled solids in the effluent. Within the anoxic/anaerobic sediments, the inputs come from solids entering through settling. Hydrolysis of solids occurs within this zone, releasing soluble products. Anoxic oxidation

or reduction of compounds occurs within the sediments and products are released to the aerobic zone in the form of soluble and gaseous compounds. Even at its simplest representation, the processes that occur within an ASB are complex.

ASBs are capable of removing phytosterols, although the extent of removal varies over time and among treatment systems (Mahmood-Khan and Hall, 2003, 2008, 2012, 2013). As noted in Chapters 4 and 5, the fate of phytosterols in a biological system is highly dependent upon the redox state encountered. Within ASBs, multiple redox zones exist, which may be broadly divided into aerobic, anoxic, sulfate-reducing and fermentative/methanogenic zones. In each zone, different biological processes occur that are important to the fate of phytosterols. Under aerobic conditions, phytosterols are degradable but some evidence suggests the degradation rate may be linked to the growth phase of the requisite microbes, with faster kinetics under stationary phase conditions (Chapter 4; Dykstra et al., 2014a). Additionally, phytosterol removal has been observed under nitrate-reducing conditions and under sulfate-reducing conditions following a significant lag, but over the course of a 112 day assay, phytosterol removal was not observed under fermentative/methanogenic conditions (Chapter 5; Dykstra et al., 2014b). From the results of these studies, it is clear that the biodegradability of phytosterols varies significantly between redox zones. Thus, the fate of phytosterols in ASBs depends on the distribution of phytosterols in the wastewater (i.e., phytosterols associated with solids vs. the soluble component) and the settling characteristics of the wastewater. As hydrophobic molecules, phytosterols have a tendency to adsorb to solids and may be carried into anoxic/anaerobic zones. On the other hand, plant materials in wastewater may release bound phytosterols during degradation.

The complexity of the biological activity and incomplete mixing conditions makes it difficult to predict the fate of phytosterols during ASB treatment. Therefore, the objective of this portion of the study was to better understand the processes and biotransformation of phytosterols within an ASB treatment system by: i) determining the characteristics of pulp and paper wastewater as a whole and as fractions separated through settling; ii) comparing the steady-state performance and phytosterol removal of a simulated ASB operated at various hydraulic retention times (HRT); and iii) investigating the microbial activity within the ASB aerobic and anoxic/anaerobic zones.

6.2. Materials and Methods

6.2.1. Pulp and paper wastewater fractionation and characterization

Untreated wastewater was obtained from a pulp and paper mill located in the southeastern United States. At the time of collection, the pulp mill used the Kraft process to produce newsprint from a mixture of hardwood and softwood pulp. The wastewater was stored at 4°C until use. The raw wastewater was analyzed for pH, TS, VS, total and soluble COD, nitrate, nitrite, ammonia, chloride, phosphate, sulfate and phytosterols. In ASB systems, wastewater is separated through settling within the basin, exposing soluble and settleable fractions to different redox conditions. Soluble components of the wastewater remain in the aerobic zone (oxidative conditions), while settleable solids enter the sediment layer and encounter more reductive conditions. Therefore, the fate of any particular wastewater component within an ASB is dependent upon its settling characteristics. To identify the wastewater components that might be expected within ASB aerobic and anoxic/anaerobic zones, the raw wastewater was fractionated. The

untreated wastewater was separated into two fractions by settling, yielding a supernatant fraction and a settled fraction. The wastewater settling was conducted in two identical 1000 mL glass graduated cylinders, each with a diameter of $2\frac{5}{16}$ inches (5.87 cm) and a height of 14.5 inches (36.9 cm) to the 1000 mL mark. Raw wastewater was thoroughly mixed and 1 L was poured into each of the graduated cylinders. Settling was conducted at room temperature (20-22°C). The maximum absorbance of the raw wastewater was obtained at a wavelength of 590 nm; thus, absorbance measurements at a wavelength of 590 nm were used to monitor the progress of the settling. At intervals throughout the settling, an aliquot of wastewater was taken from the center of each graduated cylinder at a depth of 3 inches (7.62 cm), and the absorbance was measured. The absorbance measurements for each of the graduated cylinders were taken separately and averaged to track average settling behavior. The settling was terminated at 15.5 hours, when the absorbance measurements approached a stable value. Following settling, the supernatant was removed through siphoning. Each wastewater fraction was analyzed for pH, total solids (TS), volatile solids (VS), total and soluble COD, nitrate, nitrite, ammonia, chloride, phosphate, sulfate and phytosterols. For phytosterol measurement, the whole wastewater was extracted in triplicate using the stir-plate method (Section 3.2.3) with 100 mL sample volume and 10 mL of isooctane. After extraction for 24 hours, the isooctane was directly analyzed with GC-MS/FID, without pre-concentration. The unfiltered supernatant was extracted in triplicate using the stir-plate method with 200 mL sample volume and 50 mL of isooctane. The isooctane was then evaporated using N_2 gas and the sample residue reconstituted with 1 mL of isooctane prior to analysis with GC-MS/FID. For all extractions, cholesterol was used as a surrogate standard.

6.2.2. ASB development and maintenance

A simulated ASB was developed using the mixed liquor of an ASB located at a pulp and paper mill in the southeastern United States. The ASB comprised a 7 L rectangular (21.6 cm length x 12.1 cm width x 40.6 cm height) Plexiglas reactor with an influent stream entering one end at the top of the water column and an effluent stream exiting by gravity at the other end over a weir. The ASB was maintained at room temperature (20-22°C), fed with raw pulp and paper wastewater, amended with nitrogen and phosphorus as NH_4Cl (30 mg N/L) and KH_2PO_4 (10 mg P/L), respectively, and adjusted to pH 6.5 with 2 N HCl. The feed was refrigerated at 3°C and was delivered to the ASB system with a positive displacement FYI Lab Pump at an average rate of 315 mL/d for a 22.2 d HRT. The upper zone of the ASB was aerated with pre-humidified, compressed air delivered through glass pipets at a depth of 4 cm from the water column surface. Dissolved oxygen (DO) was maintained at or near saturation throughout the water column (>8 mg/L). Aeration was tapered to achieve a quiescent zone for settling of solids prior to discharge of the treated wastewater. A layer of sediment was allowed to accumulate on the bottom of the ASB to simulate the various redox zones present in full-scale systems. DO was depleted within 1 cm of the water/sediment interface, indicating anoxic/anaerobic conditions were present within the sediment (Giles, 2012). The ASB was operated with a 22.2 d HRT for a period of 15 months, with the development of the ASB during the first several months. During the development phase, a sediment layer accumulated and turned black in color due to precipitation of metal sulfides, indicating active sulfate reduction. After 15 months of continuous operation, the ASB pumping rate was changed and the ASB operated with a HRT of 11.1 d for 6.3 months. The pumping

rate was then changed again and the ASB was operated with a HRT of 5.6 d for 6.6 months. The total duration of ASB operation was approximately 28 months.

6.2.3. ASB performance at various HRTs

The average HRT of an ASB is from 3 to 10 days, but may be as long as 28 days in some cases (Sackellares et al., 1987; Lewis et al., 2012). To investigate the effect of the HRT on the system performance, the pumping rate was changed and the system was allowed to reach steady state operation. Pumping was changed such that the initial HRT of 22.2 d was adjusted to 11.1 d and then to 5.6 d. At each HRT, the ASB was allowed to reach steady state, typically more than 3 HRTs, as determined by monitoring effluent pH, COD and nitrogen species. The ASB effluent and sediment at steady state were analyzed for pH, total suspended solids (TSS), volatile suspended solids (VSS), total and soluble COD, chloride, ammonia, nitrate, nitrite, phosphate, sulfate and phytosterols.

6.2.4. ASB aerobic and anoxic/anaerobic activity

To better understand the microbial activity occurring in the aerobic and anoxic/anaerobic zones of the ASB, a sample was taken from each zone while the ASB was operating at 5.6 d HRT. The samples were incubated for 96 h and analyzed periodically for pH, total and soluble COD, electron acceptors, gas production (anoxic/anaerobic) and phytosterols. To obtain a relatively homogeneous sample from the aerobic zone of the ASB, the water column was siphoned, leaving 1 inch of liquid above the sediment as part of an “interfacial” zone estimated to extend 1 inch into the sediment. The siphoned liquid was mixed with a Teflon-coated stir bar to homogenize the sample. A portion of the mixed sample was used to measure the oxygen uptake rate (OUR) and an

additional 2 L was placed in a 4 L glass reactor vessel. The reactor was maintained at room temperature (20-22°C) under mixing and aerated with pre-humidified, compressed air. A sample was drawn at the time of the transfer and at various times throughout the incubation period and analyzed for pH, ammonia, nitrate, nitrite, total and soluble COD, TSS, VSS and phytosterols. The ASB sediment activity was assessed under anoxic/anaerobic conditions. The sediment located below the interfacial zone was siphoned from the reactor into a helium-flushed vessel, where it was mixed before transferring 1.5 L to a 2.5 L helium-flushed, glass reactor. The anoxic/anaerobic reactor was maintained at room temperature (20-22°C) under mixing. Gas production and composition were measured periodically throughout the incubation. Initial and periodic samples were taken and analyzed for pH, gas composition, ammonia, nitrate, nitrite, sulfate, total and soluble COD, TSS, VSS, VFAs and phytosterols.

