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Physicochemical and microbiological effects of long- and short-term winery wastewater application to soils

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ABSTRACT

Application of winery wastewaters to soils for irrigation of various crops or landscapes is a common practice in the wine industry. In this study, we sought to investigate the effects of this practice, by comparing the physicochemical and microbiological soil properties in paired sites that differed in having had a history of winery waste application or not. We also compared the effects of a single application of untreated winery wastewater, to application of treated winery wastewater (sequencing batch reactor) and pure water to eliminate the effects of wetting alone. Long-term application of winery wastes was found to have significant impacts on soil microbial community structure, as determined by phospholipid fatty acid analysis, as well as on many physicochemical properties including pH, EC, and cation concentrations. ^{13}C NMR revealed only slight differences in the nature of the carbon present at each of the paired sites. A single application of untreated winery wastewater was shown to have significant impacts upon soil respiration, nitrogen cycling and microbial community structure, but the treated wastewater application showed no significant differences to wetting alone. Results are discussed in the context of sustainable winery wastewater disposal.

1. Introduction

Sustainable management of soil and water resources is of increasing global concern. Irrigation of agricultural lands with wastewaters, following varying levels of treatment, is increasing around the world [1–3]. With projections of increased incidence and severity of drought [4], such practices are likely to become even more common. Wineries are no exception to this, generating 3–5 kL wastewater/tonne of grapes crushed [5]. The ultimate fate of WWW is dependent upon the exact location and situation of individual wineries; some wineries are able to discharge to municipal sewerage, however application of WWW is soil is very common in certain geographical areas [6]. Land disposal of winery wastewaters (WWW) is a potentially sustainable management practice, and could also be of significant benefit in water-poor winegrowing regions of the world, where it could be further utilised as an irrigation water source. There are, however, concerns regarding the contaminants that are present in untreated WWW, as well as those that may persist in the treated WWW with respect to their effects on soils, crop growth, and consumers of the crop [7]. Based on these concerns, there is a need to investigate the agricultural impacts of irrigation with WWW. WWWs vary depending on the specific winemaking operations taking place at any given time, as well as treatment processes in use (reviewed in [8]). Consequently, potential reuse opportunities for WWW need to be evaluated with this considerable variation in WWW composition in mind.

WWW arises mostly from cleaning operations and spillage within the winery, and therefore typically consists of wine, grape juice and solids (vintage season only) and cleaning agents (e.g. NaOH and KOH). The wastewater in most wineries contains high concentrations of organic compounds, predominantly sugars, organic acids (acetic, tartaric, malic, lactic, propionic), alcohols, esters and polyphenols [9] (see [8], for recent review). Inorganic ions present are predominantly potassium and sodium, with low levels of calcium and magnesium, although the concentrations of both organic and inorganic constituents vary with differences in winemaking operations over time, as well as between individual wineries [10–12]. The temporal variation in WWW composition makes management especially difficult, with sometimes quite dramatic differences occurring between vintage and non-vintage periods [13]. Given the combination of moderately high levels of both organic material and sodium/potassium salts in WWW, the

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long-term impacts of land application must be carefully considered to ensure that long-term damage to agricultural systems does not occur [14]. Furthermore, regulation of WWW management differs between sites and countries, with no best practices widely accepted.

The application of WWW to soils leads to the addition of organic material. Whilst moderate application of soluble organic carbon leads to increased soil fertility through conversion to soil organic matter [15], organic overloading can lead to blockage of soil pores [16] and can, therefore, be detrimental to soil health. In addition, introduction of high levels of salts to agricultural soils can lead to increased soil salinity and sodicity, which increases the risk of dispersion [17]. Whilst it has been shown that WWW can be phytotoxic [18,19], the impacts of WWW (untreated or otherwise) application on soil biological and physicochemical properties have not been investigated in detail.

