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Physicochemical properties and trace organic compounds in a dairy processor's aerobic bioreactor

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ABSTRACT

Wastewater samples were taken from an aerobic bioreactor, operated by a dairy processor in southeastern Australia to reduce nutrient and pollutant loads. Samples were taken over a two-year period, to determine whether trace organic compounds or physicochemical analyses of the wastewater could be used to discriminate the water taken before, during and after processing of the wastewater in the bioreactor. Multivariate analyses of the physicochemical data suggested that nitrate, pH and total dissolved nitrogen best described the infeed wastewater entering the bioreactor, while organic and particulate phosphorus concentrations where predominantly responsible for describing the composition of the content of the bioreactor. Gas chromatography-Mass spectrometry data of organic compounds within the wastewater samples were also analysed via multivariate analyses. The analyses found that the compound 4-nitrophenol was associated with ammonia concentrations and mixed liquor wastewater. Therefore, 4-nitrophenol may possibly be used to act as an indicator of anaerobicity in aerobic bioreactors.

Keywords

Dairy processing wastewater: aerobic bioreactor; 4-nitrophenol; GC-MS; multivariate statistics

1. Introduction

In Australia, dairy processing facilities annually consume on average 386 megaliters of potable water and produce 452 megaliters wastewater (Allinson and Dyer, 2007). Wastewater from dairy processors can be some of the most polluted in the food industry with up to ten liters of wastewater produced per liter of processed milk

(Vourch et al., 2008). Consequently it is common for dairy processors to segregate and treat wastewater prior to disposal or reuse (Wilkinson et al., 2007). The wastewaters generally contain residual milk and milk derivatives (e.g. casein, whey), along with cleaning and buffering agents used within the processing plant. Milk residues consist of proteins, carbohydrates and fats, which account for a large proportion of the chemical oxygen demand (COD) in dairy factory wastewaters (Demirel et al., 2005). For example, milk fats (35-500 mg/L) and proteins (210-560 mg/L) were detected in dairy effluent wastewaters when investigating typical digestion problems in anaerobic digesters (Perle et al., 1995). Without prior treatment, these compounds can adversely affect municipal waste treatment systems receiving dairy processing wastewaters.

Operating an aerobic bioreactors is one method of pre-treating dairy processing waste and lessening COD, total phosphorus (TP) and total nitrogen (TN) concentrations Compared to anaerobic bioreactors, aerobic bioreactors provide for more complete degradation of the effluent found in dairy factory waste (Chan et al., 2009). However, compounds in dairy processing wastewaters vary over time in both composition and concentration and it is not uncommon for the load on the bioreactor to double compared to what would be expected from an average production run (Demirel et al., 2005). These changes can affect the various microbial species in the bioreactor and decrease waste treatment efficiency. Importantly, these changes have resulted in bioreactors becoming anaerobic and producing odorous compounds (Chan et al., 2009).

Odor reduction is an important issue as many dairy processors are located near urban centres (Demirel et al., 2005). Aerobic bioreactors minimize odors as organic compounds are reduced to carbon dioxide and water. This contrasts with anaerobic bioreactors where methane and sulphurous compounds are produced as end products (Chan et al., 2009). To maintain an appropriate microbial community, in aerobic bioreactors oxygen is continuously added, usually by pumping air to a distributor at the bottom of the system (Garcia-Ochoa and Gomez, 2009).

Burra Foods Pty. Ltd., located in the Gippsland region of Victoria, Australia is a dairy processor that specializes in the production of cheese, fresh milk concentrates, food preparations (e.g. milk protein and fat blends), specialty milk powders and fresh dairy products (e.g. ice cream and gelato base). The company uses sequential batch aerobic bioreactors to minimize the COD of their wastewater prior to discharge to the local municipal treatment facility. In the past five years, Burra Foods has reduced potable water use from 28 kL to 13 kL per tonne of milk solids, due in part to segregation of the clean wastewater stream, composed of milk condensate and rinse water, from the more organically rich trade wastewater stream. Initial analyses of the trade wastewater stream, during a period in which a greater than average organic loading was introduced into the bioreactor, revealed that compounds such as fatty acids may hinder performance of the bioreactor (Heaven et al., 2011).

Metabolomics has mainly been used in plant science and biomedical investigations, though it has recently been used in the food industry (Surowiec et al., 2011). Metabolomics often requires multivariate analyses due to the large number of samples and/or metabolites. Wastewater researchers have used multivariate analyses but

generally only in regards to physicochemical parameters such as nitrogen (N) or phosphorus (P) concentrations. For example, effluent wastewater was sampled from different factories along the Yantra River in Bulgaria (Stefanov et al., 1999). Using multivariate analyses, it was determined that pollution entering the river from the factories could be grouped into organic pollutants (Biological oxygen demand (BOD), COD, suspended solids) and nutrient pollutants (ammonia, organic N). In India, multivariate analyses were used for analysing effluent from pipes discharging into the Gomti River (Singh et al., 2005). Wastewater streams were attributed to seasonal inputs and industry classes using a combination of multivariate and univariate statistics. In Australia, multivariate statistics was used to distinguish human from non-human faecal pollution in wastewater treatment systems in a mixed land use catchment (Carroll et al., 2009).

The goal of this research was to use metabolomic techniques to identify markers for aerobic bioreactor system performance at Burra Foods. Multivariate analyses were applied to both physicochemical analytes and trace organic compounds simultaneously so that compounds and processes could be correlated.

2. Materials and Methods

2.1 Sample collection

Samples were collected from the wastewater treatment plant at Burra Foods (38º 25’ 42” S, 145º 49’ 05” E) over a period of 24 months (Table S1). Each set of samples followed a batch of wastewater as it passed through the entire treatment cycle prior to being sent offsite.

Burra Foods used sequential batch aerobic bioreactors to treat its trade waste stream, containing liquid waste organic materials collected from the site, prior to discharge to the local municipal treatment facility (Fig. S1). Wastewater from the processing of milk was divided into three categories depending on COD: dilute (<300 mg/L), direct (300-700 mg/L) and heavy wastewater (>700 mg/L). COD for the dairy wastewater varies with the seasons but averages between 300-1100 mg/L.

Heavy wastewater was sent to digesters to reduce the COD to <700 mg/L. Liquid from the digesters went to an aerobic bioreactor detailed below or the final effluent tank. Solids from the digester were trucked away as fertilizer for disposal. Dilute wastewater is sent to a reverse osmosis plant where the permeate was sent to a 50-kL clean water tank and the retentate was sent to a 260-kL up-front tank or the heavy wastewater stream depending on the total waste loadings of the bioreactor. The direct wastewater, called the infeed, was also sent to the up-front tank. Sodium hydroxide or carbon dioxide were added to the infeed wastewater before the batch (wastewater and buffering compounds) was transferred to a 50-kL equalisation tank. The equalisation tank provided a mixing zone for wastewater and buffering compounds to prevent direct contact of these chemicals with the microbes within the bioreactor. Infeed wastewater samples were taken from a tap between the up-front and equalisation tanks. Once the infeed wastewater was approximately the pH of the wastewater in the bioreactor (pH ~ 8), the wastewater was fed to one of three sequenced batch bioreactors (495-kL capacity).

The solution within the bioreactors contained recycled sludge from previous batches along with any wastewater that had been sent for digestion. Once the bioreactor was

filled with infeed wastewater, the wastewater was aerated intermittently over an eight-hour period, followed by a two-hour rest stage where solids were allowed to settle. This cycle was repeated until the COD was at a discharge concentration of <500 mg/L as set by the regional urban water authority. It should be noted that after a single cycle, the wastewater had on average COD of 40-80 mg/L and was held this low to promote stability of the microbial population in the aerobic bioreactor. Mixed liquor samples were taken after the bioreactor was at full capacity and had been in the aeration stage for approximately one hour. These samples are representative of only the initial digestion of the infeed by the bioreactor. The samples were collected from a tap located approximately 30 cm from the bottom of the bioreactor at the approximate height of the aerators.