6.2.5. Wastewater bioassays

It is useful to understand the ability of the microbial communities in the aerobic and anoxic/anaerobic zones of the ASB to degrade the two wastewater fractions. The objective of this assay was to estimate the ultimate biodegradability of pulp and paper wastewater under aerobic and anoxic/anaerobic conditions in a batch system. ASBs are designed to encourage both biological treatment and settling of wastewater. The settling process removes wastewater solids from the aerobic zone and integrates them into the anoxic/anaerobic settled zone. To approximate the settling process, raw pulp and paper wastewater was settled into two fractions: a settled fraction and a supernatant fraction. Inoculum from the aerobic zone and the anoxic/anaerobic zone of the ASB was used to assess the ultimate biodegradability of the supernatant and settled wastewater fractions,

respectively. The aerobic inoculum was biomass from a 500 mL, well-mixed sample from the aerobic zone of the ASB, taken as described in Section 6.2.5. The sample was centrifuged at 4,000 rpm for 20 min, the supernatant was discarded, wastewater supernatant was added to the biomass pellet to 100 mL and vortexed. Wastewater supernatant was combined with 100 mL aerobic inoculum, ammonium chloride (60 mg N/L) and phosphate buffer (372 mg P/L; pH 7.2), for a total liquid volume of 2 L. The reactor was aerated with pre-humidified, compressed air and maintained for 28 d at room temperature (20-22°C) under mixing. Samples were taken periodically and analyzed for pH, total and soluble COD, nitrate, nitrite, ammonia, TSS, VSS and phytosterols. Anoxic/anaerobic inoculum was taken from the mixed sediment of the ASB as described in Section 6.2.5. The settled wastewater fraction, 40 mL of anoxic/anaerobic inoculum, ammonium chloride (60 mg N/L) and phosphate buffer (372 mg P/L; pH 7.2) were combined in a 2.5 L helium-flushed glass reactor, for a total liquid volume of 2 L. The reactor was maintained for 28 d at room temperature (20-22°C) under mixing. Gas production and composition were measured periodically throughout the incubation. Liquid samples were analyzed for pH, total and soluble COD, sulfate, ammonia, VFAs, TSS, VSS and phytosterols.

6.3. Results and Discussion

6.3.1. Bioenergetics

The bioenergetics of phytosterol metabolism under various redox conditions were calculated. The standard Gibbs free energies of formation ($\Delta G_f^{o\prime}$) of β-sitosterol,

stigmasterol, campesterol, and cholesterol were calculated using the group contribution method developed by Mavrovouniotis (1990). The estimated standard Gibbs free energy of formation for β-sitosterol, stigmasterol, campesterol and cholesterol are 94.98, 173.64, 87.87 and 54.81 kJ/mol, respectively. Four redox conditions were considered: aerobic oxidation, nitrate reduction, sulfate reduction and methanogenesis. Under each condition, the free energy of reaction was calculated for the mineralization of β-sitosterol, stigmasterol, campesterol and cholesterol, without considering cell growth (Table 6.1). The free energy per electron equivalent (eeq) of reaction varied only slightly among the different compounds. Phytosterol metabolism is theoretically possible under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions. Aerobic oxidation and nitrate reduction are the most energetically favorable reactions, yielding an average of -105.0 and -98.5 kJ/eeq, respectively. As expected, sulfate reduction and methanogenesis yield far less energy with an average of -5.5 and -2.8 kJ/eeq, respectively. Although theoretically feasible, phytosterol biotransformation via methanogenesis has not been reported. An assay conducted under methanogenic conditions with a culture acclimated to pulp and paper wastewater and fed an ethanolic phytosterol solution did not show any signs of phytosterol biodegradation over 112 d (Section 5.3.1.3). Phytosterol fate is highly dependent on the redox conditions encountered. In complex treatment systems such as ASBs, phytosterols may be exposed to a range of redox conditions, making it difficult to predict their overall fate.

Table 6.1. Sterol metabolism reactions and standard Gibbs free energy of reaction ($\Delta G_r^{o\prime}$).

Sterol	Conditions / Reaction	$\Delta G_r^{o\prime}$ kJ/mol	$\Delta G_r^{o\prime}$ kJ/eeq
	Aerobic Oxidation		
βS	$C_{29}H_{50}O + 41O_2 \rightarrow 29CO_2 + 25H_2O$	-17,218	-104.99
SS	$C_{29}H_{48}O + 40.5O_2 \rightarrow 29CO_2 + 24H_2O$	-17,060	-105.31
CS	$C_{28}H_{48}O + 39.5O_2 \rightarrow 28CO_2 + 24H_2O$	-16,588	-104.99
Ch	$C_{27}H_{46}O + 38O_2 \rightarrow 27CO_2 + 23H_2O$	-15,933	-104.82
	Nitrate Reduction		
βS	$C_{29}H_{50}O + 32.8NO_3^- + 32.8H^+ \rightarrow 29CO_2 + 41.4H_2O + 16.4N_2$	-16,149	-98.47
SS	$C_{29}H_{48}O + 32.4NO_3^- + 32.4H^+ \rightarrow 29CO_2 + 40.2H_2O + 16.2N_2$	-16,004	-98.79
CS	$C_{28}H_{48}O + 31.6NO_3^- + 31.6H^+ \rightarrow 28CO_2 + 39.8H_2O + 15.8N_2$	-15,558	-98.47
Ch	$C_{27}H_{46}O + 30.4NO_3^- + 30.4H^+ \rightarrow 27CO_2 + 38.2H_2O + 15.2N_2$	-14,942	-98.30
	Sulfate Reduction		
βS	$C_{29}H_{50}O + 20.5SO_4^{2-} + 30.8H^+ \rightarrow 29CO_2 + 25H_2O + 10.3\ H_2S + 10.3HS^-$	-888.9	-5.42
SS	$C_{29}H_{48}O + 20.3SO_4^{2-} + 30.4H^+ \rightarrow 29CO_2 + 24H_2O + 10.1H_2S + 10.1HS^-$	-929.9	-5.74
CS	$C_{28}H_{48}O + 19.8SO_4^{2-} + 29.6H^+ \rightarrow 28CO_2 + 24H_2O + 9.9H_2S + 9.9HS^-$	-856.4	-5.42
Ch	$C_{27}H_{46}O + 19.0SO_4^{2-} + 28.5H^+ \rightarrow 27CO_2 + 23H_2O + 9.5H_2S + 9.5HS^-$	-798.0	-5.25
	Methanogenesis		
βS	$C_{29}H_{50}O + 16H_2O \rightarrow 8.5CO_2 + 20.5CH_4$	-449.4	-2.74
SS	$C_{29}H_{48}O + 16.5H_2O \rightarrow 8.8CO_2 + 20.3CH_4$	-495.7	-3.06
CS	$C_{28}H_{48}O + 15.5H_2O \rightarrow 8.3CO_2 + 19.8CH_4$	-432.9	-2.74
Ch	$C_{27}H_{46}O + 15.0H_2O \rightarrow 8.0CO_2 + 19.0CH_4$	-390.6	-2.57

βS = β-sitosterol; CS = campesterol; SS = stigmasterol; Ch = cholesterol

6.3.2. Pulp and paper wastewater fractionation and characterization

The results from the characterization of the pulp and paper wastewater are given in Table 6.2. To approximate the solids separation that occurs within an ASB, the wastewater was fractionated through settling, as discussed in Section 6.2.5, above. The settling curve is shown in Figure 6.1. The characteristics of the supernatant and settled fractions are also given in Table 6.2. As expected, the TS, VS and total COD were highest in the settled fraction. Soluble COD was higher in the supernatant as it contains more soluble material. Because phytosterols are hydrophobic substances with low solubility and relatively high octanol/water coefficient values (Table 2.1), they are expected to be largely associated with solids (Makris and Banerjee, 2002). In a study of untreated wastewater from a mill processing both softwood and hardwood in Finland, β-sitosterol was mainly associated with solids (34%) and colloids (66%) (Leiviska et al., 2009). Indeed, the settled fraction, which contained the greatest amount of solids, had the highest concentration of phytosterols. The settled fraction represented only 6.5% of the total wastewater by volume but contained 54% of the total solids, 63% of the total COD and 58% of the total phytosterols. By contrast, the supernatant fraction, which represented 93.5% of the total volume, contained 46% of the total solids, 37% of the total COD and 42% of the total phytosterols. These results illustrate the significant phytosterol partitioning to solids due to sorption and the incorporation of phytosterols within the cell structure of plant fibers. As the majority of solids in an effective ASB are either degraded or accumulate within the bottom sediment layers, a significant portion of phytosterols are likely to be carried into anoxic or anaerobic sediment along with the solids. Sediments in

ASBs may therefore act as a phytosterol reservoir and may release bound or internal phytosterols during solids degradation.

