Biotransformation potential of phytosterols under anoxic and anaerobic conditions

C. M. Dykstra, H. D. Giles, S. Banerjee and S. G. Pavlostathis

ABSTRACT

The biotransformation potential of three phytosterols (campesterol, stigmasterol and β-sitosterol) under denitrifying, sulfate-reducing and fermentative/methanogenic conditions was assessed. Using a group contribution method, the standard Gibbs free energy of phytosterols was calculated and used to perform theoretical energetic calculations. The oxidation of phytosterols under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions was determined to be energetically feasible. However, using semi-continuously fed cultures maintained at 20–22 °C over 16 weekly feeding cycles (112 days; retention time, 21 days), phytosterol removal was observed under nitrate-reducing and sulfate-reducing conditions, but not under fermentative/methanogenic conditions. Under sulfate-reducing conditions, stigmast-4-en-3-one was identified as an intermediate of phytosterol biotransformation, a reaction more likely carried out by dehydrogenases/isomerases, previously reported to act on cholesterol under both oxic and anoxic (denitrifying) conditions. Further study of the biotransformation of phytosterols under anoxic/anaerobic conditions is necessary to delineate the factors and conditions leading to enhanced phytosterol biodegradation and the development of effective biological treatment systems for the removal of phytosterols from pulp and paper wastewaters and other phytosterol-bearing waste streams.

Key words | β-sitosterol, campesterol, methanogenesis, nitrate reduction, stigmasterol, sulfate reduction

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INTRODUCTION

Phytosterols are naturally occurring compounds which regulate membrane fluidity and cross-membrane transport in eukaryotic organisms; they also serve as hormone precursors in plants. The most common phytosterols are campesterol, stigmasterol and β-sitosterol, the latter being the most abundant in most plant species. Phytosterols are released from wood during the pulping process and a small fraction enters the wastewater stream during washing of the pulp. The concentration of phytosterols in pulp and paper wastewaters varies depending on the pulping method used, the species of wood being pulped, and the amount of water being used by the mill. Typical total phytosterol concentration in untreated pulp and paper mill wastewater varies from 0.3 to 3.4 mg/L (Xavier *et al.* 2009). Endocrine system disturbances have been observed in fish downstream of pulp and paper mill wastewater outfalls (Hewitt *et al.* 2008). Phytosterols are suspected of contributing to the endocrine-disrupting potential of pulp and paper mill effluents. Exposure to phytosterols affected steroid production (Gilman *et al.* 2003) and induced vitellogenin production in aquatic organisms (Hewitt *et al.* 2008). There is also evidence that some of the observed endocrine disruption is due to microbial transformation products of the phytosterols rather than the phytosterols themselves (Denton *et al.* 1985).

Aerated stabilization basins (ASBs) are common biological treatment systems in North American pulp and paper mills. Due to incomplete mixing, ASBs contain several redox zones, ranging from aerobic in the upper water column to sulfate-reducing and methanogenic in the benthal zones. Phytosterols are sparingly soluble and hydrophobic and thus adsorb to organic matter in aquatic systems. Therefore, phytosterols may be exposed to either aerobic or anaerobic environments. The removal of phytosterols from wastewaters by full-scale pulp and paper mill treatment systems has been examined by several studies (Cook *et al.* 1997;

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Mahmood-Khan & Hall 2003). Activated sludge units and ASBs can remove >90% of all phytosterols. However, removal in these systems may be due to biotic and abiotic transformation of the phytosterols or to adsorption and sequestration to sludge and sediment. Phytosterols have been found to accumulate in sludge and sediment. Pulp mill activated sludge contained 10–32 mg/L total sterols (Mahmood-Khan & Hall 2003).