Once the mixed liquor wastewater COD was acceptable for discharge, the wastewater was transferred to two decant tanks (50-kL capacity each). These tanks were plumbed in parallel, to further gravity settle the solids from the liquid (4-6 h). The resultant supernatant was sent to the final effluent tank, where it was buffered and discharged to the sewer. **Supernatant** wastewater samples were collected from a tap as the wastewater was en-route from the decant tanks to the final effluent tank.

Wastewater samples (20 L) were collected in 20-L polypropylene containers and stored at < 4 C until analyzed. All materials used for processing samples for physicochemical and GC-MS analyses (e.g. hosing and valves) that came in contact with the samples were pre-rinsed with 1% Extran MA03 (Merck, Kilsyth, Australia), 10% HCl (AR Grade, Ajax Chemicals, Taren Point, Australia), deionized water and finally excess sample prior to use.

2.2 Sample Analyses

Water samples were analyzed for total solids (TS) by drying samples to constant weight at 105 C. Electrical conductivity (EC) was measured using a Model 900C conductivity meter (TPS Pty. Ltd., Brisbane, Australia). A Lachat Quickchem Series 8000 flow injection analyzer (DKSH Australia Pty. Ltd., Hallam, Australia) was used for the analyses of: dissolved reactive phosphorus (DRP); total dissolved phosphorus (TDP) and total phosphorus (TP); total dissolved nitrogen (TDN) and total nitrogen (TN); nitrate (NO_3^-); and ammonia (NH_3). The analyses were used to derive estimates for particulate phosphorus (PP = TP – TDP), organic phosphorus (OP = TP – DRP) and particulate nitrogen (PN = TN – TDN) and indicators of the general performance of the bioreactor (NO_3/TN, NH_3/TN, NH_3/NO_3, OP/DRP, DRP/TP and OP/DRP). Physiochemical analyses results were reported as mean standard deviation.

For GC-MS analyses, the samples were pre-concentrated using solid phase extraction (SPE) cartridges. Pre-filtering of samples was performed using muffled (450 C) Schleicher & Schuell GF6 filter paper (PerkinElmer, Rowville, Australia). The filtrate was then passed *via* a siphon onto 6 mL Bond Elut PPL (a styrene divinyl benzene-type solid adsorbent phase with a nominal pore size of 150 Å) solid phase extraction cartridges (Varian Inc., Mulgrave, Australia) preconditioned with 10 mL methylene chloride, then 10 mL methanol (HPLC Grade, Merck, Kilsyth, Australia) using a Vac Elut 20 extraction manifold (Varian Inc., Mulgrave, Australia). Cartridges were replaced once the flow rate was reduced to less than 0.1 mL/min. No more than 5 L was processed on any individual cartridge.

Organic compounds were extracted from the SPE cartridges. The SPE cartridges were air dried (30 min) on a Vac Elut 20 extraction manifold under vacuum. An internal standard (400 L of 2,4,6-tribromophenol, 300 g/mL in methylene chloride, Novachem, Collingwood, Australia) was added to each cartridge. After 30 min, methylene chloride (3 5 mL) was used to extract compounds from each cartridge. The extracts were combined and dried with anhydrous sodium sulfate. The extracts were separated into basic and acidic components using 5M sodium hydroxide followed by 1:1 (v:v) concentrated sulphuric acid in water. The acidified fraction was extracted into methylene chloride, pre-concentrated to approximately 50 L *in vacuo* and placed in GC-MS vials (Agilent, Forest Hill, Australia). Samples were evaporated to dryness *in vacuo*.

Samples were derivatized online using a Gerstel MPS2 autosampler (Gerstel GmbH and Co.KG, Mülheim an der Ruhr, Germany). Forty µL bis(trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane (Thermo Fisher Scientific, Rockford, IL) was added to each sample and incubated in a heated agitator at 37°C for 60 min at 750 rpm. Samples were allowed to rest at room temperature for one hour prior to injection of 1 µL for GC-MS analysis.

The GC-MS instrument (7890A GC and 5975C MSD; Agilent Technologies, Santa Clara, CA) was equipped with a split-splitless injector operated at 250°C and samples were injected at a split ratio of 1:25. A Varian FactorFour capillary column, VF-5ms (30 m × 0.25 mm i.d. and 0.25-µm film thickness + 10m EZ-Guard column), was used to effect separation using ultra-high purity helium carrier gas pressure programmed to

a constant flow (1.06 mL/min). The column oven was programmed to hold the temperature at 35°C for 2 min, increase to 325°C at 25°C/min, and hold for a further 5 min. The transfer line to the mass spectrometer was heated to 280°C and the quadrupole was operated at 150°C. In MS mode, the scan range was 50 to 600 amu with 9 scans/s. Tentative identities were assigned to compounds based on their retention time and mass spectral data using AnalyzerPro (SpectralWorks, Runcorn, UK) and Agilent ChemStation (Agilent Technologies Australia, Forest Hill, Australia). Mass spectra were compared with the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health 2005 mass spectral library (NIST/EPA/NIH 2005 mass spectral library, Gaithersburg, MD), with all computer spectral matches (minimum $R^2 \geq 80\%$) checked manually.

2.3 Statistical Analyses

Initial statistical analyses involved identifying significant relationships between sample types or between sample dates for data collected from physicochemical analyses. Means, standard deviations, ANOVA ($P < 0.05$), coefficient of variation and boxplots were calculated using Genstat Thirteenth Edition (www.vsni.co.uk). The R Statistical Computing Package (http://www.r-project.org/) was used for the Wilcoxon rank sum test. A Mixed Model analysis was also used for comparison with the Wilcoxon rank sum test using Genstat, 13th edition.

For multivariate statistical analyses of the GC-MS dataset, log transformation of the data was used to deal with the GC-MS chromatograms heteroscedastic nature (where noise is increasing with the signal intensity). Initially, the average value of each data point was taken over all the replicates at each sample date and type. Data were filtered

by keeping only those metabolites that were found in more than 60% of the samples in each group (infeed, mixed liquor and supernatant wastewater samples). Similarly, samples were deleted if more than 60% of the metabolites were missing in them. Data were normalized by the median intensity of log-concentrations in each sample, taken across chemical compounds before performing further analysis. This normalization in effect adjusted all samples to a common sample concentration and focused interest upon the pattern of relative concentrations between chemical compounds (Weckwerth, 2006).

Multivariate analyses were performed on the combined GC-MS analyses and physicochemical measurements, a data matrix consisting of over 5000 elements. These elements included contaminants from sampling equipment and from within the analytical apparatus, peaks from compounds with a mass spectral match of $R^2 < 80\%$, and other unexplained artefacts that were removed prior to examination of correlations between identified compounds and the physicochemical analyses.

Statistical analysis software Unscrambler (CAMO Software, St Peters, NSW, Australia) was used for Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA).

PCA was used to further analyse the GC-MS data. PCA is a data transformation technique used to approximate multidimensional data sets using a lower number of dimensions (Everitt et al., 2001). In PCA, a data set of interrelated variables is transformed to a new set of variables called principal components (PCs) in such a way that the PCs are uncorrelated and the first few PCs retain most of the variation present

in the original data set. The first PC is a linear combination of the original variables and defines an axis of maximal variation through the multidimensional data. The second PC is also a linear combination of the original variables defining the maximal axis perpendicular to the first. A graph of the first two PCs provides a two-dimensional approximate representation of the original multi-dimensional data. PCA is an unsupervised technique where knowledge of prior groups is not required and is useful to explore potential grouping of samples in an experiment.

PLS-DA is a supervised classification method that was used to further identify compounds and physicochemical analyses of interest (Everitt et al., 2001). This method has proved to be robust for high-dimensional data and is useful for metabolomics data analysis (Surowiec et al., 2011). PLS-DA uses a multiple linear regression technique to find the direction of maximum covariance between a data matrix (X) and its class grouping or predictor (Y). Both X and Y are reduced to principal components, and then the components of X are used to predict the scores on the Y components. The predicted Y components scores are then used to predict the actual values of grouping X.