The distribution of the three phytosterols within the two wastewater fractions varied slightly between the supernatant and settled fractions. β-sitosterol showed a greater affinity towards the supernatant fraction, comprising 92.0% of the total phytosterols in this fraction. Stigmasterol and campesterol made up 5.8% and 2.3% of the total phytosterols in the supernatant fraction. In comparison, the total phytosterols in the settled fraction consisted of 73.4% β-sitosterol, 14.3% stigmasterol and 12.3% campesterol. The total mass of phytosterols per mass of total solids was 136.1 μg/g and 158.6 μg/g for the supernatant and settled fractions, respectively. In both the supernatant and settled fractions, β-sitosterol was the primary phytosterol, followed by stigmasterol and campesterol. These results show that the phytosterol distribution between the wastewater supernatant and settleable fractions is similar.

Table 6.2. Characteristics of whole wastewater, settled and supernatant wastewater fractions.

Parameter	Whole WW	Supernatant	Settled
pH	8.95 ± 0.02[a]	9.91 ± 0.02	8.83 ± 0.02
TS (g/L)	3.6 ± 0.03	1.8 ± 0.02	30.2 ± 0.3
VS (g/L)	1.2 ± 0.01	0.43 ± 0.004	12.4 ± 0.1
Total COD (mg/L)	2,501 ± 66	1,001 ± 113	24,078 ± 125
Soluble COD (mg/L)	865 ± 31	849 ± 6	741 ± 35
Nitrite (mg N/L)	ND[b]	ND	ND
Nitrate (mg N/L)	ND	0.09 ± 0.003	ND
Ammonia (mg N/L)	ND	ND	ND
Chloride (mg Cl/L)	77.2 ± 0.4	80.1 ± 0.6	53.1 ± 0.1
Phosphate (mg P/L)	18.6 ± 0.1	10.0 ± 0.1	22.1 ± 0.1
Sulfate (mg S/L)	113.0 ± 0.4	122.9 ± 0.3	114.4 ± 0.2
Total phytosterols (µg/L)	541 ± 20	245 ± 26	4,790 ± 32
β-sitosterol (µg/L)	439 ± 20	226 ± 25	3,514 ± 32
Stigmasterol (µg/L)	58 ± 2	14 ± 4	685 ± 5
Campesterol (µg/L)	44 ± 1	6 ± 1	590 ± 2

[a]Mean ± standard deviation (n = 3); [b]ND, not detected

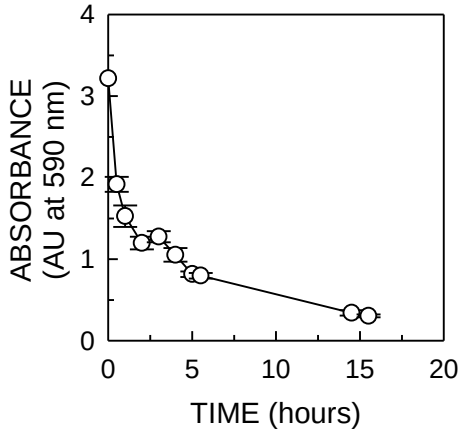


Figure 6.1. Absorbance measurements of wastewater supernatant during settling.

6.3.3. ASB performance at various HRT values

The ASB influent, and steady-state effluent and sediment characteristics at HRTs of 22.2, 11.1 and 5.6 d are given in Tables 6.3 and 6.4, respectively. TSS loading rates at HRTs of 22.2, 11.1 and 5.6d were 44, 89 and 178 mg/L-d, respectively; phytosterol loading rates were 10, 21 and 42 μg/L-d at the respective HRTs. The pH of both the effluent and the sediment remained stable across all three HRTs, an indication of internal buffering of the system. The effluent quality declined only slightly at shorter HRTs; total COD removal declined from 88% to 84% and soluble COD removal declined from 76% to 64% between an HRT of 22.2 d and 5.6 d. Nitrification occurred at each HRT; nitrate increased slightly across the system from 0.12 mg N/L in the influent to 1.7-13.63 mg N/L in the effluent, while ammonia was not detected. TSS and VSS in the effluent also were similar across all HRTs (17-31 mg/L TSS; 14-17 mg/L VSS). These results demonstrate the ability of the ASB system to accommodate a wide range of loading rates while producing a relatively consistent effluent.

Although shortening the HRT had only a moderate effect on the effluent quality, the sediment characteristics changed significantly. The total COD of the sediment increased from 8,037 mg/L at the longest HRT (22.2 d) to 18,762 mg/L at the shortest HRT (5.6 d). The soluble COD in the sediment also increased from 86 mg/L to 284 mg/L between the two HRT values. The COD increase is indicative of the accumulation of wastewater solids due to the increased loading rate at lower HRT values. The TSS and VSS concentrations decreased when the HRT was shortened from 11.1 d to 5.6 d. The decrease in solids concentration and the corresponding increase in soluble COD were due to the production of a less dense sediment layer composed of light-colored wastewater

fibers that had not yet been degraded. Gases produced within the sediment also contributed to a looser sediment layer with a lower solids and a higher soluble concentration. Indeed, the thickness of the sediment layer, which had been at a steady state when operating at 22.2 d HRT, noticeably increased at a lower HRT, indicating a buildup of solids consistent with the higher solids loading rate. At the shortest HRT, sulfate was also present at a higher concentration.

The total effluent phytosterol concentration increased as the ASB HRT decreased (Table 6.3). The longest HRT tested (22.2 d) was most effective in terms of phytosterol removal (94%), while the total phytosterol removal at 11.1 d and 5.6 d HRTs was 82% and 83%, respectively. The total phytosterol concentration in the effluent was nearly the same at both 11.1 d and 5.6 d HRTs. In contrast, the total phytosterol concentration in the ASB sediment was significantly greater at the shorter HRTs (Table 6.4). Thus, the HRT had a far more significant effect on the sediment phytosterol concentration than on the effluent phytosterol concentration. At a relatively long HRT, phytosterols in the aerobic zone have a greater opportunity to be oxidized. Additionally, organic solids stay longer in the anoxic/anaerobic zones and as a result of solids degradation, solids-associated phytosterols are released. At the longest HRT, the released phytosterols could have had time to enter the aerobic zone and be oxidized. At the shortest HRT, the rate of solids accumulation in the sediment far exceeded their degradation rate, thus leading to a relatively lower rate of phytosterols release from the sediment to the overlaying water column, and a significantly increased phytosterol sediment concentration.

The phytosterol distribution in the ASB effluent and sediment were different at the three HRTs tested, as shown in Figure 6.2. Within the effluent, β-sitosterol

represented the smallest fraction of the total phytosterols at the shortest HRT (Figure 6.2A). However, at the longest HRT, β-sitosterol comprised 40% of the total phytosterols. As β-sitosterol is the most abundant phytosterol in plant material, a release of significant quantities of β-sitosterol may occur during solids degradation within the ASB sediment layer. Releases of stigmasterol and campesterol are also likely, although smaller amounts of these phytosterols are usually present in plant material. At longer HRTs, there may be a greater degradation extent of solids and thus, a greater release of β-sitosterol relative to the other phytosterols, as measured in the effluent.

Stigmasterol was the most abundant of the three phytosterols in the ASB effluent at HRTs of 22.2 d and 11.1 d, and was the second most abundant at the shortest HRT (5.6 d) (Figure 6.2A). Although plant material tends to contain more β-sitosterol, stigmasterol may be present in a higher concentration in the effluent due to a greater release from solids and/or the preferential removal of other phytosterols within the ASB. The latter explanation is consistent with previous reports which found that stigmasterol was a less suitable substrate for cholesterol oxidase than β-sitosterol (Doukyu, 2009; MacLachlan et al., 2000). At the shortest HRT (5.6 d), campesterol represented the largest fraction of phytosterols in the effluent but was only a minor component at HRT values of 11.1 d and 22.2 d.

The sediment phytosterol distribution was also different at each of the HRTs tested, as shown in Figure 6.2B. In contrast with the effluent, β-sitosterol represented the largest fraction of the phytosterols at both the shortest and longest HRT values. When operating at a moderate HRT (11.1 d), β-sitosterol, stigmasterol and campesterol each represented a nearly equal fraction of the total phytosterols. The relative phytosterol

profile at both the shortest (5.6 d) and longest (22.2 d) HRTs were very similar (Figure 6.2B). This similarity is notable when compared to the expanded shares of stigmasterol and campesterol observed at a moderate HRT (11.1 d). The variation in the observed fractions is likely the result of a number of complex processes taking place within the ASB, including phytosterol release, sorption, desorption and biotransformation. The results show that the HRT has a significant effect on the processes that affect phytosterol release and removal in ASBs. In order to optimize phytosterol removal, it is first necessary to understand the processes involved in phytosterol appearance and disappearance within an ASB.