Many species are capable of transforming phytosterols aerobically. The initial reactions in the degradation of phytosterols and cholesterol are thought to be catalyzed by the same enzymes due to their structural similarity. The complete mineralization of cholesterol has been observed under both aerobic and denitrifying conditions (Harder & Probian 1997). The first step in the microbial cholesterol degradation pathway is oxidation to cholest-5-en-3-one followed by isomerization to cholest-4-en-3-one, reactions catalyzed by either of two classes of dual-function enzymes: cholesterol oxidases, which require molecular oxygen as electron acceptor, or cholesterol dehydrogenases/isomerases (Uhia *et al.* 2011). Side chain degradation is then initiated by the hydroxylation of C27 or C25 followed by ring cleavage (Chiang *et al.* 2008). The anaerobic degradation of cholesterol, however, has received less attention. The hydrogenation of cholesterol to cholestanol is known to occur in the mammalian gut. In experiments with sewage sludge and lake sediment, the only observed transformation of cholesterol was hydrogenation of 0.5% of the original cholesterol mass to cholestanol (Gaskell & Eglington 1975). In contrast to available information for the biotransformation of cholesterol, the biodegradation of phytosterols is less well understood, especially under anoxic and anaerobic conditions.

The objective of this study was to assess the biotransformation potential of three phytosterols (campesterol, stigmasterol and β-sitosterol) under denitrifying, sulfate-reducing and fermentative/methanogenic conditions.

MATERIALS AND METHODS

Wastewater and chemicals

Untreated wastewater was collected from a southeastern US pulp and paper mill, characterized, and stored at 4 °C until use. The mill used Kraft pulping to produce newsprint from a mixture of hardwood and softwood. The wastewater characterization included: pH, total suspended and volatile suspended solids (TSS/VSS), total and soluble chemical oxygen demand (COD), total and soluble carbohydrates, nitrate, ammonia, chloride, phosphate, sulfate, total and soluble phytosterols. The wastewater was fractionated by settling at room temperature (20–22 °C), yielding a supernatant fraction and a fraction containing settled solids. Settling for 15.5 hours was considered sufficient based on preliminary settling tests which lasted longer. In addition, settling was chosen as opposed to other means of solids separation (e.g. centrifugation) to better simulate process conditions at the pulp and paper mill wastewater treatment plant. Cholesterol (≥99%) was obtained from Sigma Aldrich (St Louis, MO). A phytosterol mixture assayed as 25.2% campesterol, 25.4% stigmasterol and 41.3% β-sitosterol was purchased from Purebulk.com (Roseburg, OR). Stock solutions were prepared in ethanol at 8 and 4.57 g/L for cholesterol and the phytosterol mixture respectively. The aqueous solubility of phytosterols at 25 °C and pH 7 is reported as 0.5–2 μg/L and their octanol–water partition coefficient (log K_{ow}) is 9.2–9.7 (Giles 2012).

Stock cultures

A culture, developed with mixed liquor obtained from the aeration basin of a pulp and paper wastewater treatment plant, was maintained at room temperature (20–22 °C) under alternating aerobic/anoxic conditions. The culture was fed pre-settled, untreated pulp and paper mill wastewater, supplemented with salts, NH_4Cl at 60 mg N/L, phosphate buffer, and trace metals (Giles 2012). The resulting feed characteristics were: pH, 7.3 ± 0.2; total COD, 351 ± 5 mg/L; soluble COD, 300 ± 3 mg/L. Nitrate, produced as a result of nitrification, was removed by incorporation of a one-day anoxic stage into the weekly operating cycle. The culture was maintained with a hydraulic retention time (HRT) of 4.2 days and a solids retention time (SRT) of 21 days.

A sulfate-reducing culture and a fermentative/methanogenic culture were developed with lagoon sediment from a pulp and paper plant, fed settled solids removed from the untreated pulp and paper mill wastewater, supplemented with phosphate buffer, NH_4Cl at 120 mg N/L, and anaerobic culture media (Giles 2012). The sulfate-reducing culture feed was also supplemented with Na_2SO_4 at 1.5 g S/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. The carry-over sulfate concentration from the pulp and paper mill wastewater in the methanogenic feed was 33 ± 1 mg S/L. Both cultures were maintained at room temperature (20–22 °C) with an HRT/SRT of 21 days.