3. Results and Discussion

Comparing infeed and supernatant wastewater samples, the bioreactor reduced all physicochemical analytes except NH_3 (Table 1, S2). Chemical analytes varied heavily between sampling dates as demonstrated by most coefficients of variation being > 50% and some (e.g. infeed DRP) > 100%. Only TP, TS and NH_3 concentrations were higher in the mixed liquor wastewater samples compared to the infeed wastewater

samples reflecting the recycled "heavy" wastewater and microbial sludge that is present in the bioreactor (Fig. 1).

Using ANOVA, it was found that between seasons, only DRP, TDP and EC concentrations were significantly different (Fig. 1). Seasonal variation in milk composition had been observed previously and can be attributed to differences in feed composition (Christian et al., 1999) and stage of lactation (Auldist et al., 2010). In the Gippsland region, farmers generally calve their herds from late winter to early spring so that peak milk production coincides with maximum feed availability. There are only a few herds that calve in autumn in response to the slightly higher milk prices offered. Seasonal variation in wastewater composition may therefore have been expected. The lack of such variation probably reflects the variability in the wastes the bioreactor receives which depend on the products being manufactured and the selective use of milk derivatives (i.e. cream) and other additives acquired from outside Burra Foods' farm milk suppliers.

3.1 Phosphorus analyses

Physicochemical analyses of P, TP (and associated OP and PP measurements) were statistically different between sample types due to the relatively high concentrations of phosphorus in the mixed liquor wastewater samples (Fig. 1). TP concentrations increased in mixed liquor because of increased PP (PP concentrations: infeed = 3.5 ± 1.6 mg/L; mixed liquor = 17.3 ± 12.3 mg/L) and OP (OP concentrations: infeed = 7.1 ± 3.1 mg/L; mixed liquor = 21.0 ± 13.7 mg/L). These results are attributable to P contained in the sludge used to seed the reactor to maintain the concentration of mixed liquor suspended solids at 4000-4500 mg/L and the sludge volume index at

100-150 mL/g. The sludge was preferentially sampled when the mixed liquor was extracted from near the bottom of the reactor. Overall, P concentrations decreased by approximately 24% as wastewater passed through the bioreactor.

3.2 Nitrogen analyses

N concentrations decreased by approximately 50% as wastewater passed through the bioreactor (Table 1); however, only NO_3 and TDN concentrations, and the ratio NO_3/TN (oxidative capacity of bioreactor), were statistically significant between sample types. Average concentrations increased from mixed liquor wastewater samples to supernatant wastewater samples for TDN (32% increase) and NO_3 (53% increase). This is in keeping with mixed liquor being sampled from low in the bioreactor where nitrification would be reduced and denitrification enhanced compared to more aerobic areas (Münch et al., 1996). Under such operating conditions an aerobic bioreactor should produce low concentrations of odorous compounds (Mudliar et al., 2010).

3.3 Other physicochemical analyses

Infeed TS was reduced by >50% while the salt load, as measured by EC, was reduced by 23%. The TS concentration was significantly lower between the supernatant wastewater sample and the other two wastewater types reflecting the sampling position for the mixed liquor wastewater and the role of the decant tanks in sequestering solids. The average pH in the infeed wastewater samples was basic (10.5 ± 1.6) suggesting that buffering agent was being sampled before complete mixing with the wastewater had occurred in the equalisation tank. Wastewater pH was

stabilised to approximately 8.5 in both mixed liquor and supernatant wastewater streams.

3.4 GC-MS analyses

Forty-nine compounds were tentatively identified as being present in the wastewater samples and grouped into different classes depending on functional groups (Table 2, S3). Most compounds are attributable to milk (i.e. fatty acids) or milk derivatives (e.g. indoles are formed from microbial breakdown of proteins and amino acids). Most fatty acids were present at the highest average concentrations in mixed liquor with the exceptions of octadecanoic acid that was highest in the infeed, and hexadecanoic, 2-hydroxyhexanoic, 9-hydroxydecanoic, 10-hydroxydecanoic, 2-octenoic and oleic acids which were higher in the supernatant. Dicarboxylic acid compounds were also found to be relatively higher in concentration in the bioreactor mixed liquor compared to the infeed or supernatant, and supports previous research that these compounds are either from materials used to upgrade the bioreactor in 2008 or lubricants and greases used to maintain the wastewater treatment system (Heaven et al., 2011).

Two phenolic compounds were identified which are from classes of compounds characterised as USEPA priority pollutants (Santana et al., 2009). The first compound, *p*-cresol, was found in all the wastewater samples and is produced from biodegradation of amino acids. It has been used as a marker compound for unprocessed wastewater contaminating lakes in Germany (Schüssler and Nitschke, 1999). On average, concentrations of *p*-cresol decreased by up to 66% from the infeed wastewater samples to the supernatant wastewater samples; however, on several occasions the concentrations of *p*-cresol in the mixed liquor wastewater samples were

an order of magnitude higher than infeed or supernatant wastewater samples. This suggests that *p*-cresol was produced in the bioreactor by anaerobic bacteria, at least at the time of sampling or is associated with the sludge rather than the liquid phase in the bioreactor.

The other pollutant, 4-nitrophenol, was intermittently detected in infeed and mixed liquor samples but was present in all supernatant samples, though at lower concentrations than in other wastewater samples taken on the same date. The presence of 4-nitrophenol is known to inhibit bacteria in bioreactors (Sponza and Kuscu, 2011). The source of 4-nitrophenol is difficult to ascertain as it is known to be produced from a variety of methods as for example, microbial products, as an airborne environmental pollutant, as a product of herbicide manufacture (see (Salehi et al., 2011) and references therein).

Another compound detected in the wastewaters that could adversely affect the bioreactor was dehydroabietic acid. This compound is often found in pulp mill effluent as it is a pesticide used by trees as a defence against insects (Patoine et al., 1997). It is unknown how dehydroabietic acid could get into dairy processing wastewater streams though the flora around the dairy processor is predominantly pine, a prime source of the compound. Dehydroabietic acid has been known to affect methanogenic anaerobic microbes in an anaerobic sludge bed reactor dealing with 12 g COD/L/d, two orders of magnitude more (120-400 mg COD/L/d) than what the wastewater system processes at Burra Foods (Patoine et al., 1997) contained. Dehydroabietic acid was usually at highest concentration in the mixed liquor

wastewater; however, during the summer of 2008-2009, it was only detected in the supernatant, perhaps indicating the seasonal nature of this compound.

3.5 Multivariate analyses

The analyses of the physicochemical data using PCA showed no clear separation between wastewater types with the first two principal components (PC-1, 33%; PC-2, 18%) explaining just over half the variation (Fig. 2). The PCA loadings reveal that the variation was mainly due to a separation of P from N measurements. Exceptions were DRP and ratio DRP/TP that were opposed to the TP analyses (TP, OP/TP, OP, PP and OP/DRP) on the loading plot. This suggests a possible inverse relationship between inorganic P and total P. This would be consistent with DRP being incorporated into the microbial biomass. For N, the PCA loading chart reveals a similar inverse relationship between NO_3 and NH_3 with NO_3 and NO_3/TN on the opposite side of the plot to NH_3, NH_3/TN and NH_3/NO_3. Again this suggests that mineralisation and nitrification are occurring (Münch et al., 1996). In contrast, PN was found to have no correlation with NO3- and NH3 as indicated on the PCA loading plot by PN being at right angles to both measurements. PN in dairy wastewater is likely to consist of undissolved macromolecular proteins from milk (such as caseins) or breakdown products from proteins from the bioreactors' microbial biomass (Lucey et al., 1996).