For WWW to be sustainably disposed of on land, the impacts upon soil structure and essential processes such as nitrogen and carbon cycling need to be considered on both long- and short-term timeframes. Long-term application of municipal wastewater has been shown to impact upon mycorrhizal associations [20], and olive mill wastewater has been shown to impact on microbial community structure, in particular the ammonia oxidising bacteria [21]. The addition of organic and inorganic material would be expected to effect physicochemical changes on the soil, which would in turn impact upon soil microbial community structure, as has previously been shown following organic soil amendment [1,22,23]. Organic amendments provide an additional carbon source to microorganisms, and in this way, are likely to stimulate growth. In contrast, some organic molecules can also be toxic, and therefore are likely to reduce microbial growth. The inorganic components of WWW are also known to impact upon soils, with elevated EC and decreased pH values noted in previous studies [6].

One of the major difficulties in ascertaining long-term impacts of wastewater applications on soils is the fact that it often takes a number of years for effects to become evident. This may be compounded by progressive growth in the scale of these industrial processes over time; such is the case for a number of facilities found in the wine industry. As WWW is often applied to vines this leads to a risk to high value vineyards and also possibly grape and wine quality. Changes to microbial populations have been shown to be an early warning signal of ecosystem perturbations (Friedel et al. [22]), and therefore, of potentially unsustainable practices. Microorganisms play vital roles in nutrient and energy cycling, and are thus a critical component of any functioning ecosystem. The stability of a microbial population may be defined in terms of its resistance (ability to resist change) and resilience (ability of to recover from disturbance) [24]. Changes in soil microbial community structure following WWW application may provide valuable insight into the long-term sustainability of WWW application to soils. In this study, phospholipid fatty acid (PLFA) analysis has been used to assess the microbial community present under different WWW application regimes. In addition, ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy was utilised to assess the nature of the organic carbon present in the soils.

In this study, the short- and long-term effects of WWW application on the physicochemical and biological properties of vineyard soils were evaluated. Specifically, we assessed the effect of:

1. Long-term WWW application (30 years) on soil physicochemical properties and the chemical nature of carbon in soils; and
2. Short-term WWW application on soil microbial community dynamics and soil physicochemical properties.

Table 1
Characteristics of winery wastewaters used in field study.

Property (all in mg/L)	WWW	TWWW
Na	99	112
K	240	150
TOC	2,100	48
COD	13,000	610
Total phenols	0.19	<0.04

(Abbreviations: WWW, winery wastewater; TWWW, treated winery wastewater; TOC, total organic carbon; COD, chemical oxygen demand).

2. Methods

2.1. Study design

Physicochemical effects of long-term winery waste application to soils.

Field studies were conducted near Coldstream (Lat. 37.68°, Long. 145.43°, 83 m ASL) about 45 km ENE of Melbourne, Victoria, Australia, in May–June 2009. The region has cool winters (July mean maximum 13.2 °C, mean minimum 3.7 °C) and warm to hot summers (January mean maximum 27.7 °C, mean minimum 11.7 °C). Impacts of long-term land application of winery wastes on soil physicochemical properties were assessed on adjacent paired sites.

The surface soils at the site are dark grey-brown (10YR3/1 moist) silty clay loams of uniform texture to at least 1 m and formed on Holocene alluvium deposited by the Yarra River. The soil profile is classified as an Oxyaquic Haplustoll [25].

The first site had received winery wastes (liquid and solid) over a period of ca. 30 years, and is hereafter referred to as the acclimatised (acc.) site. The second site had never received any winery waste material, and is referred to hereafter as the non-acclimatised (n. acc.) site. The two sites are separated by a drainage ditch which ensured that there was no spill-over of wastewater into the n. acc. site. Vegetation at the sites was dominated by common weed and pasture species, including *Bromus* sp. and *Phalaris* sp. in the acc. site, and *Poa pretensis* and *Lolium* sp. in the n. acc. site.