Semi-continuous cultures

Assessment of the biotransformation potential of the three phytosterols was carried out under nitrate-reducing, sulfate-reducing and fermentative/methanogenic conditions using three semicontinuously fed cultures, prepared with inocula from the above-mentioned three stock cultures. The nitrate-reducing culture was prepared by transferring 150 mL of settled mixed liquor collected at the end of the anoxic period from the mixed culture described above, which was maintained under alternating aerobic/anoxic conditions. In order to avoid transferring wastewater solids, the sulfate-reducing and fermentative/methanogenic cultures were prepared by transferring 150 mL of supernatant obtained after the respective stock cultures were allowed to settle for a day. The three cultures were maintained at room temperature (20–22 °C) in 2.2 L glass bottles with a liquid volume of 1.5 L, wasted/fed every 7 days with culture media (see above), an ethanolic mixture of the three phytosterols and either sodium nitrate (nitrate-reducing culture) or sodium sulfate (sulfate-reducing culture). It should be noted that, after the initial inoculation, pulp and paper wastewater was not fed to the three cultures throughout their incubation period. The initial concentrations immediately after feeding, ignoring residuals, were: ethanol, 1,100 mg COD/L; nitrate, 400 mg N/L; sulfate, 670 mg S/L; and total phytosterols, 3.2 mg/L. The resulting retention time of all three cultures was 21 days. The following parameters were monitored: pH, soluble COD, gas production and composition, electron acceptors (i.e. nitrate and sulfate), volatile fatty acids (VFAs), and phytosterols. In order to assess the long-term fate of the phytosterols in the three semicontinuously fed cultures under static (i.e. non-feeding) conditions after 16 feeding cycles (112 days), the three cultures were batch incubated for 7 months and then re-analyzed. In parallel to the three semicontinuously fed cultures, sacrificial abiotic controls, prepared in 160 mL serum bottles similarly to the three active cultures and amended with 200 mg/L of sodium azide, were batch incubated at room temperature (20–22 °C) and periodically assayed for the phytosterol concentration over the duration of this assay.

Analytical methods

pH, TSS, VSS, COD, and ammonia were measured following procedures outlined in *Standard Methods* (APHA 2012). Carbohydrates were measured by the anthrone method. Anions were quantified by ion chromatography. Total gas was measured with a digital pressure transducer. Gas composition and VFAs were determined by gas chromatography with thermal conductivity detection and flame ionization detection (FID), respectively. Analysis of phytosterols involved liquid–liquid extraction with iso-octane, followed by gas chromatography/mass spectrometry (GC/MS). The extraction efficiency was above 95%. Cholesterol was used as a surrogate standard. Analysis of phytosterols and cholesterol was performed using an Agilent 7890 GC unit with FID and MS detectors (Agilent Technologies, Santa Clara, CA, USA). The column used was a 30 m × 0.25 mm × 0.10 μm Zebron ZB-5HT (Phenomenex, Torrance, CA, USA), which terminated in a Dean's switch for simultaneous collection of FID (quantification) and MSD (identification) data. MS analysis was conducted by electrospray ionization (EI) with positive ion polarity at 70 eV fragmentation voltage and a mass scan range of m/z 50–500. MSD spectra were compared to standards and those in the NIST library. The GC injection port was held at 325 °C and 23.4 psi using a splitless MS-certified inlet liner. Helium was used as carrier gas with a flow rate of 2.4 mL/min. The injection volume was 1 μL. The oven temperature was initially held at 90 °C for 1 min, ramped to 250 °C at 20 °C/min and held for 1 min, then ramped again to 310 °C at 6 °C/min and held for 2 min before termination of the run.