Initially, PLS-DA was used to identify which physicochemical parameters were best at predicting wastewater type (Fig. 3). The first factor predicted 32% of the separation of the wastewater samples and accounted for 27% of the variation while the second factor predicted 17% of the separation and accounted for a further 26% of the variation. Most P analyses (with the exception of DRP, DRP/TP and TDP) clustered

around the mixed liquor predictor. The predictor for infeed wastewater had its biggest contributors from NO_3, NO_3/TN, TDN, and pH analyses. This presumably relates to the denitrification of nitrate in the bioreactor. Nitrate and dissolved N compounds are probably clustered around the infeed wastewater due to nutrients from residual milk. The PLS-DA also shows that the analyses involving NH_3 describe mixed liquor and supernatant wastewater types roughly equally with NH_3/NO_3, NH_3/TN and NH_3 clustered together between both predictors. Mixed liquor or supernatant analyses were removed as predictors to see which output would show the greatest variation in comparison to the infeed wastewater samples. Using just infeed and supernatant analyses as predictors did not produce a clear separation between the two types of wastewater samples (Fig. S2); however, after removing the supernatant analyses as a predictor and including the trace organic compound analyses, PLS-DA showed a clear clustering and separation between infeed and mixed liquor wastewater sample types (Fig. 4, top). Focussing on just infeed and mixed liquor provided stronger predictions of the variation than with PLS-DA of all three wastewater types (Fig. 3). The first factor predicted 19% of the separation and 67% of the variation between the two wastewater types, and the second factor predicted a further 9% of the separation and another 24% of the variation.

The PLS-DA loading plot revealed that the greatest proportion of separation between the two wastewater types came from physicochemical analyses rather than trace organic compounds (Fig. 4, bottom). Dissolved nutrients in the wastewater stream were found to be the best for discriminating infeed wastewater samples. Nitrate measurements (NO_3, NO_3/TN) were well correlated with the infeed wastewater samples as would be expected for dairy waste that contains a high percentage of

amino acids from milk. Other dissolved components of the physicochemical analyses (DRP, TDP and TDN) are also in the same quadrant as infeed wastewater samples. For P analyses, TP and PP and associated analyses clustered around the mixed liquor wastewater predictor; however, there were some differences between PLS-DA of the three wastewater types (Fig. 3) and when just infeed and Mixed liquor were used as predictors (Fig. 4). For instance, NH_3 was found in the upper left quadrant adjacent to both infeed and mixed liquor wastewater predictors, indicating little correlation between either type of wastewater sample; however the related ratio NH_3/NO_3 that indicates reductive versus oxidative activity of the bioreactor had moved relative to the initial PLS-DA of all three wastewater types (Fig. 3, between the mixed liquor and supernatant predictors) to be clustered with the phosphorus analyses next to the mixed liquor wastewater predictor (Fig. 4, bottom). This emphasizes that ammonia is forming in the bioreactor (average for mixed liquor: 2.5 1.7 mg/L) rather than being transported from the infeed wastewater (average for infeed: 1.5 1.3 mg/L). Likewise, DRP/TP, which was situated between infeed and supernatant wastewater samples in the PLS-DA of all three wastewater types, was the closest point to the infeed wastewater predictor when only two wastewater types were modelled. The means for DRP concentrations are similar for infeed wastewater samples (9.4 ± 10.3 mg/L) when compared to mixed liquor (8.1 ± 4.5 mg/L) or supernatant (7.1 ± 3.9 mg/L) wastewater samples therefore the correlation between infeed and DRP/TP is because of TP. Evidence for TP being the cause of the correlation is that OP (= TP – DRP) is also correlated to the mixed liquor predictor due to the average TP (29.1 ± 14.0 mg/L) of the mixed liquor samples being greater than three-fold more concentrated than DRP (8.1 ± 4.5 mg/L).

Most organic compounds located on the PLS-DA loading chart were in quadrants adjacent to both wastewater types and therefore were not strong contributors to either wastewater predictors (Fig. 4, bottom); however, several compounds were in the same quadrant as either wastewater types, indicating some correlation. Besides the phenolic compound *p*-cresol, compounds that correlated with the infeed wastewater predictor were either fatty or benzoic acids. The fatty acids included nonanoic and octanoic acid. Nonanoic acid is a minor component of milk fatty acids (Moate et al., 2007) and forms when cheeses are made *via* bacterial processes (Mulet et al., 1999). Octanoic acid, a component of milk fatty acids, was also loosely correlated to the infeed wastewater predictor (Moate et al., 2007). Hydroxylated fatty acids, 3-hydroxyoctanoic, 2-hydroxyhexanoic and 2-hydroxyheptanoic acids, were also correlated with the infeed wastewater. This suggests that some oxidation of milk fatty acids may have occurred prior to wastewater entering the bioreactor, so it is reasonable to suspect these compounds are from milk residues, though whether this occurred within the cow or during milk processing could not be determined (Kandel et al., 2006). Likewise, the benzoic acid-like compounds correlated with the infeed wastewater may be attributed to residue milk waste that has entered into the wastewater treatment plant. For example, the conversion of cyclohexanecarboxylic acid to benzoic acid occurs in the liver of mammals, and both compounds have been found in milk residue (Baltes et al., 1952) or as an additive from the processing of dairy products like yogurt or cheese. Similarly, 4-hydroxybenzoic acid has been detected in milk from cows eating ryegrass silage, a common practice used on Australian farms (Besle et al., 2010), while, hydrocinnamic acid is an intermediate of the degradation of phenylalanine to benzoic acid (Sieber et al., 1995).

In contrast to the infeed wastewater, the mixed liquor wastewater predictor was correlated with fewer trace organic compounds. Besides 9-tetradecenoic acid, the compounds were very similar in structure (Fig. 5). Each compound consists of a central phenyl group with a hydroxyl and/or carboxylic acid ligand plus an extra ligand.

The USEPA priority pollutant 4-nitrophenol was found to have the strongest correlation with the mixed liquor wastewater predictor for all trace organic compounds. Being in the same quadrant and clustered with OP/DRP and TP suggests that 4-nitrophenol may be present due to degradation of organophosphate compounds that were formed within or entered the bioreactor and/or 4-nitrophenol is recalcitrant to degradation in the bioreactor (Sponza and Kuscu, 2011). As 4-nitrophenol is also closely correlated to the ratio NH_3/NO_3, further research may be warranted into determining if 4-nitrophenol could be used as a marker for anaerobicity of the aerobic bioreactor. Different species of anaerobic bacteria working in a concerted manner are required to degrade nitro-aromatic compounds (Kulkarni and Chaudhari, 2007). The correlation between NH_3/NO_3 and 4-nitrophenol could then be due to nitro-aromatic compounds acting as a feedstock for the anaerobic bacteria, thus contributing to the bioreactor's anaerobicity.

The other aromatic compounds found in the same quadrant as the mixed liquor wastewater predictor are probably degraded milk products. They may be break down products from polyphenols that have been detected in milk and degraded by the bacteria in the bioreactor (Besle et al., 2010). In the case of benzaldehyde, the compound could be unchanged from dairy farms as this compound is an indicator of

ryegrass or silage-fed cows. While one of the compounds, salicylic acid, is a well known pharmaceutical and could be a contaminant in the wastewater treatment system, the compound is also a common plant biosynthetic metabolite (Hayat et al., 2010).

The fatty acid 9-tetradecenoic acid was the only compound with some correlation to the mixed liquor wastewater predictor that was not structurally related to the other compounds. 9-tetradecenoic acid has antibacterial qualities, making it recalcitrant to microbial degradation (Desbois and Smith, 2010). This compound may be a representative of the initial infeed wastewater organic components that survived after one hour in the bioreactor at the time of the mixed liquor wastewater sampling. Evidence for this is that the average concentrations of 9-tetradecenoic acid were similar between all three wastewater types (Table S3).