Soils were collected from the acc and n. acc sites at four random locations within a ca. 25 m^2 area using an auger (6 cm diameter), from the 0–5 cm, 5–15 cm, and 15–30 cm soil layers. These soil samples were air-dried, and sieved to <2 mm diameter. An additional four samples from each site were collected from the surface layer (0–7 cm) and stored immediately at −20 °C for analysis of microbial communities by PLFA analysis (see below). Bulk density rings were also used to collect soil samples from the mid-points of each of the 0–5 cm, 5–15 cm, and 15–30 cm soil layers.

2.1.1. Study design: short-term effects of WWW application to acclimatised soils

Twelve experimental plots (1.5 m × 1.5 m) were randomly arranged in four blocks, in a ca. 15 m^2 area on the acc. site. Plots were protected from rainfall via free-standing plastic coverings. At the time of plot establishment, one PVC collar (10 cm) was inserted into each plot in a random location to allow for undisturbed soil respiration measurements to be taken throughout the course of the experiment (see below). The experimental plots were irrigated with one of three water treatments:

1. Untreated winery wastewater (WWW hereafter), collected prior to treatment from the winery on-site;
2. Treated winery wastewater (TWWW hereafter), collected after sequencing batch reactor treatment from the winery on-site; or
3. Distilled water (dH_2O hereafter).

Key characteristics of the WWW and TWWW are given in Table 1 (note that soil characteristics are as described in Table 2 for acc.

Table 2
Physicochemical soil properties in acclimatised and non-acclimatised vineyard soils.

Analyte	Units	Guideline[a]	Acclimatised			Non-acclimatised			ANOVA significance		
			0–5 cm	5–15 cm	15–30 cm	0–5 cm	5–15 cm	15–30 cm	Soil	Depth	Soil × depth
EC (1:5 extract)	μS/cm	150	289 (55)	156 (9.5)	106(15)	233 (35)	132 (9.0)	96 (4.7)	NS	***	NS
pH (1:5 extract)		6.5	5.6 (0.1)	5.6 (0.3)	5.0 (0.1)	4.9 (0.04)	4.7 (0.03)	4.9 (0.02)	***	NS	**
OM	%	>4.5	12.0 (0.7)	8.3 (1.1)	5.2 (0.2)	15.3 (1.1)	5.2 (0.07)	4.3 (0.07)	NS	***	**
C:N	ratio	10–12	10.9 (0.1)	11.0 (0.3)	11.0 (0.1)	11.5 (0.1)	11.1 (0.1)	10.8 (0.1)	NS	NS	NS
Bulk density[b]	g/cm		0.78	1.22	0.99	0.98	1.06	1.12	-	-	-
K	mg/kg	190	727 (18)	314 (98)	177 (54)	496 (8.7)	174 (60)	156 (32)	**	***	NS
Na	mg/kg	60	139 (12)	137 (16)	120 (11)	78 (6.2)	112 (11)	143 (18)	NS	NS	**
Mg	mg/kg	200	859 (17)	849 (65)	847 (58)	729 (53)	714 (9.6)	830 (43)	*	NS	NS
Ca	mg/kg	2150	4275 (55)	3565 (197)	2090 (656)	2249 (241)	1408 (186)	1455 (44)	***	**	*
Al	mg/kg	45	17 (7.6)	79 (6.0)	227 (67)	95 (50)	318 (14)	307 (14)	***	***	NS
Zn	mg/kg	5	12 (5.3)	4 (2.9)	0 (0.2)	2 (0.9)	0 (0.1)	0(0.1)	***	***	**
Mn	mg/kg	22	25 (14)	10 (4.4)	3 (1.3)	12 (2.2)	9 (3.0)	6 (2.8)	NS	**	**
Fe	mg/kg	22	376 (19)	344 (32)	238 (15)	417 (23)	243 (4.6)	179 (17)	***	***	***
Cu	mg/kg	2.0	2.8 (1.3)	1.4 (0.9)	0.3 (0.2)	0.2 (0.03)	0.1 (0.05)	0.2 (0.03)	***	**	**
B	mg/kg	1.7	1.5 (0.2)	0.9 (0.2)	0.7 (0.1)	1.0 (0.3)	0.6 (0.4)	0.4 (0.04)	**	***	NS