RESULTS AND DISCUSSION

Wastewater characteristics

The chemical composition of the untreated pulp and paper wastewater is given in Table 1. Fractionation of the wastewater resulted in the following mass distribution (%) between the supernatant and the settled solids fractions, respectively: total solids, 46/54; volatile solids, 33/67; total COD, 37/63; total phytosterols, 42/58. In terms of individual phytosterols, their mass distribution was as follows: in the supernatant, 92% β-sitosterol, 6% stigmasterol, 2% campesterol; in the settled solids fraction, 74% β-sitosterol, 14% stigmasterol, 12% campesterol. Based on these data, it appears that a significant portion of the phytosterols is associated with the organic fraction, both dissolved and solid. Thus, the lack of or slow degradation of phytosterols in the aerobic zone of ASBs, especially the solids-associated fraction of phytosterols, combined with settling of solids leads to the transfer of phytosterols to bottom sediments where anaerobic conditions prevail.

Table 1 | Characteristics of pulp and paper mill wastewater used in this study

Parameter	Value	Parameter	Value
pH	10.0 ± 0.1[a]	Chloride, mg/L	18.8 ± 3.3
Total suspended solids, g/L	0.66 ± 0.01	Phosphate, mg P/L	ND[b]
Volatile suspended solids, g/L	0.27 ± 0.01	Sulfate, mg S/L	43.6 ± 6.8
Total COD, mg/L	903 ± 102	Total β-sitosterol, μg/L	69.2 ± 7.5
Soluble COD, mg/L	279 ± 20	Soluble β-sitosterol, μg/L	22.8 ± 4.6
Total carbohydrates, mg/L	250 ± 24	Total stigmasterol, μg/L	22.6 ± 2.3
Soluble carbohydrates, mg/L	9.7 ± 3.3	Soluble stigmasterol, μg/L	5.0 ± 0.3
Nitrate, mg N/L	0.24 ± 0.04	Total campesterol, μg/L	9.3 ± 2.4
Ammonia, mg N/L	ND	Soluble campesterol, μg/L	2.1 ± 1.0

[a]Mean ± standard deviation ($\eta = 3$).
[b]ND, not detected.

Energetics of phytosterol degradation

A group contribution method (Mavrovouniotis 1991) was used to estimate the Gibbs free energy of the sterols. The estimated Gibbs free energies for cholesterol, campesterol, stigmasterol and β-sitosterol are 54.81, 87.87, 173.64 and 94.98 kJ/mol, respectively. The Gibbs free energy for sterol reduction half-reactions is between 25.99 and 26.59 kJ/electron equivalent (eeq). The Gibbs free energy for the oxidation of each sterol under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions were calculated. As an example, results for β-sitosterol are shown in Table 2 for each redox condition ($\Delta G_r'$, kJ/eeq at pH 7 and 298 K). Based on these results, biodegradation of sterols is theoretically feasible under each of the four redox conditions examined.

Biotransformation potential of phytosterols under different electron-accepting conditions

Figure 1 shows the time course of the electron acceptor (i.e. nitrate or sulfate), soluble COD, pH, gas production (i.e. headspace pressure), and total phytosterols in the nitrate-reducing, sulfate-reducing, and fermentative/methanogenic cultures over 16 feeding cycles (112 days). Figure 2 shows the concentration of each phytosterol in the three cultures after each feeding cycle over the duration of this assay. Very similar patterns for all phytosterols were observed at each electron-accepting condition. Abiotic, sodium azide-amended controls prepared similarly to the three active cultures and batch incubated over the duration of this assay did not result in any decrease of phytosterol concentration.