3.6 Wilcoxon rank sum test and Mixed Model analysis

The non-parametric Wilcoxon rank sum test was used to compare the physicochemical and GC-MS data sets without the assumption of normality. This test was used rather than the more typical Student's T-test as GC-MS data are typically not normally distributed due to the absence or low concentrations that can and often do occur and the potentially highly variable input from dairy processor infeed wastewater which is typical for the industry (Demirel et al., 2005). Note that as the GC-MS data were sample-normalised, the Wilcoxon test was designed to detect differences between sample types in the relative abundance of each chemical compounds. The physicochemical data was also sample normalised through calculating ratios of the measurements of P and N (i.e.: NO3/TN, NH3/TN,

NH3/NO3, OP/DRP, DRP/TP and OP/TP, Supplementary Table 1). The test was performed to compare and corroborate the findings from the multivariate analyses with comparisons considered significantly different at P 0.05 (Fig. 6). The Wilcoxon test was also compared with a Mixed Model Analysis to investigate whether there were any seasonal interactions with sample type (Table S4). Unlike the Wilcoxon rank sum test, the Mixed Model analysis allowed for the investigation of significant differences in the wastewater samples between seasons, between sample type, and the interaction of season sample type.

Generally for the Wilcoxon rank sum test, significant differences between wastewater types followed the clustering of physicochemical analyses around the wastewater predictors in the PLS-DA. Only PP was significantly different between all three wastewater types ($P < 0.001$ for both infeed and supernatant wastewater samples versus mixed liquor wastewater samples) with mixed liquor wastewater samples having a greater average concentration of PP than infeed or supernatant wastewater samples ($P = 0.035$ for infeed versus supernatant wastewater samples) (Table 1). All other phosphorus measurements were significantly different between mixed liquor wastewater samples versus infeed and supernatant wastewater samples, further evidence that phosphorus analyses are the greatest contributors to the mixed liquor wastewater samples as seen in the PLS-DA analyses (Fig. 3, 4). Likewise, the Wilcoxon rank sum test found that the ratio for inorganic phosphorus (DRP/TP) was also significantly different ($P = 0.01$) for both infeed and supernatant wastewater samples versus mixed liquor wastewater samples. This corroborates the PLS-DA data (Fig. 3, 4) where DRP/TP was inversely correlated with the mixed liquor predictor. For infeed wastewater sample concentrations, pH, NO_3 and TDN, and the ratio NH_3/NO_3 were significantly different between infeed versus mixed liquor and

supernatant wastewater samples, the same parameters that were correlated with infeed wastewater predictor in the PLS-DA; however an exception was the ratio NO_3/TN which was only significantly different between infeed and mixed liquor wastewater samples (P = 0.005). This difference between the PLS-DA and Wilcoxon rank sum test may be due to the large variation in individual sample concentrations, especially in the mixed liquor and supernatant samples for NO_3 (Table 1, Coefficient of Variation for nitrate: mixed liquor = 204%; supernatant samples = 170%).

The Mixed Model analysis identified similar significant differences between physicochemical parameters as had been found with the Wilcoxon rank sum test (Fig. 6). The only significant difference between sample types that was found contrary to the Wilcoxon rank sum test was for TDP (P = 0.041). The only seasonal effect was for TDP (P = 0.004) and TP (TP = 0.013). Boxplots shows that winter samples for TDP (Fig. 1) and TP (Fig. S3) had lower mean concentrations compared to the other seasons. There were also significant season sample type interactions for TP, pH, NH_3 and TDP, emphasizing the highly variable wastewater solutions that were sampled.

Using the Wilcoxon rank sum test for the trace organic compounds only 9-tetradecenoic acid was significantly different between the supernatant water samples and the infeed or mixed liquor wastewater samples. Interestingly, 9-tetradecenoic acid was not significantly different between infeed and mixed liquor wastewater samples, thus providing further evidence of its recalcitrance to degradation by the bioreactor microbes due to its antibacterial activity (Desbois and Smith, 2010).

Another five trace organic compounds were significantly different between the infeed and mixed liquor samples only using the Wilcoxon rank sum test. The compounds *p*-cresol, 2-hydroxyheptanoic acid and nonanoic acid, were also correlated with the infeed wastewater samples in the PLS-DA loading plot (Fig. 4, bottom). Sebacic acid, which is extracted from castor oil plants and used as a plasticizer, can be assumed to have come from the bioreactor construction material rather than environmental sources (Ogunniyi, 2006). The last compound, 3-phenyllactic acid, is a potent antibacterial agent produced within dairy products (Dieuleveux et al., 1998). Except for *p*-cresol, there is an increase in average concentration of the compounds when going from the infeed to the mixed liquor samples. They then decrease again when going from mixed liquor to the supernatant samples. This seems to suggest then that these compounds have sources within the mixed liquor wastewater, either from recalcitrance to degradation and concentration in the sludge, production of the compounds from microbes in the bioreactor or leaching from the treatment plant (e.g. pipes), as in the case of sebacic acid.

4. Conclusion

Applying multivariate analyses to dairy processing wastewater samples has revealed that nutrient P and N have their greatest contribution to the infeed and mixed liquor sections of the wastewater treatment process respectively. Most identified trace organic compounds are independent of the composition of the infeed or mixed liquor wastewater stream, though compounds like *p*-cresol are loosely related to infeed wastewater, while others like 4-nitrophenol are related to the mixed liquor wastewater samples. In particular, the clustering of 4-nitrophenol and the NH_3/NO_3 ratio may allow for anaerobicity of the bioreactor to be monitored using this compound as a

marker. Generally, this study shows that multivariate statistical analyses, commonly used in metabolomics, may be used to identify compounds within wastewater systems that can indicate problems occurring within bioreactors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Fig. 1. Boxplots of selected physicochemical analyses of the dairy processing wastewater sampled at Burra Foods.

Fig. 2. Principle Component Analysis (PCA) of physicochemical data of wastewater samples (Red = infeed; Green = mixed liquor; Blue = supernatant). PCA loading relating physicochemical analyses to wastewater samples is depicted as red lines leading to the physicochemical parameter from the origin (Note: software depicts forward slash as a full stop e.g. DRP/TP = DRP.TP).

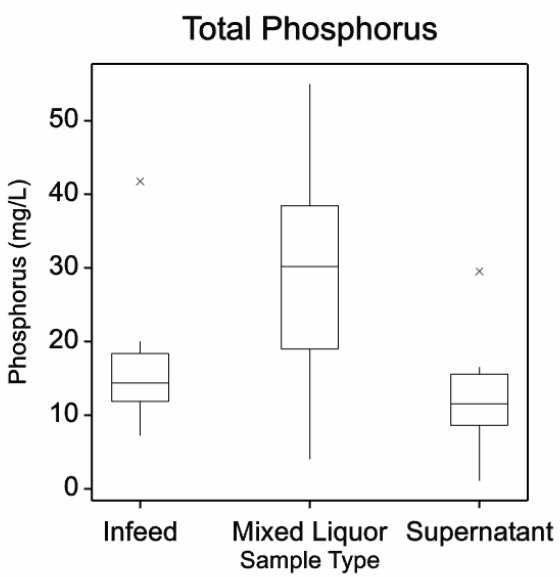
Fig. 3. Partial Least Squares-Discriminant Analysis (PLS-DA) loading plot of physicochemical data plotted with each wastewater type.

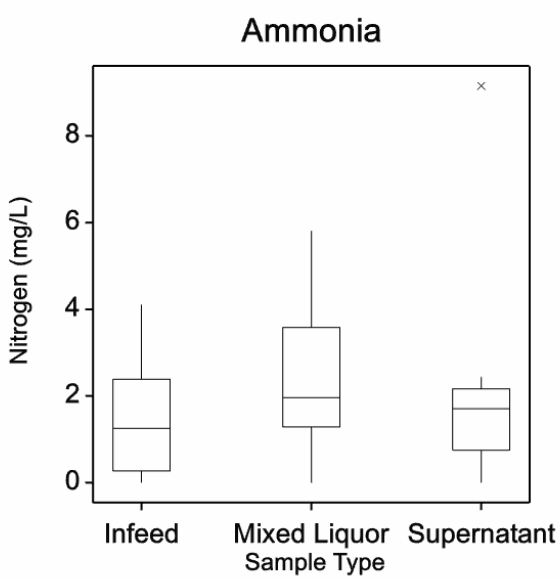
Fig. 4. (top) PLS-DA scores plot of infeed (red square) and mixed liquor (green square) wastewater samples. (bottom) Loading plot of PLS-DA depicting what physicochemical analyses and trace organic compounds contribute to separation of infeed and mixed liquor wastewater samples. Letters represent compound classes defined in Table 2.