Values are means ± standard errors; $n = 4$ unless otherwise indicated.
Stars indicate relative levels of significance, with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.
(Abbreviations: EC, electrical conductivity; ANOVA, analysis of variance).
[a] Guideline values from analytical testing laboratory, www.scu.edu.au/schools/esm/eal.
[b] $n = 1$; no standard error term, or statistical analysis performed.

soils). The experimental plots were irrigated with 8 L of the appropriate water treatment using hand-held watering cans, over an eight-hour period. This approach, which was equivalent to application of 36 mL/ha, was consistent with industrial application rates at the site.

One day prior to water application (−1 days, hereafter), and 1, 3, 7 and 16 days after water application, four soil cores were collected from the top 7 cm of each experimental plot, using a 6 cm diameter auger. These four cores were then combined in the field to produce a composite plot sample at each sampling time, and then stored at 4 °C until further analysis (see below).

2.2. Chemical analysis

2.2.1. Solid-state ^{13}C NMR of soils

The nature of the soil organic matter in the soils collected from all depths from the acc. and n. acc. sites (long-term experiment only) was analysed by solid-state ^{13}C NMR as has been described previously [26]. Briefly, replicate soil samples (four from each soil/depth combination) were pooled to obtain a composite sample, and then air-dried and sieved (2 mm) prior to HF digestion. Carbon-13 cross polarisation with magic angle spinning (^{13}C CPMAS) NMR spectra were obtained at a frequency of 100.6 MHz using a Varian Unity INOVA 400 NMR spectrometer (Varian, Walnut Creek, CA, USA). Samples were packed in 7 mm diameter cylindrical zirconia rotors with Kel-F rotor end-caps and spun at the "magic angle" (54.7°) at 6500 ± 100 Hz in a Doty Scientific supersonic MAS probe (Doty Scientific, Colombia, SC, USA). Free induction decays (FIDs) were acquired with a sweep width of 50 kHz; 1216 data points were collected over an acquisition time of 12 ms. All spectra were zero-filled to 8192 data points and processed with a 100 Hz Lorentzian line broadening and a 0.010-s Gaussian broadening. Chemical shifts were externally referenced to the methyl resonance of hexamethylbenzene at 17.36 ppm. Spectra represent the accumulation of 4000 scans and were acquired using a 90° ^{1}H pulse of 5–6 μs duration, a 1 ms contact time and a 1 s recycle delay.

2.2.2. Soil physicochemical analyses

Analysis of sulphate, nitrate, ammonium and phosphorus were determined using a modified Morgan extract [27]. Electrical conductivity and pH were determined using a 1:5 soil:water extract (3A1 and 4A1, [28]). Percent organic matter was measured by combustion (modified standard method 6B3; [28]), and total C and N determined instrumentally (LECO CNS analyser). Micronutrients (Zn, Mn, Fe, Cu and B) were determined following a DPTA and hot $CaCl_2$ extraction (12A1, [28]). Ca, Mg, K, Na and Al were determined using an ammonium acetate equivalent extraction (15B1, [28]). Concentrations of H (15G1, [28]) was measured on a KCl extract to enable calculation of CEC (15J1, [28]). All physicochemical analyses were performed by the Environmental Analysis Laboratory (EAL) at Southern Cross University (SCU) (www.scu.edu.au/schools/esm/eal). Soil samples for bulk density measurement were oven-dried (105 °C) until a constant mass was achieved.

