Using metabolomic methods in the Victorian Dairy industry to understand the importance of organic nitrogen: from factory to farm.

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Abstract
Metabolomic techniques were used to identify metabolites that are produced and used in the dairy industry. Organic nitrogen (OrgN) compounds were identified in dairy factory wastewater streams. OrgN metabolites were predominantly found in the effluent stream and successfully segregated within the factory from recycled water streams used for irrigation and replenishment of a nearby waterway. Tolytriazoles were identified in the effluent waste water. Due to their recalcitrant nature, they have the potential to act as a marker of downstream pollution. A time based study of the dairy factory bioreactor waste waters identified another potential marker of factory productivity. The OrgN metabolite, 4-nitrophenol was found to be correlated with increasing anaerobicity of the bioreactor. The methodologies optimised from this research were used to identify OrgN metabolites in soil samples from farms in the main dairy regions of Victoria. Amino acids were the largest component of all metabolites identified. Several metabolites (e.g. cytidine) were found to be significantly changes in concentration in response to increasing potassium fertiliser application rates. These metabolites may be related to microbial or plant biochemical metabolic pathways. Microbial community analyses showed similar trends in regards to microbes (archaea, bacteria) associated with N metabolite production.

Keywords
Dairy factory, Dairy farm, Metabolites, Organic nitrogen, Microbes, Mass spectrometry

Introduction
The dairy industry is one of Victoria's largest exporters. The nexus of cows, plants and soil means that the dairy industry is a large user and producer of nitrogen (N). Inorganic N, N compounds that do not contain carbon atoms, is of major interest in the industry. They are generated as products of attempts to improve such things as pasture yield or animal productivity (i.e. more milk). Inorganic N compounds include ammonia (NH_3) and nitrate (NO_3^-) found in the ground or waterways, and gaseous products like nitrous (N_2O). However, there are also a great variety of organic N compounds (OrgN) whose identity and role is less elucidated.

Metabolomic methodologies were undertaken to identify OrgN. Untargeted analyses are a mainstay of metabolomics, where maximising the number of compounds (metabolites) collected with the minimal amount of adulteration to the sample is desired. Compared to a targeted analyses (e.g. analysing samples just for N containing compounds), this technique allows for associated metabolite data to be collected that can provide insight to a compound's interactions in the soil. Mass spectrometry combined with chromatography (liquid (LC-MS) or gas phase (GC-MS)) was used throughout all studies, and compared with physicochemical data using bioinformatics, multivariate statistical techniques that have recently come to prominence due to the increase in computing power available to analyse large data sets.

This paper details the research that has been recently undertaken to examine the various potential sources and sinks of OrgN in the dairy industry. Sampling for OrgN has been undertaken from numerous parts of the dairy industry including soil from dairy farms, wastewater streams from a dairy factory, and recycled water from the factory used to irrigate a nearby recreation oval. One goal of the research was to compare our findings with what is already found in the environment. That has included drinking, rain and water from a creek close to the dairy factory. The research presented focuses on the OrgN metabolites that were found, and the implications of the analyses.

Method

Initial sampling took place at a milk factory in Korumburra (Verheyen et al., 2009). The majority of the samples were collected from this factory over three years (Heaven et al., 2011, Heaven et al., 2012). The waste water samples were taken from: a) condensate, water taken from the milk after it has been dried to powder; b) "clean" water, composed of condensate and potable water; c) effluent, water taken from an aerobic bioreactor used to digest the waste products prior to disposal to municipal sewers. For comparison, samples were also from related water sources from around the factory, including potable water and water from the nearby Foster Creek that feeds the town of Korumburra.

A follow up study investigated the utility of metabolomic techniques in identifying biogeochemical transformation in soils from dairy farms (Heaven et al., 2015). A farm from each of the three main dairy regions of Victoria, Gippsland, Northern Irrigation Region (NIR) and South West Victoria was sampled. In combination with another experiment, plots had been prepared with various concentrations of N, phosphorus (P) and potassium (K) applied.