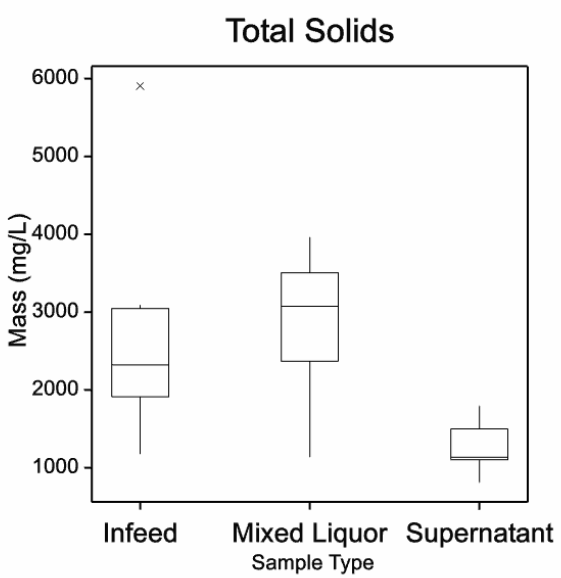
Fig. 5. Structures of compounds clustered in the same quadrant as the mixed liquor wastewater predictor in the PLS-DA loading plot (Fig. 4, bottom). Top (from Left to Right): benzaldehyde, 4-hydroxybenzaldehyde, 4-hydroxybenzonitrile, salicylic acid, 4-nitrophenol; Bottom: 9-Tetradecenoic acid.

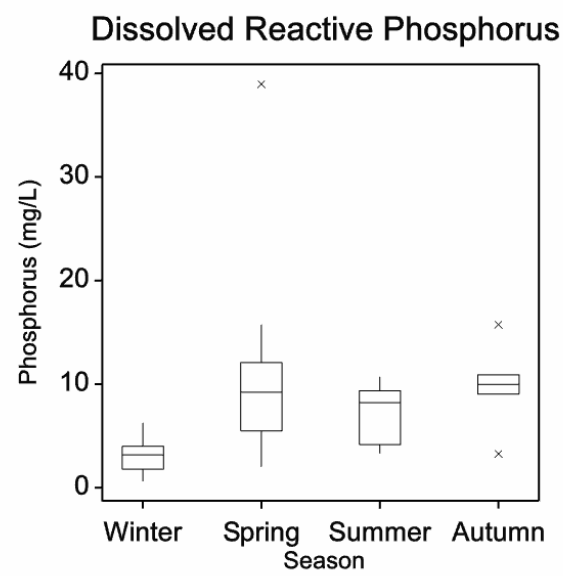
Fig. 6. Wilcoxon rank sum test detailing compounds and physicochemical analyses that were significantly different between wastewater types ($P \leq 0.05$; between infeed and mixed liquor wastewater samples = open diamond; between infeed and supernatant wastewater samples = open square; between mixed liquor and supernatant

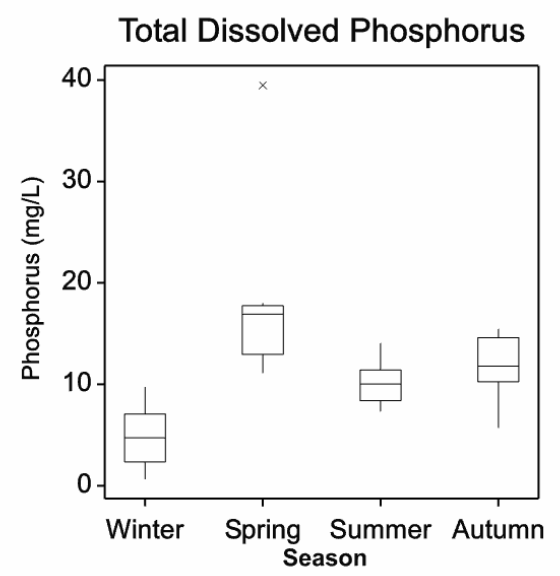
wastewater samples = open triangle). Bottom axis arranged in order of significance in regards to infeed and mixed liquor wastewater samples.