2.2.3. Inorganic N analysis

Nitrate and ammonium concentrations were determined on all soil samples collected from the short-term study. Triplicate soil samples (30 g moist soil) were taken, extracted with 2 M KCl, and inorganic N content determined colorimetrically using a modification of Miranda et al. [29] for NO_3^- (plus NO_2^-) and Forster [30] for NH_4^+. All spectrophotometric analyses were performed in Greiner 96 well plates, and analysed using a TeCan Evo Spectrophotometer (TeCan, Germany). Gravimetric moisture was determined after drying approximately 50 g moist soil samples at 105 °C for 48 h.

2.3. Biological analysis

Soil respiration was measured *in situ* in the short-term experiment at each sampling time point using a portable soil respiration unit (6400-09 Soil CO_2 Flux Chamber) coupled to a LiCor 6400 photosynthesis unit (Li-Cor, Lincoln, NE, USA).

PLFA analysis was performed on soils collected from both short- and long-term experiments (0–7 cm soil layer only, see above). Aggregates of approx 5 mm in diameter were collected and transferred to tubes for storage at −20 °C. Soil samples were extracted in duplicate, using a chloroform methanol solvent [31] modified to incorporate a citrate buffer [32], followed by transesterification of the polar lipid fraction containing the phospholipids [33]. Separation, quantification and identification of PLFA were done using a Varian CP 3800 gas chromatograph (Varian, Walnut Creek, CA, USA), fitted with a 5%phenyl 95%methylsiloxane column (Varian, USA) [34]. Peaks were identified and quantified by comparing with Supelco Bacterial Acid Methyl Ester (BAME) standard mix (product

number 47080-U, Supelco, USA), as has been described previously [35].

2.4. Statistical analysis

In the long-term study, differences between sites and depths were compared using mixed model analysis of variance (ANOVA) in SPSS 19.0 (SPSS Inc, Chicago, Illinois, USA). The mixed model approach allowed for repeated measures to be taken into account as required. Where the ANOVA revealed significant differences, individual means were compared using Tukey's HSD test [36].

In the short-term study, soil physicochemical and respiration data were also analysed using a two-way mixed model ANOVA, with water treatment and sampling time as factors in the analysis, in SPSS 19.0 (SPSS Inc, Chicago, Illinois, USA). As in the long-term study, where the ANOVA revealed significant differences, individual means were compared using Tukey's HSD test [36].

PLFA profile data from both the short and long-term study were first analysed using a multivariate analysis of variance (MANOVA), and, where the MANOVA result was significant based on Wilks-Lambda testing, canonical variate analysis (CVA) was performed using JMP version 8.0.2 (SAS Institute Inc., Cary, NC, USA). CVA is a form of discriminant analysis that maximises between group variation whilst minimising within group variation, and therefore maximises the ability to discriminate between groups in PLFA analysis [37]. Multivariate analysis was performed using data from all peaks identified at all sampling times. Canonical Correspondence Analysis (CCA) was used to investigate any relationships between the PLFA profiles and the environmental variables measured, however, no correlations were observed, and data is therefore not presented.

3. Results

3.1. Long-term application of winery wastes

Soil physicochemical properties differed between the acc. and n. acc. soils, irrespective of depth (Table 2, i.e. site main effect). Specifically, the concentrations of K and Mg were significantly ($p < 0.05$) higher in the acc. than n. acc. soils. Conversely, the concentrations of Al were significantly ($p < 0.05$) higher in the n. acc. soils. Other heavy metals, specifically Zn, Fe, Cu and B, were generally significantly increased in the acc. soils at all depths, in comparison to the n. acc. soils. Concentrations of Mn followed the same trends, but the differences were not significant due to high sample variability. Soil physicochemical properties also changed with depth, irrespective of site (i.e. depth was the main effect). The concentrations of K, and soil EC significantly ($p < 0.05$) decreased down the soil profile, and Al concentrations increased with increasing depth.