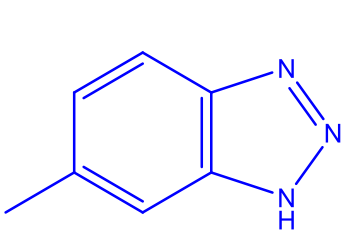
Physicochemical analyses of the samples consisted using flow injection analyses for water samples. Soil analyses (e.g. pH, EC, Total N) were conducted using traditional techniques at DEDJTR Ellinbank and Macleod laboratories. The untargeted analyses of metabolites used facilities at Federation University, Churchill and Metabolomics Australia, University of Melbourne. GC-MS was used for the dairy factory study. LC-MS analyses were conducted for the dairy farm study. The dairy farms soils were also analysed using microbial community analyses. These samples had DNA extracted at AgriBio by DEDJTR staff using standard microbial community analyses.

Discussion

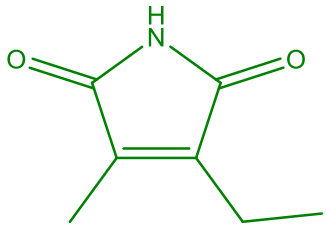
Dairy Factory

Initial sampling of the factory wastewaters identified that nitrogen concentrations were highest in the effluent, indicating that the aerobic bioreactor was struggling to digest all the N containing waste products. Evidence for this was a high correlation of total dissolved N vs. P ($R^2 = 0.99$) indicating a similar source of both nutrients (i.e. CN phosphoproteins). NH_3 were the largest component of inorganic N in the effluent, providing further evidence that anaerobic processes were dominating in a supposedly aerobic bioreactor.

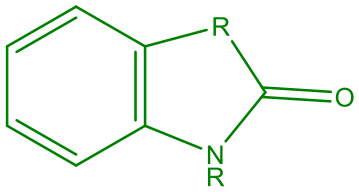
Table and Scheme 1. N metabolites and their concentrations in the dairy factory wastewater streams and other water streams



5-tolyltriazole



3-ethyl-4-methyl-1H-pyrrole-2,5-dione



2H-indol-2-one*

	Concentration (µg/kg)		
	5-tolyltriazole	3-ethyl-4-methyl-1H-pyrrole-2,5-dione	2H-indol-2-one (*2 isomers)
Clean		22	
Condensate		19	
Foster Creek		3.6	0.350
Effluent	730	32	330
Potable		31	

(N = Nitrogen atom; O = Oxygen atom; H = hydrogen atom; R = Methyl group or hydrogen atom)

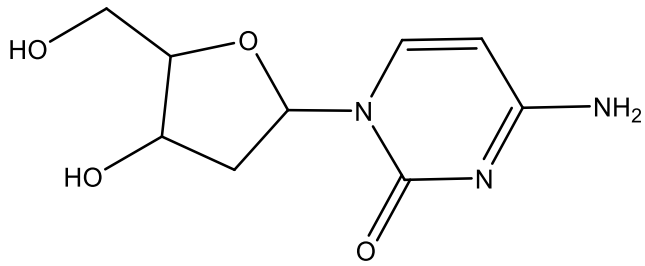
GC-MS identified only a single metabolite common to all water streams, both within and external to the dairy factory (Table and Scheme 1). The metabolite 3-ethyl-4-methyl-1H-pyrrole-2,5-dione is an analogue of maleimide, an aroma metabolite found in many plant species. Many indole compounds, such as 5-hydroxyindole (9.8 mg/kg), 1,3-dihydro-2H-indol-2-one (2.7 mg/kg), and 2,3-dihydro-1H-indole-1-

carboxaldehyde (23 µg/kg) were also identified. These metabolites are likely breakdown products of the amino acid tryptophan. Other N containing compounds of note were a veterinary drug, Dihydro-methoxy-methyl-1H-1,5-benzodiazepin-2-one, found in the effluent (54 µg/kg) and clean water (18 µg/kg) streams, and 5-tolyltriazole. This latter compound is a recalcitrant compound used as an anti-corrosion agent that could be used as a marker of downstream pollution.