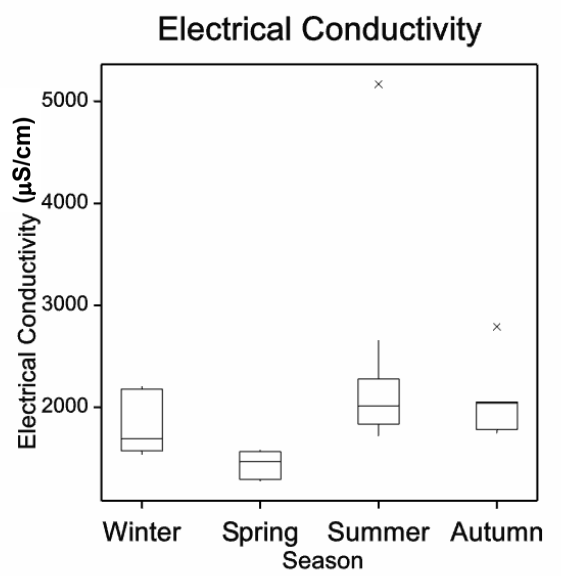


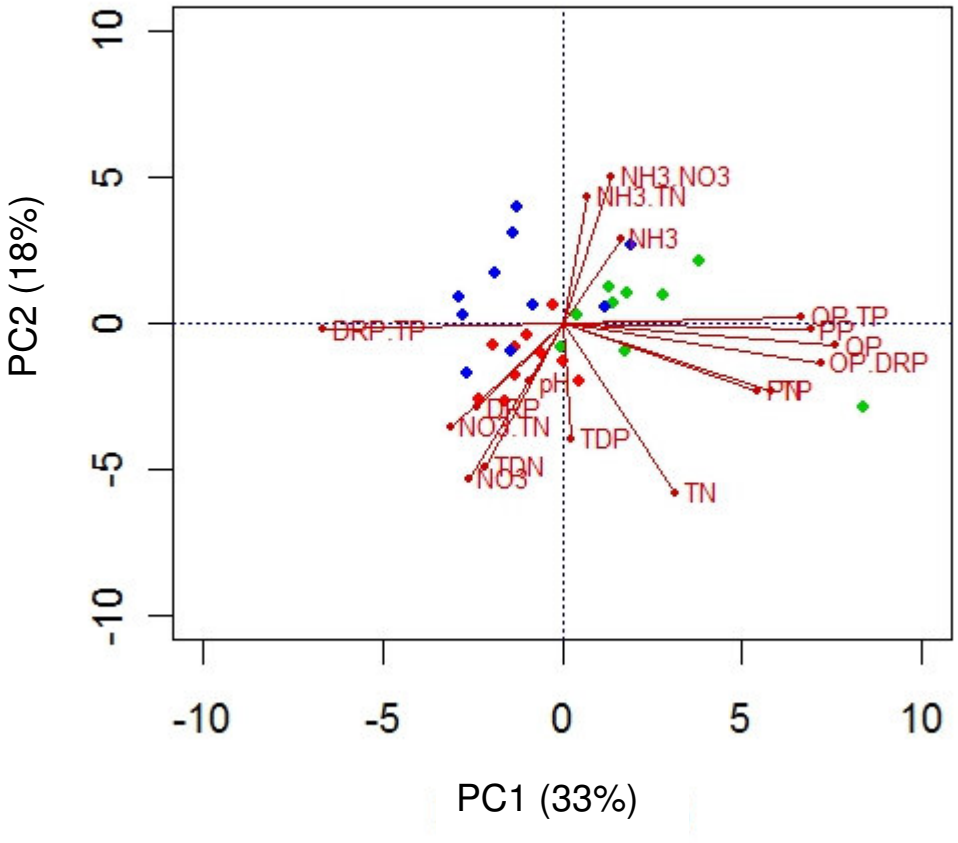


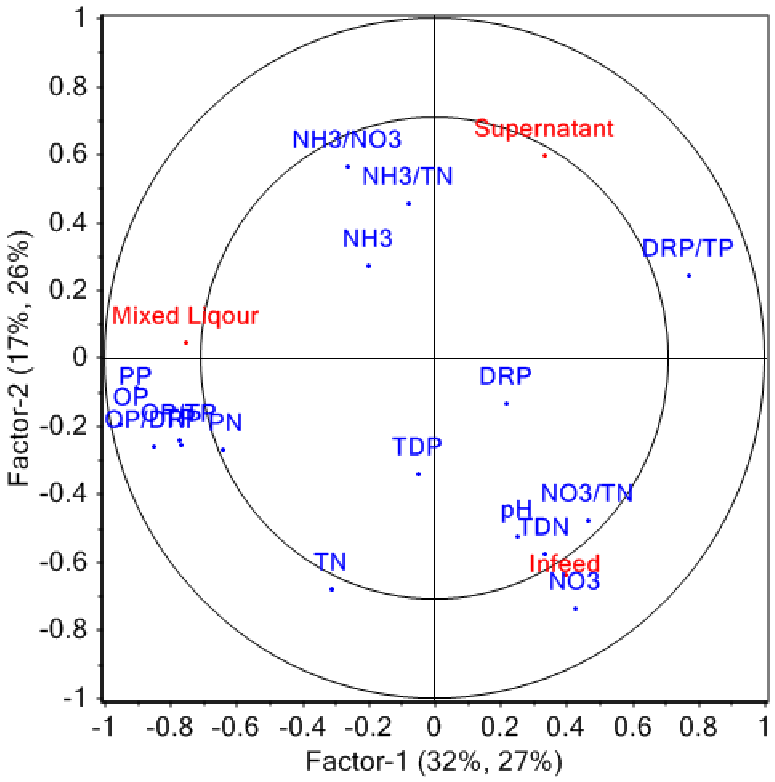


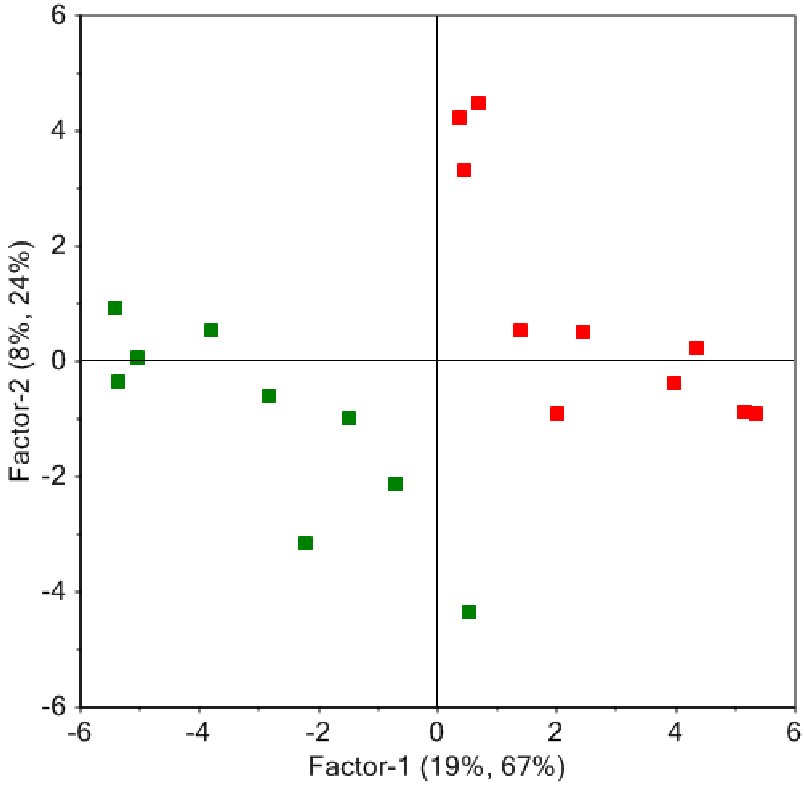


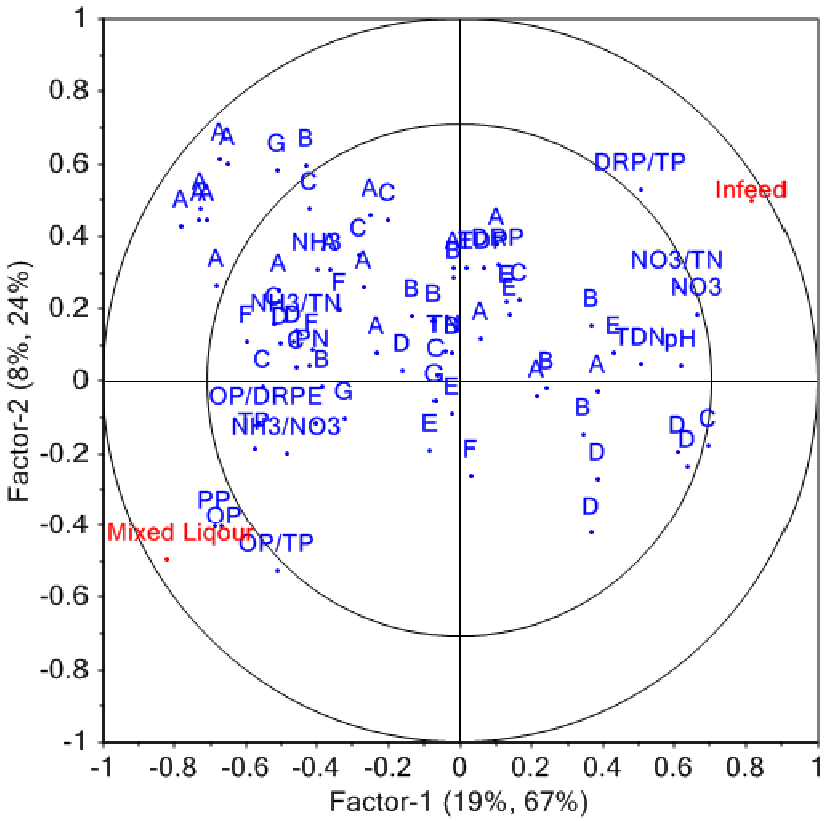


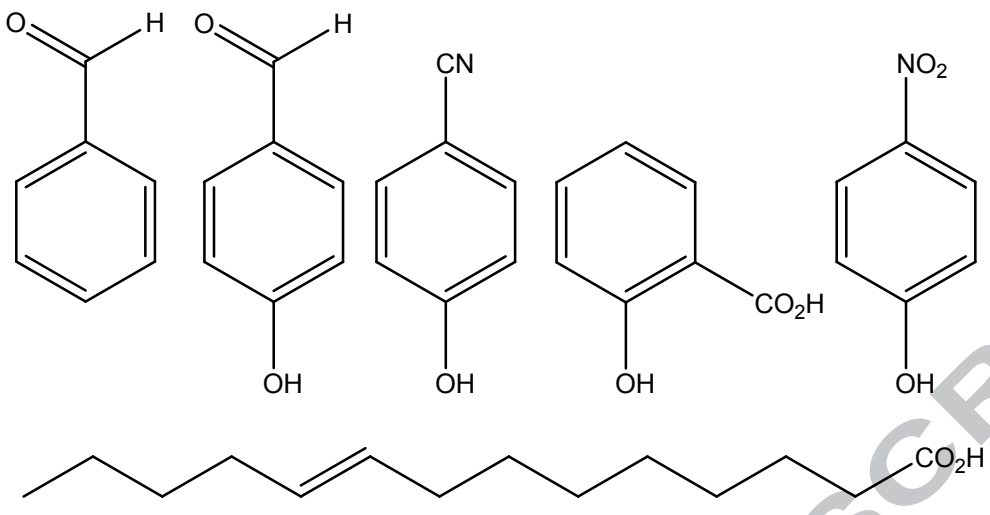


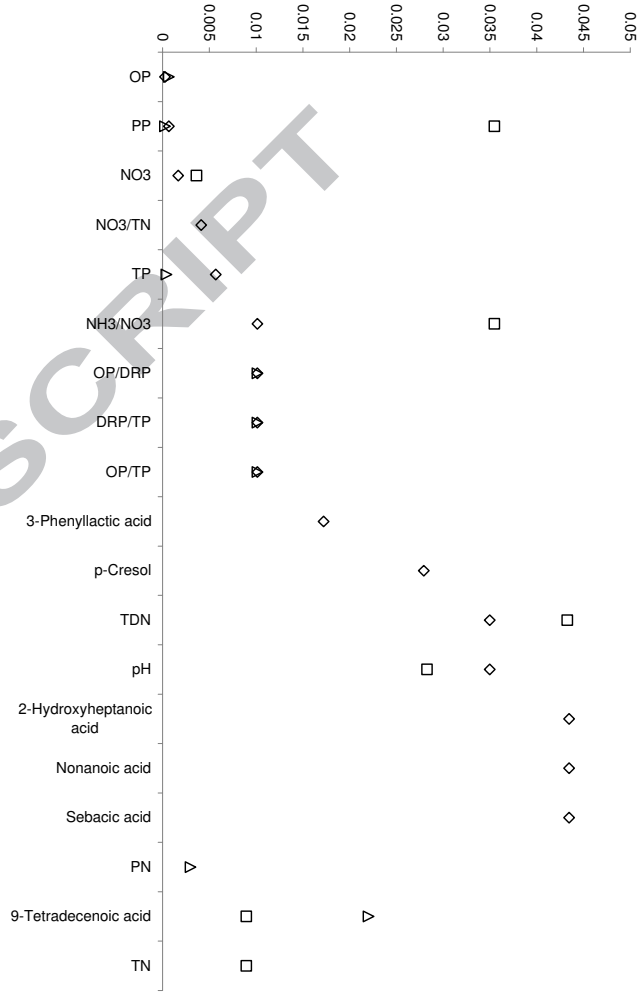












Highlights

- Dairy processing wastewater is analysed for indicators of bioreactor performance
- Physicochemical and GC-MS analyses is combined using metabolomic techniques
- Phosphorus concentrations is related to bioreactor mixed liquor
- Nitrogen concentrations are related to infeed into mixed liquor
- 4-nitrophenol is correlated to analyses relating to anaerobicity (e.g. NH_3/NO_3)

Table 1

Average, Standard Deviation (SD) and Coefficient of Variation (%CV) of chemical properties analysed of the wastewater sampled at Burra Foods dairy processor from August 2008 to July 2010.

	Chemical Property[a]									
Sample	DRP mg/L	TDP mg/L	TP mg/L	TDN mg/L	TN mg/L	NH_3 mg/L	NO_3 mg/L	TS mg/L	EC S/cm	pH
Infeed										
Average	9.4	13.0	16.5	47.7	55.9	1.5	19.6	2641	2273	10.3
SD	10.3	9.3	9.2	24.9	22.5	1.3	14.3	1240	1077	1.6
%CV	109	72	56	52	40	85	73	47	47	15
Mixed Liquor										
Average	8.1	11.7	29.1	16.2	48.3	2.5	3.1	2893	1781	8.5
SD	4.5	4.8	14	17.7	45.7	1.7	6.3	918	300	0.5
%CV	56	41	48	109	95	70	204	32	17	6
Supernatant										
Average	7.1	10.3	12.2	21.4	25.9	2.1	5.9	1268	1758	8.5
SD	3.9	5.2	7.6	25.2	25.2	2.5	10.1	299	295	0.3
%CV	55	50	62	118	97	121	170	24	17	4

[a]DRP = Dissolved Reactive Phosphorus, TDP = Total Dissolved Phosphorus, TP = Total Phosphorus, TDN = Total Dissolved Nitrogen, TN = Total Nitrogen, TS = Total Solids, NH_3 = Ammonia, NO_3 = Nitrate, EC = Electrical Conductivity

Table 2

Compounds tentatively identified within wastewater samples from Burra Foods dairy processor sampled from August 2008 to July 2010 (Mass spectral match: R^2 80%, NIST/EPA/NIH 2005 mass spectral library).

	Tentative Identity	Average Retention Time (min)	Average Concentration (count, $\times 10^4$)		
			Infeed	Mixed Liquor	Supernatant
A	**Fatty Acids** (saturated)				