The concentration of organic matter, total C, total N, and Ca all differed between both sites and with depths, as indicated by a significant two-way interaction ($p < 0.05$) in the analysis; generally the concentrations of these species decreased with depth, with slightly differing patterns between the two sites. The pH decreased with depth in the acclimatised soil, whilst remaining fairly constant in the non-acclimatised soil. Conversely, H concentration remained fairly constant in the acclimatised soil and increased with depth in the non-acclimatised soil. Sodium concentrations showed completely opposite trends in the two soil types, decreasing with depth in the acclimatised soil whilst increasing with depth in the non-acclimatised soil.

^{13}C NMR spectra (Fig. 1) of soils from both acc. and n. acc. sites were generally similar. Characteristic prominent signals can be seen for alkyl carbon (0–45 ppm), and in the O-alkyl region, which includes contributions from carbohydrates, lignin, tannin and humic substances (45–110 ppm). A peak in the 100–110 ppm region is characteristic of anomeric carbons of carbohydrates. Aryl (110–140 ppm) signals are observed in all spectra, with a small peak due to O-aryl C (140–160 ppm) clear in only the spectra of the top (0–5 cm) soil layer. A prominent carboxyl resonance appears in the 160–190 ppm region of all spectra.

The most consistent variation with soil depth is an increase in the relative strength of the aromatic resonance, especially for the n. acc. soil. This is consistent with previous studies [38]), and can be attributed to the greater recalcitrance of aromatic structures to microbial degradation. The relative intensity of alkyl to O-alkyl peaks, which is commonly used as a measure of the degree of organic matter decomposition [39] did not vary greatly with depth and was surprisingly greatest at the intermediate depth (5–15 cm). There were no consistent differences between spectra of acc. and n. acc. soils. The general similarity of the NMR spectra is consistent with similarity in C/N ratios, which vary through a very narrow range of 10.7–11.5 (Table 2).

To assess shifts in soil microbial communities, PLFA profiles from both the acc. and n. acc. soils were also compared. Whilst the treatments appeared to separate on inspection, no significant differences were revealed in the multivariate analysis (MANOVA) due to a small sample:variable ratio (data not shown).

3.2. Impacts of a short-term application of winery wastewater to acclimatised soils

3.2.1. Physicochemical properties

Soil moisture content changed significantly in all treatments over time, with the maximum moisture content measured on the first day after water application, followed by a return to pre-irrigation levels three days after the irrigation event (Table 3). Concentrations of both nitrate and ammonium differed between water treatments through time (Fig. 2), as indicated by a significant two-way interaction ($p < 0.05$). Application of WWW resulted in an increase in soil ammonium concentrations, especially 7 days after treatment, whereas application of TWWW or dH_2O did not result in any significant differences to ammonium concentration over the timecourse. Similarly, soil nitrate concentrations reached their peak at 7 days, with WWW and TWWW both showing significantly elevated concentrations compared with the dH_2O treatment; the concentration of soil nitrate was not significantly affected by dH_2O application.

3.2.2. Biological analysis

Soil respiration generally increased one day after irrigation (Fig. 3), although only significantly ($p < 0.05$) so in the WWW treatment. Irrespective of changes between sampling times, there were also some differences in soil respiration profiles between the watering treatments, with WWW treatment resulting in a significantly different respiration pattern to treatment with dH_2O.

Analysis of PLFA profiles revealed distinct patterns in microbial communities between watering treatments (pooled over sampling times) (Fig. 4) and between sampling times (pooled over irrigation treatments) (Fig. 5). The microbial communities (as determined by PLFA profiles) in soils collected from soils irrigated with the different treatments were distinctly different (Fig. 4). There were distinct associations of PLFA markers with different treatments. Treatment with dH_2O was most strongly associated with i15:0, a common bacterial signature marker [40], and fungal markers 18:1w9(cis) and 18:2w6 [41]. TWWW is associated with cy19:0 and cy17:0, previously reported as being characteristic for anaerobes [41] and 16:1w9, another common bacterial signature marker. WWW treatment is associated with bacterial signature markers 18:0, 2-OH 14:0, and 15:0 [41].