Table 6.3. Steady state ASB influent and effluent characteristics at various HRT values.

Parameter	Influent	Effluent at HRT:		
		22.2 d	11.1 d	5.6 d
pH	6.5 ± 0.02[a]	7.44 ± 0.02	7.48 ± 0.02	7.53 ± 0.02
TSS (mg/L)	846 ± 19	31 ± 1.5	29 ± 1.4	17 ± 0.3
VSS (mg/L)	321 ± 6	16 ± 0.1	17 ± 0.7	14 ± 0.4
Total COD (mg/L)	834 ± 22	99 ± 4	106 ± 10	130 ± 3
Soluble COD (mg/L)	288 ± 10	69 ± 7	64 ± 12	103 ± 15
Nitrite (mg N/L)	ND[b]	ND	ND	ND
Nitrate (mg N/L)	0.12 ± 0.01	8.7 ± 0.2	13.6 ± 1.0	1.7 ± 0.04
Ammonia (mg N/L)	30	ND	ND	ND
Chloride (mg Cl/L)	101.7 ± 0.1	155.6 ± 1.6	246 ± 18.9	139.2 ± 1.5
Phosphate (mg P/L)	16.21 ± 0.03	19.9 ± 2.5	25.9 ± 3.0	50.6 ± 3.0
Sulfate (mg S/L)	37.7 ± 0.1	49.0 ± 0.9	66.5 ± 4.6	39.8 ± 0.7
Total phytosterols (µg/L)	198.0 ± 2.7	11.3 ± 3.3[c]	35.6 ± 3.9	34.1 ± 3.7
β-sitosterol (µg/L)	156.1 ± 2.5	4.5 ± 1.2[c]	6.7 ± 0.8	2.4 ± 0.2
Stigmasterol (µg/L)	22.6 ± 0.8	4.9 ± 3.0[c]	23.2 ± 3.8	12.6 ± 3.4
Campesterol (µg/L)	19.3 ± 0.7	1.9 ± 0.7[c]	5.7 ± 0.6	19.1 ± 1.4

[a]Mean ± standard deviation (n = 3); [b]ND, not detected; [c]Values reported by Giles (2012) for the same system.

Table 6.4. Steady state ASB sediment characteristics at various HRT values.

	Hydraulic Retention Time		
Parameter	**22.2 d**	**11.1 d**	**5.6 d**
pH	6.42 ± 0.02[a]	6.76 ± 0.02	6.76 ± 0.02
TSS (mg/L)	NR[b]	67,603 ± 377	33,657 ± 878
VSS (mg/L)	NR	18,641 ± 388	11,131 ± 378
Total COD (mg/L)	8,037 ± 92	14,591 ± 575	18,762 ± 920
Soluble COD (mg/L)	86 ± 35	119 ± 10	284 ± 50
Nitrite (mg N/L)	NR	ND[d]	ND
Nitrate (mg N/L)	NR	0.04 ± 0.01	6.8 ± 0.2
Ammonia (mg N/L)	NR	9.3 ± 8.1	14 ± 0
Chloride (mg Cl/L)	NR	119 ± 0.5	599 ± 0.6
Phosphate (mg P/L)	NR	47.6 ± 1.6	728 ± 15
Sulfate (mg S/L)	NR	3.3 ± 0.1	61 ± 1.3
Total phytosterols (µg/L)	10,688 ± 1,207[c]	18,157 ± 280	43,838 ± 1,977
β-sitosterol (µg/L)	7,634 ± 1,148[c]	4,649 ± 122	30,618 ± 871
Stigmasterol (µg/L)	1,682 ± 370[c]	4,304 ± 113	10,334 ± 1,530
Campesterol (µg/L)	1,372 ± 54[c]	9,204 ± 225	2,886 ± 900

[a]Mean ± standard deviation (n = 3); [b]NR, not reported; [c]Values reported by Giles (2012) for the same system; [d]ND, not detected

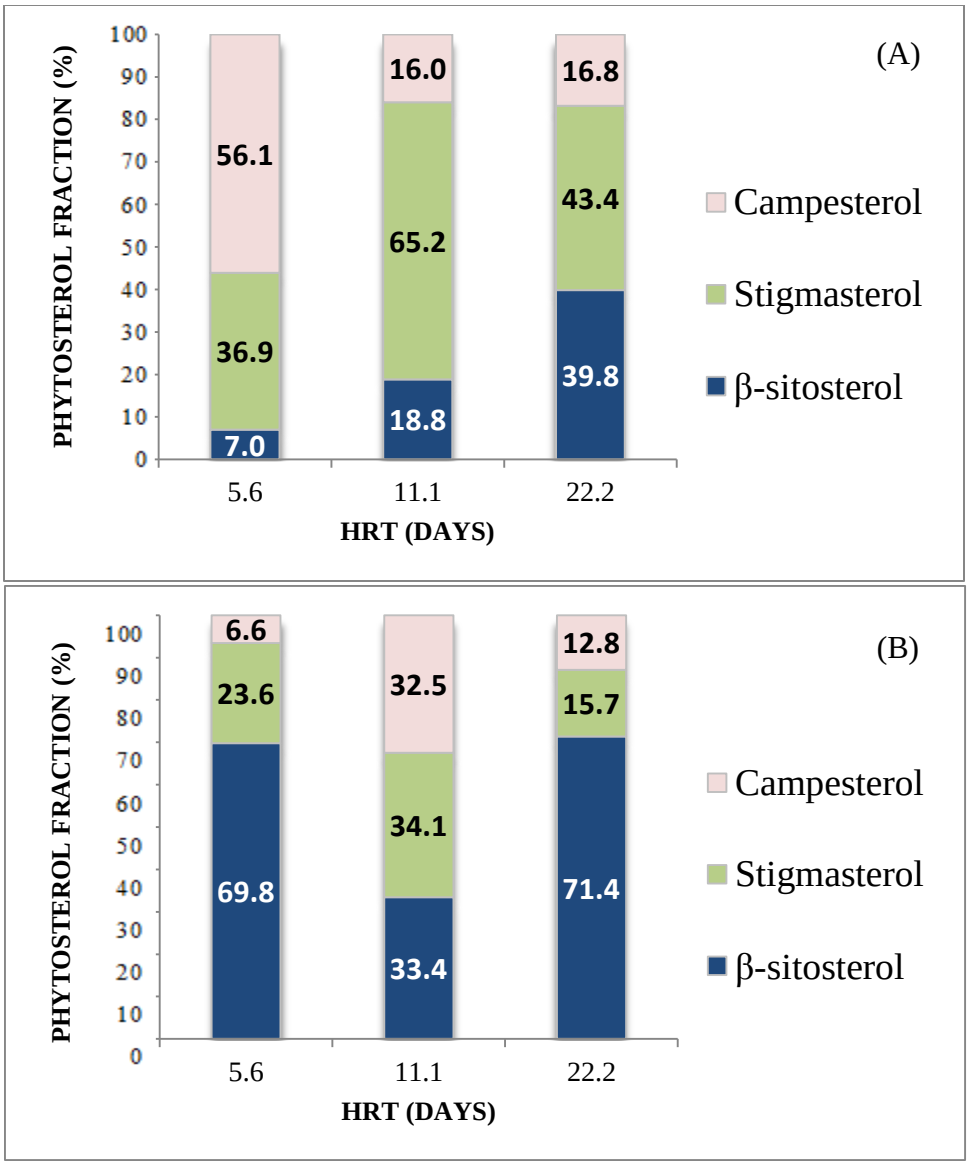


Figure 6.2. β-sitosterol, stigmasterol and campesterol fraction of the total phytosterols measured in the ASB effluent (A), and sediment (B) during the steady-state operation of the ASB at three HRT values.

6.3.4. ASB aerobic and anoxic/anaerobic activity

To better understand the processes that take place in the aerobic and anoxic/anaerobic zones of an ASB, a sample was taken from each zone of the simulated ASB and incubated over 96 h. The aerobic sample's pH increased sharply from 7.65 to 8.31 over the first 8 h and then nearly leveled out at 8.63 by the end of incubation (Figure 6.3A). The initial soluble COD was relatively low (114 mg/L) and only 27% of soluble COD was degraded at 96 h (Figure 6.3B), indicating a low level of readily degradable COD was present in the aerobic zone. Ammonia was only detected in trace amounts at the 4 h measurement (data not shown) and nitrate remained relatively stable (6.6-7.2 mg N/L) over the incubation period (Figure 6.3C). In an ASB system, benthal feedback typically contributes ammonium to the aerobic zone (Bryant, 2010). At initial sampling, the biomass in the ASB aerobic zone had a specific oxygen uptake rate of 12.9 mg O_2/g VSS-h. Initially, VSS (39.2 mg/L) made up 73% of the TSS (53.7 mg/L). VSS increased 27.5% over the first 24 h of incubation, then remained stable, with a final VSS of 51.0 mg/L at the end of incubation (Figure 6.3D).