Nitrate-reducing conditions

For the first nine feeding cycles, the nitrate-reducing culture was very active, resulting in high removal of both COD and nitrate as well as gas production (Figures 1A1, 1A2, 1A4). From the second through the ninth feeding cycle, the total gas production ranged from 242 to 377 mL at 22 °C/cycle. During this period, the culture pH was between 7.5 and 8.9, necessitating periodic adjustment with dilute HCl. With the onset of the 10th feeding cycle, the gas production

Table 2 | Standard Gibbs free energy of β-sitosterol oxidation under different redox conditions

Redox condition	$\Delta G_r'$ kJ/eeq
Aerobic $C_{29}H_{50}O + 41O_2 \leftrightarrow 29CO_2 + 25H_2O$	− 104.99
Nitrate-reducing $C_{29}H_{50}O + 32.8NO_3^- + 32.8H^+ \leftrightarrow 29CO_2 + 41.4H_2O + 16.4N_2$	− 98.47
Sulfate-reducing $C_{29}H_{50}O + 20.5SO_4^{2-} + 30.75H^+ \leftrightarrow 29CO_2 + 25H_2O + 10.25H_2S + 10.25HS^-$	− 5.42
Methanogenic $C_{29}H_{50}O + 16H_2O \leftrightarrow 8.5CO_2 + 20.5CH_4$	− 2.74