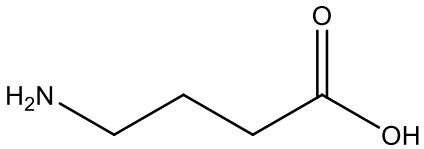
Another potential marker of bioreactor performance was identified when a time based study was undertaken of the factory. The metabolite, 4-nitrophenol, was found to correlate with physicochemical analyses of the wastewater streams related to anaerobic measurements (i.e. NH_3/NO_3^-). The strongest correlation between 4-nitrophenol and NH_3/NO_3^-) occurred in the mixed liquor wastewater, where batches of wastewater are digested. 4-nitrophenol is known to inhibit bacteria in bioreactors, its presence in the dairy factory potentially from degradation of organophosphate metabolites by microbes.
An important outcome of this research was the determination that the potential OrgN metabolites identified from this research were found in comparable concentrations upstream and upwind from the factory, or captured in the effluent stream. The GC-MS and associated physicochemical analyses were instrumental in showing that the condensate "wastewater" stream was clean enough to irrigate the nearby recreation oval, and after EPA approval, supply the nearby Foster Creek to help replenish its diminished flow.

Dairy farms
Two out of the three dairy farms were also found to have metabolites and microbial communities significantly different due to K application rates. The Gippsland soil samples had N containing metabolites concentrations of deoxycytidine halve and 4-aminobutanoate increase by 29% when K fertiliser rates were doubled (Figure 1). In the NIR, cytidine was reduced by 5% and aspartic acid concentrations quadrupled when K fertiliser rates were doubled. These metabolites appeared to be due to increases pasture growth with 4-aminobutanoate and aspartic acid associated with vitamin transformations (Pineau et al., 2008). The DNA related metabolites (cytidine and deoxycytidine) may have been reduced in concentration due to their consumption by microbes as a food source as increased fertiliser increased plant and microbial metabolism (Dell'Anno et al., 2002).

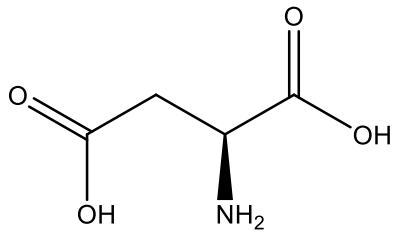
Amino acids ($R^1NH_2C{=}OR^2$: R = organic moiety) were the largest group of metabolites identified from the soil extracts. Plant based amino acids such as phenylalanine, hydroxyproline, tyrosine, taurine, L-threonine and L-cycloserine increased substantially to increasing P fertiliser, with hydroxyproline increasing concentration by an order of magnitude when P rates were doubled. Dairy related metabolites were also identified. They included cholesteryl sulfate, a major component of ruminant hooves (Wertz and Downing, 1984), and allantoin, a component of cow urine (Susmel et al., 1994) and milk.



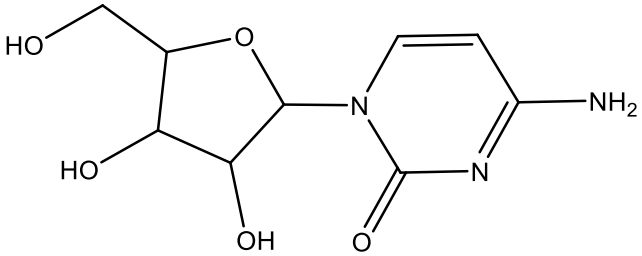
1. Deoxycytidine



2. 4-Aminobutanoate



3. Aspartic acid



4. Cytidine

Figure 1. Soil OrgN metabolites isolated from farm soils that were significantly different in concentration due to the amount of K fertiliser applied (P < 0.05).

Microbes that produce OrgN metabolites were also found to be affected by increasing fertiliser. For instance, in the case of archaea *nitrosopumilus*, that convert NH_3 to NO_3^-, increasing K fertiliser increased the concentrations of this microbe. In comparison ,the bacteria *nitrospira*, that oxidises nitrites and is a target for nitrification inhibitors, it was found that increasing concentrations of P fertiliser caused this microbe to increase in concentration. Further analyses correlating OrgN metabolites to microbes from this study is continuing.

Conclusions

OrgN is an important set of molecules that has until recently been grouped as difficult to identify in complex matrices as those found in soil and dairy factory water streams. These studies identify that the technologies used for metabolomics are suitable to explore OrgN identities and concentration of water and soil samples related to the dairy industry. In particular, the techniques are useful in studying changes in concentrations of N containing metabolites in response to a range of stimuli, be it anaerobicity of bioreactors or fertiliser application on dairy farms.

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