Total phytosterols decreased by 28.2% over the incubation period, as shown in Figure 6.3E. Initially, campesterol was the predominant phytosterol, possibly due to a more rapid biotransformation of β-sitosterol and stigmasterol. During incubation, campesterol declined by 33.3%. In comparison, stigmasterol and β-sitosterol declined by 21.9% and 16.7%, respectively. The rate of total phytosterol removal increased from 0.670 μg/L-h during the first 24 h to 3.23 μg/L-h from 24-96 h, possibly due to the release of bound cholesterol oxidase as the culture entered stationary phase. A similar accelerated phytosterol removal was reported in an aerobic batch assay when the culture

reached stationary phase (Section 4.2.3.3; Dykstra et al., 2014a). Thus, as cell turnover increased, the release of bound cholesterol oxidase may have contributed to the accelerated phytosterol removal.

The sample from the anoxic/anaerobic zone of the ASB displayed greater microbial activity than the aerobic sample. The initial pH (6.48) was lower than that of the aerobic zone (7.65) and remained relatively stable throughout the incubation, with a final pH of 6.62 (Figure 6.4A). Unlike the aerobic zone, the anoxic/anaerobic zone contained a large amount of organic matter to fuel microbial activity; thus, greater activity was observed during incubation under anoxic/anaerobic conditions. The total COD steadily declined by 17.6% over 96 h (Figure 6.4B). The soluble COD increased by 137.7% during incubation due to the release of soluble products from the breakdown of solids (Figure 6.4C). Sulfate was present initially at 14 mg S/L but was quickly consumed and not detected after 48 h (Figure 6.4D). During the first 8 h, sulfate was depleted by 71%; during this period the greatest increase in gas production was also observed (Figure 6.4E). The initial increase in gas production was likely due in part to the production of hydrogen sulfide by sulfate reducing bacteria. The gas production rate was relatively high in the first 8 h and then continued at a steady rate, indicating steady biological activity.

The concentration of total phytosterols increased within the first 24 h and then gradually declined, with a net 105.6% increase by the end of the 96-h incubation (Figure 6.4F). The initial phytosterol increase is likely due to the release of sorbed and/or bound phytosterols associated with solids, as well as a result of solids disintegration and hydrolysis. Phytosteryl esters, which are sterols bound to other molecules via ester bonds, are hydrolyzed by lipases, producing free phytosterols (Shimada et al., 2003). Because

steryl esters are found in non-trivial amounts in plant cells, the contribution of steryl esters to the release of free phytosterols may be significant. For example, the sapwood of Scot's pine has been found to contain more than 7 times the amount of steryl esters than free β-sitosterol per gram oven dried wood (Martinez-Iñigo et al., 1999). The release of both cell-bound and sorbed phytosterols likely caused the 126% increase in total free phytosterols over the first 24 h.

A slight decrease in total free phytosterols occurred following the initial increase, indicating removal. As shown in Chapter 5, phytosterol removal is possible under sulfate-reducing conditions, although removal was only observed following a significant lag time (Section 5.3.1.2). In the present study, the three phytosterols in the mixture did not undergo proportional changes during incubation, indicating that the production and/or removal mechanisms for each phytosterol occurred at different rates. Over the first 24 h, β-sitosterol, stigmasterol and campesterol increased by 119%, 123% and 198%, respectively, indicating that campesterol may be more easily released during solids digestion. Between 24 and 96 h, β-sitosterol and campesterol decreased by 8% and 38%, respectively and stigmasterol increased by 12%. During this period, the removal of β-sitosterol and campesterol exceeded their release from the solids, while stigmasterol did not. These results suggest that stigmasterol is either released more slowly over time and/or that the rate of removal is less than with β-sitosterol or campesterol. Although little is known about the substrate specificity of enzymes in the anoxic degradation pathway, aerobic cholesterol oxidase enzymes preferentially degrade β-sitosterol over stigmasterol (Doukyu, 2009; MacLachlan et al., 2000) and this may also be true for enzymes in the anoxic pathway. Two enzymes have been identified in the initial stages of

the anoxic metabolism of cholesterol by *Sterolibacteriun denitrificans* under denitrifying conditions. The first enzyme, AcmA, catalyzed the oxidation of the hydroxyl group and the subsequent isomerization to cholest-4-en-3-one. A second enzyme, AcmB, catalyzed the oxidation to cholesta-1,4-dien-3-one, followed by the hydroxylation of C25 on the side chain using water as the oxygen donor (Chiang, et al., 2008). Enzymes that catalyze reactions on the side chain of a sterol likely vary in substrate specificity between the three phytosterols.

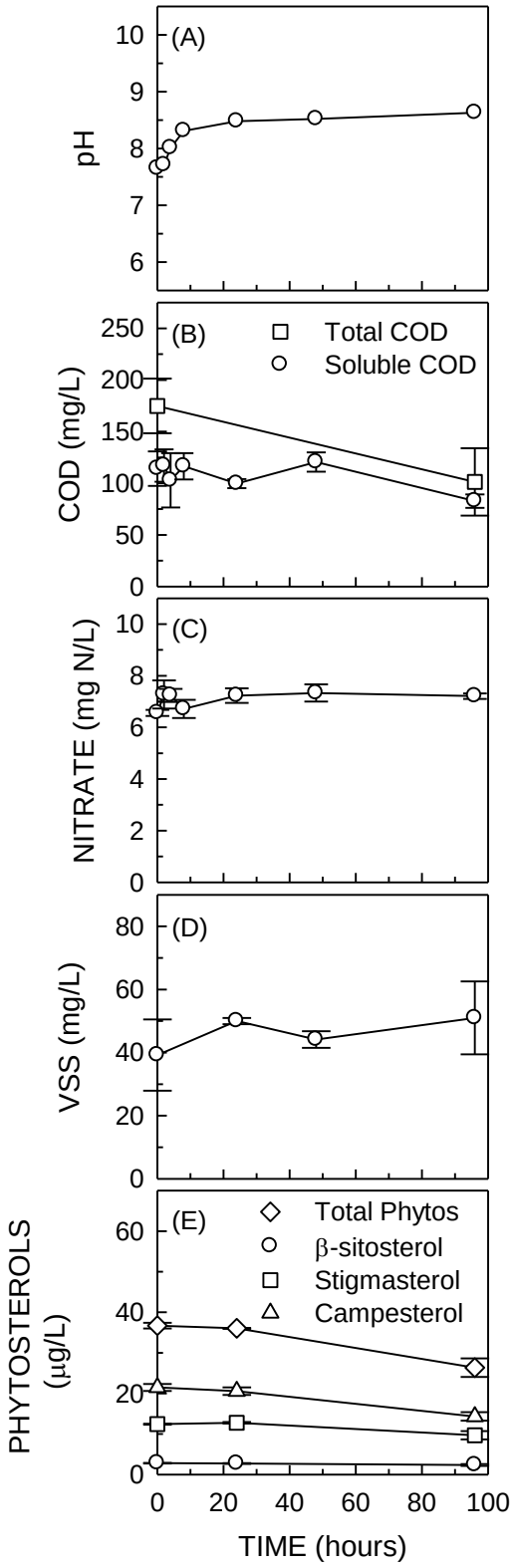


Figure 6.3. Activity of the aerobic zone of the ASB monitored by pH (A), total and soluble COD (B), nitrate (C), TSS, VSS (D), and phytosterols (E) through a 96-h batch assay.