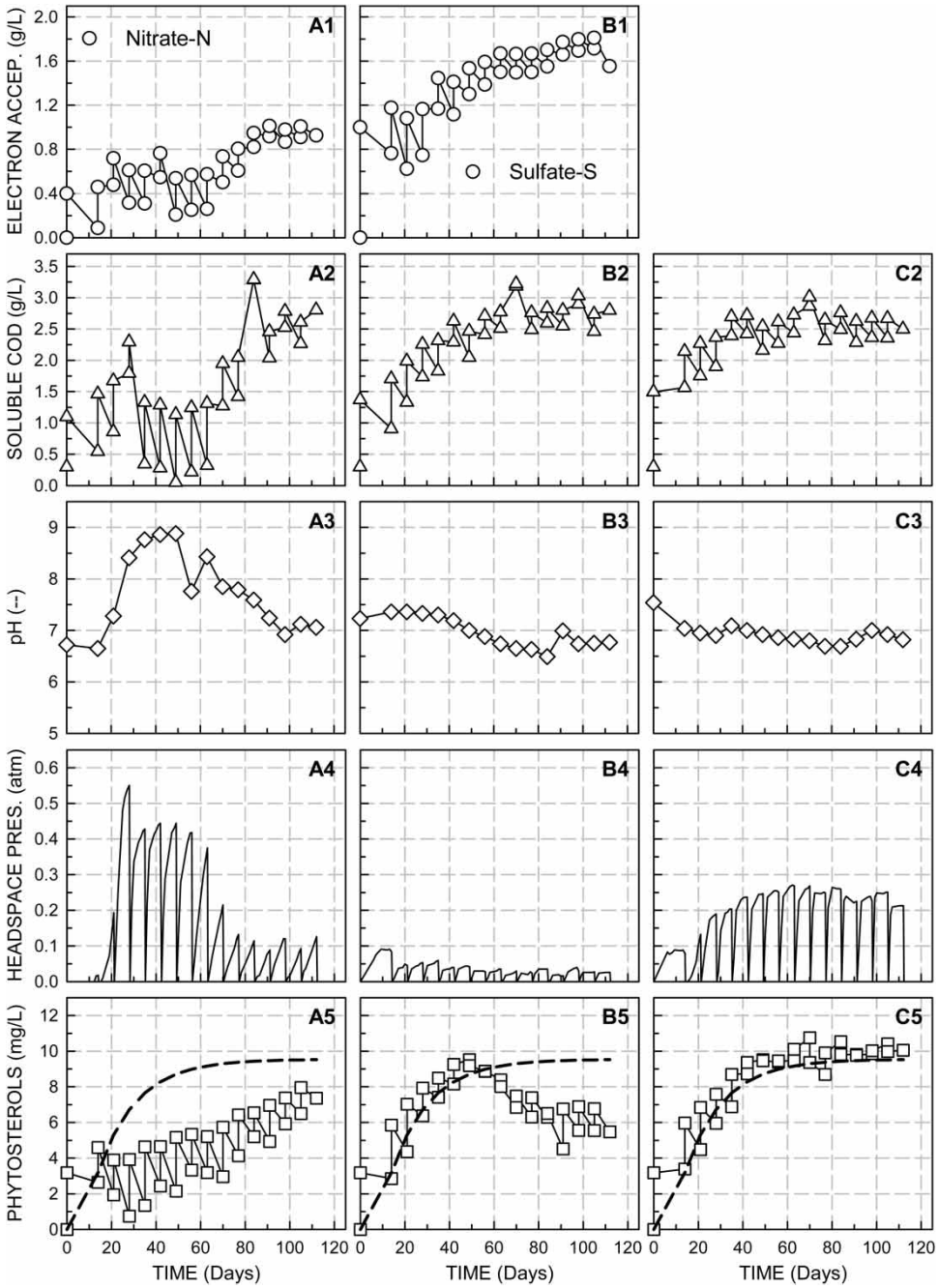

Figure 1 | Time course of (1) electron acceptor (i.e. nitrate or sulfate), (2) soluble COD, (3) pH, (4) headspace pressure, and (5) total phytosterols in semi-continuously fed nitrate-reducing (A), sulfate-reducing (B), and methanogenic (C) cultures (broken lines represent phytosterol concentrations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

decreased and the residual soluble COD gradually increased, reaching 2,800 mg/L by the end of the last feeding cycle (Figure 1A). From the 10th through the 16th feeding cycle, the total gas production decreased significantly and ranged from 53 to 88 mL at 22 °C/cycle. During these last seven feeding cycles, the culture pH was between 7.0 and 7.8. The exact reasons for the decline in nitrate-reduction activity starting at the 10th feeding cycle are not known. However, the possibility that this decline was related to inhibition by phytosterols should be dismissed based on the fact that the phytosterols concentration was between 3 and 6 mg/L long before the decline in nitrate

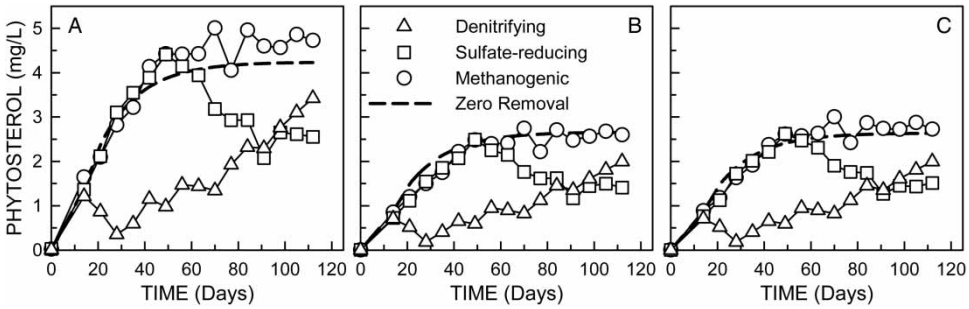

Figure 2 | Phytosterol profiles in the three semi-continuously fed cultures. A, β-sitosterol; B, stigmasterol; C, campesterol (broken lines represent phytosterol concentrations calculated taking into account wasting and feeding of the cultures and assuming complete recalcitrance of the three phytosterols).

reduction was observed. In addition, given the very low aqueous solubility of the phytosterols (≤2 μg/L), as well as the relatively high total phytosterols concentration maintained in the methanogenic culture which did not exhibit any inhibition (Figure 1C), the possibility of phytosterol-induced inhibition of the nitrate-reducing culture is further rejected.

For the first 70 days (10 feeding cycles), significant removal of all three phytosterols was observed, ranging between 69 and 88%. However, during the last seven feeding cycles, the phytosterol removal decreased and reached only 19% by the end of the 16th feeding cycle (Figure 1A5). At the end of the 16th feeding cycle, the total phytosterols concentration was 7.36 μg/L (campesterol, 2.00; stigmasterol, 1.94; β-sitosterol, 3.42). The decline in phytosterol removal is more likely related to the overall decline of nitrate reduction of this culture as discussed above. In addition, given that the organic substrate was switched from the complex pulp and paper wastewater used for the development of the mixed aerobic culture maintained under alternating aerobic/anoxic conditions (see above) to ethanol, more likely the microbial community diversity was reduced over time. Furthermore, the period of high pH in the nitrate-reducing culture could also have decreased the population of phytosterol degraders by selecting for phytosterol non-degrading microorganisms that thrive on ethanol at relatively high pH.