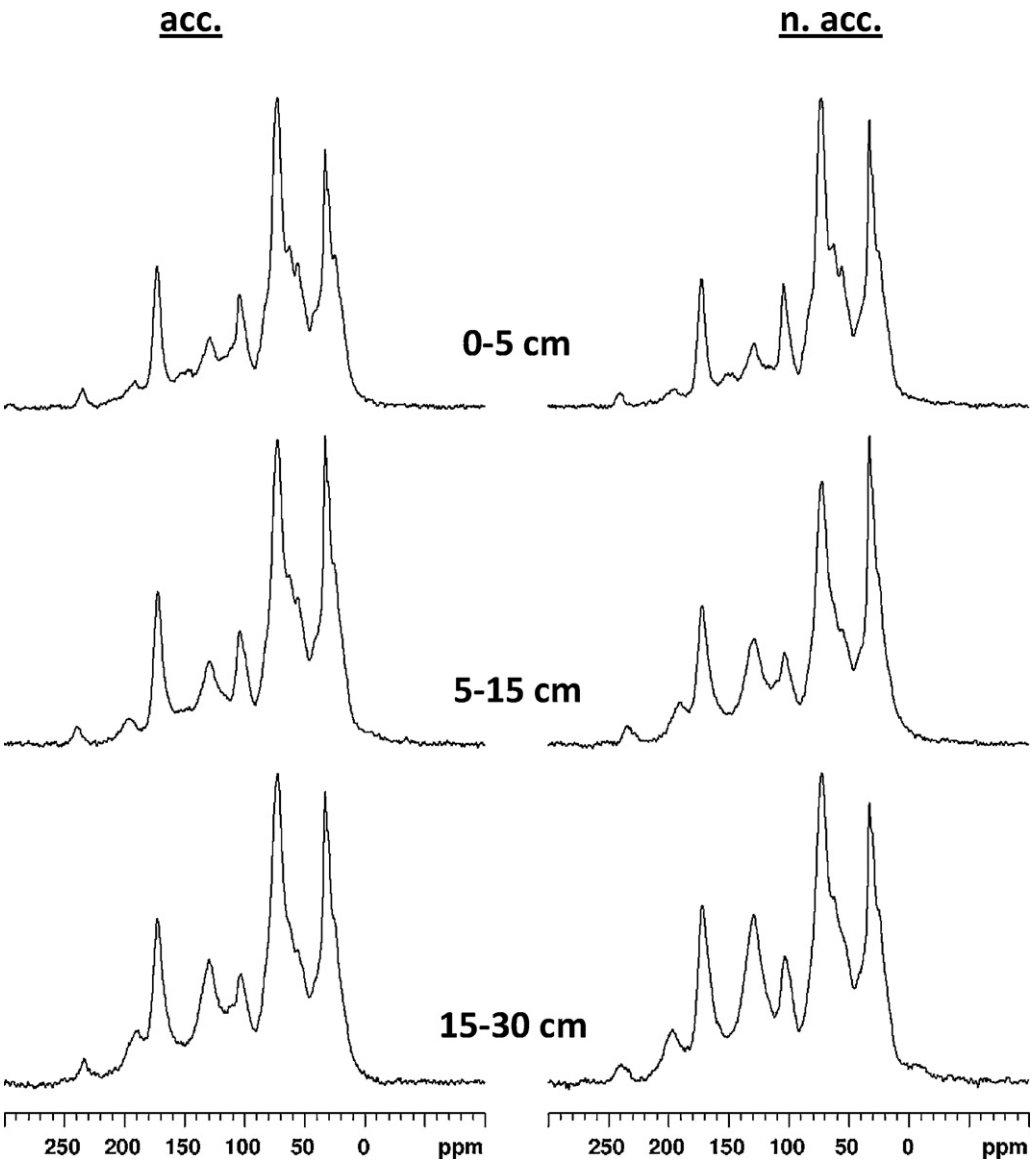

Fig. 1. NMR traces from acc. and n. acc. soils.