1	Hexanoic acid	6.46	317	640	324
2	Octanoic acid	7.68	299	388	277
3	Nonanoic acid	8.14	30	73	50
4	Decanoic acid	8.74	317	660	270
5	Dodecanoic acid	9.63	93	248	122
6	Tridecanoic acid	9.99	15	47	15
7	Tetradecanoic acid	10.48	104	370	97
8	Pentadecanoic acid	10.86	17	71	24
9	Hexadecanoic acid	11.34	69	14	125
10	Octadecanoic acid	12.06	90	23	45
B	**Hydroxylated Fatty Acids** (saturated)				
11	3-Hydroxypentanoic acid	7.46	38	76	24
12	2-Hydroxyhexanoic acid	7.46	22	15	33
13	2-Hydroxyheptanoic acid	8.18	8	20	11
14	3-Hydroxyoctanoic acid	8.33	23	35	26
15	3-Hydroxydecanoic acid	9.65	34	75	32
16	9-Hydroxydecanoic acid	10.04	6	7	8
17	10-Hydroxydecanoic acid	10.36	53	29	75
18	3-Hydroxydodecanoic acid	10.46	52	99	42
19	3-Hydroxytetradecanoic acid	11.23	11	18	12
C	**Non-Linear Fatty Acids** (saturated)				
20	Benzeneacetic acid	7.92	118	334	118
21	Hydrocinnamic acid	9.16	326	687	251
22	4-Phenylbutyric acid	9.28	35	74	21
23	3-Phenyllactic acid (unsaturated)	9.38	234	267	215
24	2-Octenoic acid	8.02	14	34	70
25	Cinnamic acid	8.60	20	37	23
26	9-Tetradecenoic acid	10.52	60	72	54
27	Palmitoleic acid	11.27	33	167	48
28	Oleic acid	11.98	9	22	23
D	**Di-carboxylic Acids** (saturated)				

29	Suberic acid	9.90	3	8	4
30	Azelaic acid	10.31	49	71	53
31	Sebacic acid	10.72	17	26	20
32	1,11-Undecanedioic acid	11.13	32	48	40
33	1,12-Dodecandioic acid	11.49	21	24	17
34	1,14-Tetradecandioic acid	12.19	11	47	19
E	**Phenols**				
35	*p*-Cresol	7.02	35	28	12
36	Cyclohexanecarboxylic acid	7.34	49	32	49
37	4-Hydroxybenzaldehyde	8.40	21	36	18
38	4-Hydroxybenzonitrile	8.46	121	198	89
39	Salicylic acid	9.03	53	70	70
40	4-Nitrophenol	9.11	27	64	14
41	4-Hydroxybenzoic acid	9.60	6	35	5
42	4-Hydroxybenzene acetic acid	9.63	13	27	2
F	**Indoles**				
43	1H-Indole-5-carboxylic acid	11.01	29	48	23
44	1H-Indole-1-acetic acid	11.09	69	146	37
45	3-Indoleacetic acid	11.10	225	737	43
46	3-Indolelactic acid	11.82	138	187	35
G	**Other**				
47	Benzoic acid	7.67	604	871	652
48	Benzaldehyde	8.37	5	31	11
49	Dehydroabietic acid	12.70	8	42	18

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