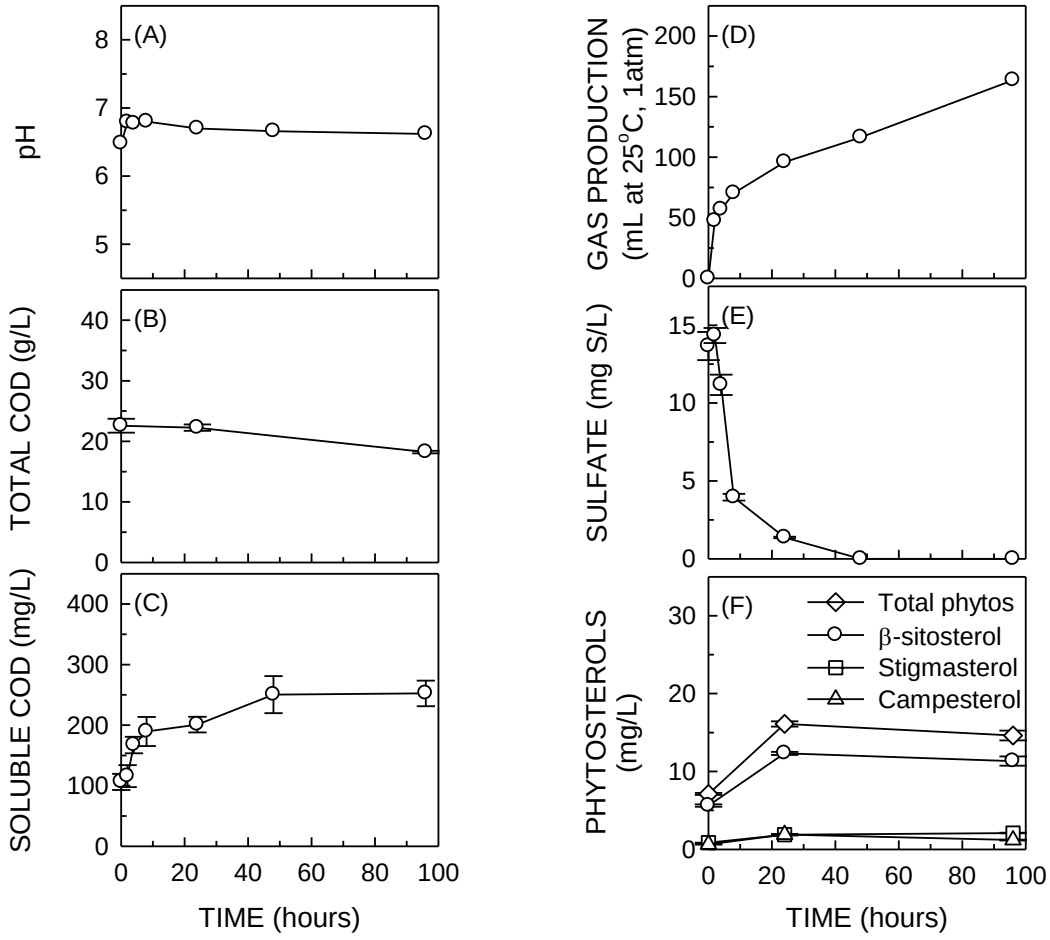


Figure 6.4. Activity of the anoxic/anaerobic zone of the ASB monitored by pH (A), total COD (B), soluble COD (C), sulfate (D), gas production (E), and phytosterols (F) through a 96-h batch assay.

6.3.5. Wastewater bioassays

To better assess the ultimate fate of pulp and paper wastewater in ASB treatment systems, aerobic and anoxic/anaerobic bioassays were conducted with wastewater fractionated by settling, using ASB aerobic and anoxic/anaerobic zones samples as inocula. The initial pH of the aerobic assay was 7.00, which increased during the 28 d incubation period to 7.45, as shown in Figure 6.4A. The initial total COD was 872 mg/L, which decreased steadily through day 10 before reaching a plateau, with a final total COD of 294 mg/L (Figure 6.4B). At the beginning of the incubation, the soluble COD was 849 mg/L, representing 97% of the total COD, indicating that most of the degradable organic material was in a soluble form. The soluble COD decreased throughout the first 10 days, reaching a final soluble COD of 203 mg/L (Figure 6.4B). Although ammonia was present throughout the incubation period, nitrification was not observed until day 18, after which the ammonia began to decline and nitrate began to gradually increase (Figure 6.4C and 6.4D). Nitrifiers are known to be sensitive to a number of compounds and it is likely that the delayed nitrification was due to inhibitory effects of compounds in the raw wastewater. Fatty acids, phenols and chlorinated compounds are commonly found in pulp and paper wastewater and are known inhibitors of nitrification (Makris, 2003; Svenson et al., 2000; Strotmann and Eglsaer, 1995). Typically, wastewater entering the ASB is diluted, thus reducing the effect of potentially inhibitory compounds in the wastewater. However, in this bioassay, no dilution occurred, exposing the nitrifiers to full strength wastewater. Towards the end of the incubation, nitrification began at a slow rate, perhaps as a result of degradation and removal of such organic inhibitors by heterotrophic bacteria.

The total phytosterols in the aerobic assay declined by 42% over the course of the 28 d incubation period (Figure 6.4E). However, the total phytosterol concentration increased by 161% within the first 10 d, primarily due to a 217% (1.02 m/L) increase in stigmasterol. By comparison, β-sitosterol increased by 23% (0.04 mg/L) and campesterol remained stable within the first 10 d. Between the 10^{th} and 28^{th} day, the total phytosterol concentration decreased by 78%, with a 34% decrease in stigmasterol and a 71% decrease in β-sitosterol. The campesterol concentration remained at 20 μg/L throughout the incubation period. These results show that stigmasterol may increase during the aerobic treatment of pulp and paper wastewater. Indeed, Cook et al. (1997) found an increase in stigmasterol ranging from 192% to 377% across four full-scale ASBs examined. One ASB was also found to have a 25% increase in β-sitosterol across the treatment system (Cook et al., 1997). Another study found ASB removal of stigmasterol to be highly dependent on the stigmasterol loading rate. When the rate was relatively low (0.18-0.19 mg/L-d), stigmasterol increased across the treatment system by 29-37%. However, at higher rates (0.60-0.62 mg/L-d), the ASB was able to remove 90% of the stigmasterol (Chamorro et al., 2009). Although the mechanisms behind the observed increase in stigmasterol are unknown, it is probable that it is due in part to the release of membrane-bound and sorbed phytosterols from solids during their disintegration and microbial hydrolysis. The observed increase and subsequent decrease in stigmasterol could explain the variability in the stigmasterol concentrations of the ASB effluent at various HRTs (Table 6.3). The highest effluent stigmasterol concentration was observed at 11.1 d HRT, while lower concentrations were found at both shorter and longer HRTs. A moderate HRT could allow for the release of phytosterols from hydrolyzed solids

without providing enough time for significant phytosterol biotransformation in the water column.

During the anaerobic assay, microbial activity was observed throughout the incubation period. The pH declined from 7.7 to 6.5 over the course of 13 d, then remained stable through the remainder of the incubation period (Figure 6.5A). The soluble COD increased from 741 mg/L to 3,185 mg/L over the course of the incubation (Figure 6.5B). The increase in soluble COD reflected the production of VFAs, primarily acetate and propionate (Figure 6.5C). At the end of the incubation, VFAs comprised 80% of the soluble COD present. Sulfate was observed for the first 6 d but was only detected in trace amounts afterward (Figure 6.5D). Gas production was moderate for the first 6 d, after which the gas production accelerated, reaching 680 mL at STP at the end of the incubation (Figure 6.5E). The majority of the gas produced was made up of CO_2 (71.5%), CH_4 (14.4%) and H_2S (4.1%); the remaining gaseous products (10.0%) were undetected. The disappearance of sulfate corresponded with the appearance of hydrogen sulfide, indicating active sulfate reduction. (Figure 6.5F). The production of methane increased towards the end of the incubation when sulfate was depleted and conditions began to favor methanogenesis. The total phytosterols increased by 93% during incubation, primarily due to a 101% increase in β-sitosterol (Figure 6.5G). The concentrations of stigmasterol and campesterol increased by 55% and 69%, respectively, but their concentrations were relatively low in comparison to β-sitosterol. The increase in phytosterols was likely due in part to the release of phytosterols from solids. Unlike the aerobic assay, the phytosterol concentrations continued to increase throughout the incubation period. Additionally, a disproportionate increase in stigmasterol was not

observed, which suggests that the production or release of significant amounts of stigmasterol in ASBs primarily occurs in the aerobic zone.

Given the conditions of this study, the supernatant wastewater fraction was readily degraded under aerobic conditions. During the incubation period, 66 and 76% of the total and soluble COD, respectively, was degraded. Additionally, 42% of total phytosterols were removed. The settled wastewater fraction incubated under anaerobic conditions was biodegraded less and the total COD declined by 1% over 28 d of incubation. The soluble COD increased by 330% during the incubation due to the production of VFAs. When the soluble COD contribution from VFAs was not considered, the soluble COD removal was 14% over the incubation period. Total phytosterols increased by 73%, which is likely due to the release of solids-associated phytosterols during the disintegration and hydrolysis of organic solids.