Phytosterol biotransformation products were not detected during the early feeding cycles when significant phytosterol removal was observed. Biodegradation of cholesterol under nitrate-reducing conditions has been previously reported (Harder & Probian 1997; Chiang *et al*. 2007, 2008). *Sterolibacterium denitrificans* metabolized cholesterol under nitrate-reducing conditions by the oxidation of the first ring and then hydroxylation of the terminal C-25 of the side chain by an oxygen-independent reaction using water as oxygen donor. Cholest-4-en-3-one was an intermediate biotransformation product of cholesterol. In the absence of oxygen, cholesterol dehydrogenases/isomerases, which utilize NAD^+ or $NADP^+$ as cofactors, carried the initial cholesterol biotransformation (Chiang *et al*. 2007, 2008). Cholest-4-en-3-one has been identified as an intermediate biotransformation product of cholesterol under both oxic and anoxic (denitrifying) conditions (Chiang *et al*. 2007, 2008; Uhia *et al*. 2011).

Sulfate-reducing conditions

The sulfate-reducing culture was consistently active throughout the 16 feeding cycles with a total gas production ranging from 20 to 42 mL at 22 °C/cycle; the pH was between 6.8 and 7.3 (Figure 1B). The residual soluble COD increased over the feeding cycles and reached 2,400 mg/L by the end of the 16th feeding cycle, representing about 25% COD removal. Most of the residual COD was VFAs, primarily acetate. Sulfate removal followed the soluble COD removal pattern and reached approximately 1,500 mg S/L by the end of the test (i.e. 22% removal). The growth and metabolic activity of the sulfate-reducing culture could have been limited by its decreasing pH, because sulfate-reducing bacteria are reported to have dramatically lower growth and sulfate removal rates at a pH below 7 (Al-Zuhair *et al*. 2008). Phytosterol-induced inhibition of the sulfate-reducing culture is dismissed on the same grounds discussed above for the nitrate-reducing culture.

For the first seven feeding cycles removal of phytosterols did not occur (Figure 1B5). With the onset of feeding cycle eight, a drastic decrease of the concentration of all three phytosterols was observed and reached between 40 and 47% removal by the end of the 16th cycle (β-sitosterol, 40%; stigmasterol, 47%; campesterol, 43%) (Figure 2). At the end of the 16th feeding cycle, the total phytosterols concentration was 5.57 μg/L (campesterol, 1.51; stigmasterol, 1.41; β-sitosterol, 2.55). The absence of phytosterol removal initially, as well as the relatively low removal of phytosterols starting

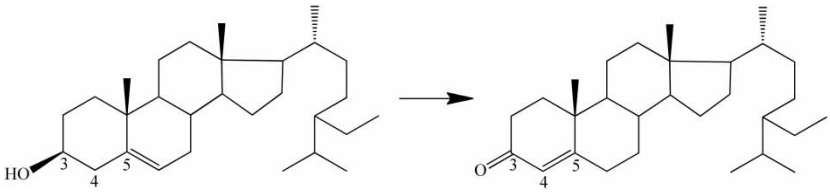

Figure 3 | Production of stigmast-4-en-3-one from β-sitosterol.

with the seventh feeding cycle may be related to a decrease of microbial community diversity associated with the switch in substrate from pulp and paper wastewater to ethanol, as well as the overall relatively low metabolic activity related to pH.