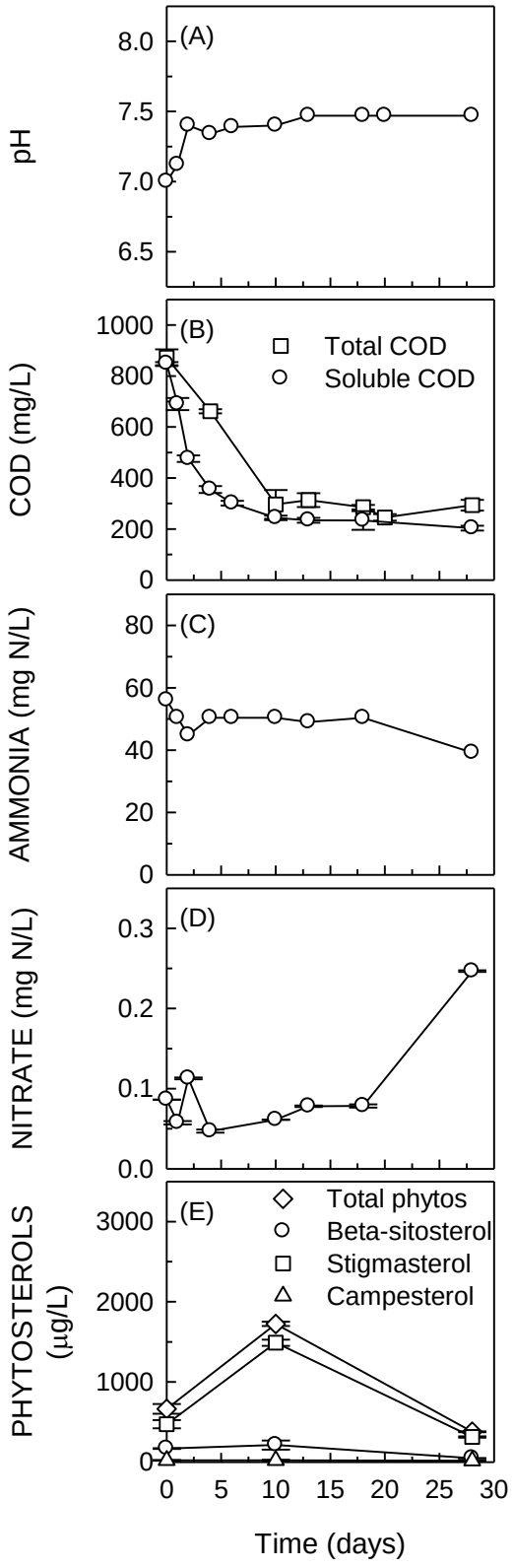


Figure 6.5. Aerobic wastewater batch assay: pH (A), total and soluble COD (B), ammonia (C), nitrate (D), and phytosterols (E).

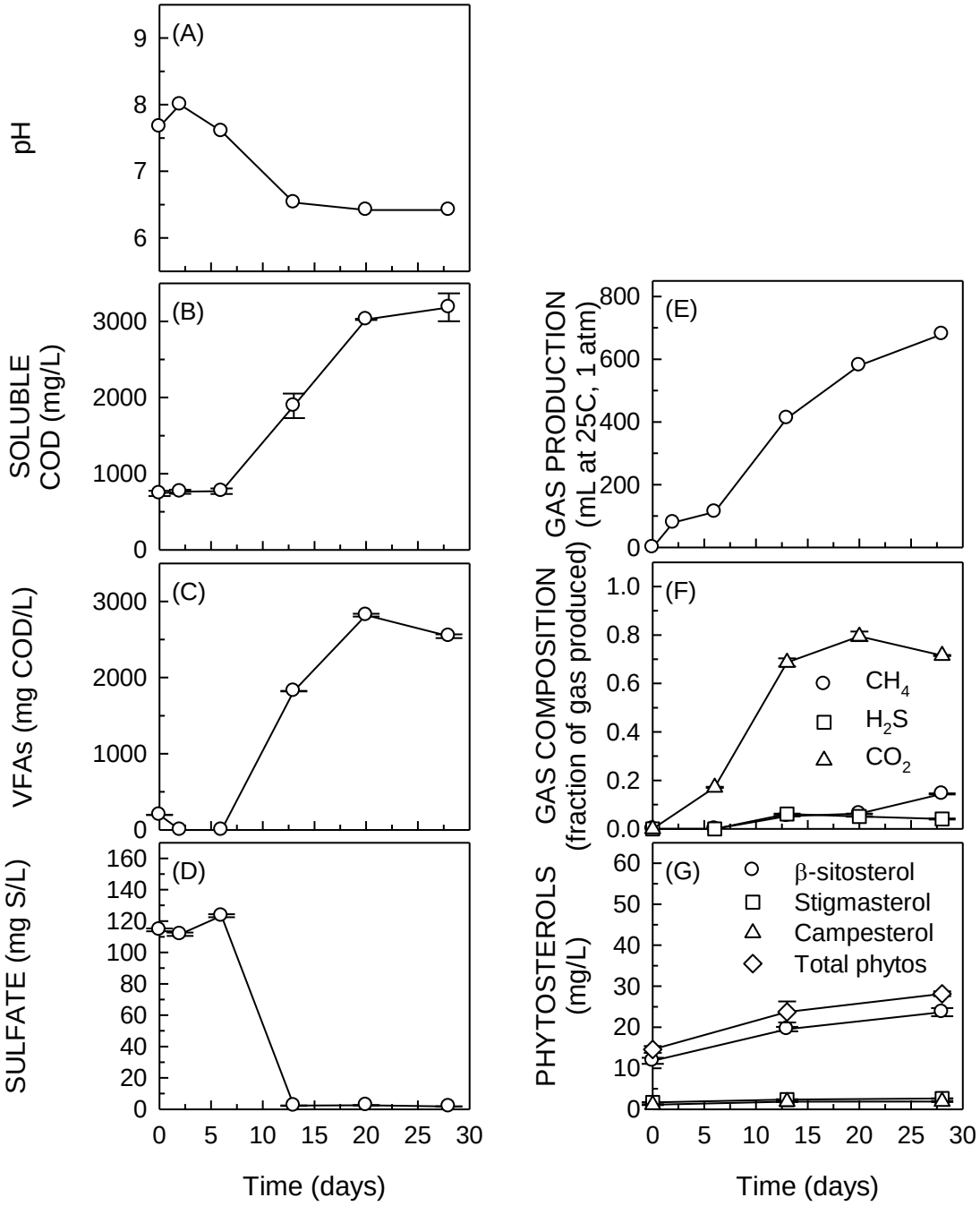


Figure 6.6. Anaerobic wastewater batch assay: pH (A), soluble COD (B), VFAs (C), sulfate (D), gas production (E), gas composition (F) and phytosterols (G).

6.3.6. Phytosterol release and biotransformation

While it is clear that ASBs remove phytosterols, the mechanisms involved are difficult to determine because of complex interactions between the water column and sediment. When considering influent whole pulp and paper wastewater and ASB effluent, the overall phytosterol removal by the ASB used in this study was 82-94% (Table 6.5). However, batch assays with the ASB sediment and the settled wastewater fraction indicated release of phytosterols associated with solids disintegration and hydrolysis. As a simplified approach, the ASB water column was considered as a well-mixed, isolated system (i.e., no interaction with settled solids) and a phytosterol mass balance was conducted. Under these assumptions, the water column achieved a total phytosterol removal of 57 to 86%, with the highest removal at the longest HRT (Table 6.5). As discussed in Section 3.2, above, the supernatant portion of the wastewater, which is most likely to remain in the aerobic zone of an ASB, carries 42% of the total phytosterols. Thus, when the influent phytosterols associated with settleable wastewater solids were not taken into account, the phytosterol removal efficiency was lower than when the whole wastewater was taken as the influent.

Although the total phytosterol removal was positive at each HRT, the relatively higher β-sitosterol removal obscured the low or even negative removal of stigmasterol and campesterol across all HRTs (Table 6.5). Considering that β-sitosterol was significantly removed from the water column at all HRTs, biotransformation and/or sorption to solids must have occurred. The water column contained a low solids concentration (< 32 mg/L), indicating aerobic biotransformation was likely the primary removal mechanism in this zone. We have previously demonstrated the biotransformation

of all three phytosterols under aerobic conditions, with β-sitosterol preferentially removed over stigmasterol and campesterol (Chapter 4; Dykstra et al., 2014a).

Using the isolated water column analysis, at the longest HRT of 22.2 d, stigmasterol and campesterol were removed by 10 and 30%, respectively. However, at the two shortest HRTs, corresponding to the highest solids and phytosterols loadings, the analysis showed an increase in stigmasterol (3-fold increase at 11.1 d HRT) and campesterol (6-fold increase at 5.6 d HRT) concentrations, leading to negative stigmasterol and campesterol removal from the water column (Table 6.5). Thus, an unaccounted-for source of phytosterols, attributed to the release of phytosterols from settled solids undergoing disintegration and microbial hydrolysis, leads to this imbalance. Indeed, anaerobic incubation of an ASB sediment sample resulted in a nearly 2-fold increase in phytosterol concentration over a period of 24 h without a significant phytosterol removal (Figure 6.4). Another way to view this foregoing analysis is to calculate the required mass rate of phytosterols released from the sediment to achieve a mass balance, assuming no losses in the water column (e.g., no biodegradation). A positive mass rate would then indicate the minimum rate of phytosterol release from the sediment. Indeed, at the two shortest HRTs of 11.1 and 5.6 d, a positive mass rate from the sediment to the water column must occur for stigmasterol and campesterol to achieve a mass balance in the water column. The calculated minimum rate of release of stigmasterol and campesterol from sediment was 0.2 and 2.7 μg/d, respectively, at an HRT of 11.1 d, and 9.0 and 20.7 μg/d, respectively, at an HRT of 5.6 d. In comparison, the total influent, including settleable solids, carried 14.2-28.5 μg/d of stigmasterol and 12.2-24.3 μg/d of campesterol into the ASB at these HRTs. Thus, at the shortest HRTs,

the release of stigmasterol and campesterol into the water column was significant. When phytosterol release from sediment solids was considered in the calculation of ASB phytosterol removal, the efficiency increased from 82-94% to 88-96%.

The observed overall removal represents the combined removal from both biodegradation and settling. To estimate the contribution of biodegradation to the overall phytosterol removal at the three HRTs, it was assumed that biodegradation took place in the aerobic water column only, that phytosterol release occurred from the sediment into the water column and that phytosterol sorption to solids in the water column was negligible. It should be noted that biodegradation of phytosterols under the anoxic/anaerobic conditions prevailing in the sediment was not significant as shown by the batch assays (Sections 6.3.4 and 6.3.5) and previously reported (Chapter 5; Dykstra et al., 2014b). Given these assumptions, across all HRTs, the average phytosterol removal due to aerobic biodegradation in the water column, solids settling and accumulation in the sediment, and effluent discharge was calculated as 51, 40 and 9%, respectively.

Table 6.5. Steady state ASB phytosterol removal (%).

Phytosterols	**HRT 22.2 d**	**HRT 11.1 d**	**HRT 5.6 d**
Observed Overall Removal			
β-sitosterol	97	96	98
Stigmasterol	78	-3	44
Campesterol	90	70	1
Total phytosterols	94	82	83
Isolated Water Column Removal			
β-sitosterol	94	92	97
Stigmasterol	10	-328	-132
Campesterol	30	-111	-607
Total phytosterols	86	57	59

6.4. Summary

Phytosterol biotransformation is highly dependent on the redox conditions encountered, as shown by assays conducted under aerobic, nitrate-reducing, sulfate-reducing and fermentative/methanogenic conditions. The bioenergetics calculations for phytosterol metabolism show that degradation of phytosterols is energetically feasible under each redox condition considered. Phytosterols are hydrophobic molecules with very low solubility that have a tendency to partition to solids, which may carry them into anoxic or anaerobic sediments in ASBs. Phytosterols incorporated within plant cells within the wastewater, may be released during cell breakdown. Raw pulp and paper wastewater was separated into two fractions by settling and although the settled fraction represented 6.5% of the volume, it contained 54% of the total solids, 63% of the total COD and 58% of the total phytosterols. A simulated ASB was set up and the steady-state

effluent and sediment characteristics were determined at HRT values of 22.2, 11.1and 5.6 d. The ASB effluent quality remained high for each HRT and the results highlighted the ability of ASBs to accommodate a large range of loading rates while producing a consistently high quality effluent. In contrast, shorter HRT values had a significant effect on the sediment, where phytosterols and non-degraded solids accumulated. The activity of the aerobic zone and anoxic/anaerobic zone of the ASB was examined. Aerobic phytosterol removal increased over time, possibly due to the release of membrane-bound cholesterol oxidase. A higher activity was observed in the anoxic/anaerobic zone where phytosterols increased across the 96 h incubation period, possibly due to the release of solids-associated phytosterols or the conversion of precursors into phytosterols. Bioassays of wastewater fractions under aerobic and anoxic/anaerobic conditions were conducted using inocula from the ASB. Phytosterols in the aerobic wastewater bioassay increased and then decreased, primarily due to the appearance and disappearance of stigmasterol. A similar increase and decrease in stigmasterol was not observed in the sediment, indicating that the increase in stigmasterol observed across some ASBs may be due to a process primarily taking place within the aerobic zone. Further research is needed to better delineate the processes within an ASB that release or produce phytosterols.

Approximately 60% of phytosterols in the pulp and paper used in this study were associated with settleable solids, which carry phytosterols into anoxic/anaerobic zones where significant biotransformation of phytosterols does not occur. ASB sediment acts as a phytosterol reservoir and as solids are disintegrated/degraded, phytosterols are released into the aerobic water column where they undergo aerobic biodegradation. In this study,

phytosterols entering the ASB were primarily removed via biotransformation, with the remaining phytosterols settling in the sediment and a small portion leaving in the effluent. Settling of solids in an ASB is important for maintaining a low, stable effluent phytosterol concentration across a range of HRTs, although sediment characteristics are significantly affected by HRT and solids loading rate. The results of this study suggest that a model can be developed using HRT, wastewater settling characteristics, and biotransformation rates as key parameters to predict overall ASB phytosterol removal and accumulation in the sediment. Further research is needed to develop and test such a model, which can then guide wastewater treatment system design and operation.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

Phytosterols are naturally produced compounds that have endocrine disrupting effects in aquatic species and are found in relatively high concentrations in wastewater from the pulp and paper industry. Pulp and paper wastewater is commonly treated in ASBs where both settling and biological treatment occur. Very little is known about how the complex and interrelated processes within ASB treatment systems affect the fate of phytosterols. This research aimed to improve understanding of the biotransformation of phytosterols within ASBs by: 1) examining phytosterol biotransformation under aerobic, nitrate-reducing, sulfate-reducing and fermentative/methanogenic conditions; and 2) investigating the performance and phytosterol removal of a continuously-fed lab scale ASB.

Phytosterol biotransformation is highly dependent upon the redox condition and occurs more readily in aerobic systems. Several conclusions were reached based on the study of aerobic phytosterol biotransformation. Phytosterols have a very low solubility in water, which limits their bioavailability and biotransformation. Heterotrophic activity drives little, if any, phytosterol solubilization. Phytosterol removal under aerobic conditions proceeds slowly until the culture reaches the stationary phase, after which phytosterol removal dramatically increases, most likely as a result of the accelerated release of cholesterol oxidase. During the stationary phase, cell turnover may contribute to the release of membrane-bound cholesterol oxidase, increasing the available enzyme concentration.

Phytosterols may also be carried into anoxic or anaerobic sediments in ASBs during settling of solids. While phytosterol biotransformation under aerobic conditions is well known, very little is understood about phytosterol biotransformation under anoxic/anaerobic conditions. The research on phytosterol biotransformation under anoxic/anaerobic conditions yielded several findings. Phytosterol biotransformation is possible under nitrate-reducing conditions, although more research is needed to determine which conditions and factors contributed to the reduced microbial activity in the later stage of the assay. Phytosterol biotransformation is possible under sulfate-reducing conditions, which is a significant finding because pulp and paper wastewater typically carries sulfate into treatment systems. Additionally, stigmast-4-en-3-one was identified as a potential intermediate in the biotransformation of β-sitosterol under sulfate-reducing conditions. These results suggest that the initial step of the pathway for phytosterol biotransformation under sulfate-reducing conditions is similar to that of cholesterol biodegradation under denitrifying conditions. Phytosterol biotransformation was not observed under fermentative/methanogenic conditions despite acclimation over the course of 16 feeding cycles (112 days). This indicates that phytosterols are recalcitrant under highly reduced conditions, which are present in sediment layers of ASBs.

To better understand phytosterol removal within actual ASB treatment systems, tests were conducted using a continuously-fed lab scale ASB. Based on the research conducted on the lab-scale ASB, it was determined that a change in the ASB HRT only minimally impacts the steady-state effluent quality but significantly changes the characteristics of the sediment. Effluent COD and solids remained relatively stable across

the HRT values tested but sediment COD values were significantly higher at shorter HRT values. ASB effluent phytosterol concentrations were highest when operating at a moderate HRT and lower when operating at either a shorter or longer HRT. At a shorter HRT, there is less of an opportunity for the release of phytosterols from solids degradation. At a longer HRT, both free phytosterols and phytosterols released from solids have an opportunity to be biotransformed. The lowest effluent phytosterol concentrations were observed at the longest HRT value.

ASBs are complex systems with competing processes of phytosterol removal and release. In an anaerobic batch biodegradation test of wastewater solids, phytosterol concentrations increased over time, indicating a release of phytosterols from solids. In an aerobic batch biodegradation test of wastewater supernatant, phytosterol concentrations increased temporarily and then decreased, with an overall net removal. These results highlight the need to account for both release and removal of phytosterols in ASB systems.

ASBs are able to remove significant levels of phytosterols through biotransformation and the settling of solids-associated phytosterols. In biotransformation assays, phytosterol removal occurred more readily under aerobic conditions. Therefore, controlling aeration and DO levels within an ASB is important. The presence of alternate electron acceptors such as nitrate or sulfate is also important to phytosterol biotransformation within an ASB treatment system.

Further research is needed to better understand the phytosterol release and removal mechanisms within ASBs. Additionally, questions remain to be answered regarding the metabolism of phytosterols under sulfate-reducing conditions. The

identification of an intermediate may assist in the future elucidation of the biodegradation pathway. Additional research is also needed to understand the role of enzyme induction and release in phytosterol biotransformation. Given a better understanding of phytosterol biotransformation, a model might be developed to predict ASB phytosterol removal using HRT and wastewater settling characteristics as additional parameters.

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