During the 14th feeding cycle of the sulfate-reducing culture, stigmast-4-en-3-one was positively identified by GC/MS (90% similarity to the stigmast-4-en-3-one in the NIST08.L Database), more likely a product of β-sitosterol biotransformation (Figure 3). A recent study has reported production of stigmast-4-en-3-one from β-sitosterol by *Rhodococcus erythropolis* under oxidative conditions (Grishko *et al.* 2012). As mentioned above, previous studies have identified cholest-4-en-3-one as an intermediate product of cholesterol biotransformation under both oxic and anoxic (denitrifying) conditions (Chiang *et al.* 2007, 2008; Uhia *et al.* 2011). It is noteworthy that *Rhodococcus erythropolis* has been reported to reduce sulfate to sulfite (Takarada *et al.* 2005). Thus, it is plausible that enrichment of certain sulfate-reducing bacteria in the present study contributed to the observed biotransformation of phytosterols.

Methanogenic conditions

The methanogenic culture was consistently active throughout the 16 feeding cycles with a total gas production ranging from 130 to 190 mL at 22 °C/cycle; the pH was between 6.8 and 7.0 (Figure 1C). The methane content of the gas produced was 66%. The residual soluble COD increased over the first four feeding cycles and remained relatively constant at about 2,500 mg/L over the remaining feeding cycles. The COD removal was about 24%. Most of the residual COD was VFAs, primarily acetate.

Throughout the 16 feeding cycles, all three phytosterols were completely recalcitrant under methanogenic conditions (Figures 1 and 2). At the end of the 16th feeding cycle, the total phytosterols concentration was 10.06 μg/L (campesterol, 2.73; stigmasterol, 2.60; β-sitosterol, 4.73). As mentioned above, hydrogenation of cholesterol to cholestanol by fermentative bacteria has been previously observed (Gaskell & Eglington 1975). However, degradation of cholesterol or phytosterols by methanogens has not been reported.

Long-term batch incubation

After the above-described three cultures were maintained for 16 feeding cycles (112 days), they were then incubated for 7 months without any wasting or feeding and then re-analyzed. Table 3 shows the data obtained after a 7-month incubation.

These results confirm that the nitrate-reducing culture was inhibited, shown by the significant nitrate and soluble COD remaining after the long-term incubation. It is noteworthy that nitrite was not detected. The ammonia concentration was comparable to that found in the other two cultures, indicating that dissimilatory nitrate reduction to ammonia did not take place in the nitrate-reducing culture. A low level of VFAs, mainly acetate also remained. The sulfate-reducing culture had a high soluble COD level, most of which was acetate. The remained high sulfate concentration was expected as sulfate was added to this culture in excess to maintain sulfate-reducing conditions. Finally, the fermentative/methanogenic culture had a low level of soluble COD and no VFAs, confirming that this

Table 3 | Results of analyses at the end of 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[c]
Nitrate (mg N/L)	141.2 ± 3.8	ND	ND
Sulfate (mg S/L)	ND	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 of batch incubation.
[b]Mean ± standard deviation ($n = 3$).
[c]ND, not detected.

culture was not inhibited. After 7 months of batch incubation, the total phytosterols concentration was significantly lower in the sulfate-reducing culture (Table 3) compared to that at the end of the 16th feeding cycle (see above). However, the phytosterols concentrations in both the nitrate-reducing and the fermentative/methanogenic cultures were practically unchanged.

CONCLUSIONS

Although oxidation of phytosterols was determined to be energetically feasible, in the present study removal of phytosterols was observed under nitrate- and sulfate-reducing conditions, but not under fermentative, methanogenic conditions. Biotransformation of phytosterols under nitrate- and sulfate-reducing conditions is promising and warrants further study to delineate the factors and conditions leading to enhanced phytosterol biodegradation, especially under sulfate-reducing conditions. Phytosterol degradation under sulfate-reducing conditions is particularly important in the context of pulp and paper wastewater treatment as the wastewater contains significant sulfate concentrations. Understanding such factors can lead to the development of effective biological treatment systems for the removal of phytosterols from pulp and paper mill wastewaters and other phytosterol-bearing wastewater streams.

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