When PLFA profiles were compared over time (irrespective of irrigation treatment) distinct patterns were also observed (Fig. 5), with clear separation between all sampling times, except for days 1 and 3. The first two dimensions of the CVA (Fig. 5) were considered significant using Bartlett's Chi-square approximation. The first two dimensions account for 81% of the variance where the majority of the separation occurs in the first dimension (61%), and is driven by days 1 and 16 at opposing ends. The PLFA profile remains quite different at $t = 16$ to $t = -1$, suggesting that it takes longer than 16 days for the effects of the water application on the soil microbial community to be overcome. It is possible to consider PLFA markers as drivers in this analysis. $t = -1$ appears to be associated with i15:0, $t = 1$ and 3 with i16:0, $t = 7$ with 16:0, and $t = 16$ with a15:0. Whilst these markers appear to be associated with different times, they are all classified as bacterial signature markers [41].

4. Discussion

4.1. Effects of long-term application of WWW

A number of significant differences were observed between acclimatised and non-acclimatised soils. The increased concentration of a number of cations (Mg, Ca, K, Na) in the acclimatised site is likely to result from these cations being present in the waste materials [9,10] and their subsequent accumulation. Concentrations of the majority of cations exceeded recommended values at both sites, especially in the surface soils, although concentrations in the acc. soil were generally higher. The concentrations of these cations is unlikely to be of concern from an agricultural perspective, as the levels are not so high as to be considered toxic [42]. Elevated concentration of heavy metals (Zn, Fe, Cu and B) in the acc. soils is of concern due to the fact that restrict plant growth

Table 3
Effects of short-term application of winery wastewaters on soil moisture, carbon and nitrogen pools.

Time	−1	1			3			7			16			Mixed model ANOVA		
Treatment		WWW	TWWW	dH_2O	WWW	TWWW	dH_2O	WWW	TWWW	dH_2O	WWW	TWWW	dH_2O	Treatment	Time	Time × treatment
Soil moisture (%)	26.3 (0.5)	29.7 (2.0)	29.7 (0.4)	26.9 (0.9)	28.1 (1.3)	29.5 (0.5)	28.7 (0.7)	26.9 (0.9)	27.5 (1.2)	27.1 (1.2)	25.6 (0.5)	26.1 (0.6)	25.3 (0.4)	NS	**	NS
C:N ratio	11.5(0.08)	11.0(0.08)	11.2(0.29)	10.9(0.58)	10.9(0.11)	10.3(0.33)	10.9(0.39)	10.9(0.13)	10.8(0.33)	10.9(0.15)	11.1(0.25)	10.8(0.15)	11.2(0.19)	NS	NS	NS
NO_3–N (μg/g dry soil)	2.7 (0.3)	9.3 (0.8)	7.2 (0.8)	3.6 (0.2)	6.0 (0.8)	6.0 (1.1)	2.7 (0.2)	20.2 (0.7)	12.3 (0.8)	2.4 (0.1)	4.0 (0.4)	3.3 (0.2)	2.5 (0.5)	**	***	***
NH_4–N (μg/g dry soil)	2.4 (1.3)	1.1 (0.5)	6.0 (3.3)	9.6 (2.1)	4.8 (1.2)	3.7 (2.0)	2.9 (1.7)	20.2 (6.3)	7.9 (3.7)	6.7 (2.6)	5.8 (1.5)	1.7 (0.4)	3.1 (1.5)	NS	*	*

Values in data table represent mean values, with figures in parentheses being standard errors ($n = 4$).
(Abbreviations: WWW, winery wastewater; TWWW, treated winery wastewater; dH_2O, distilled water control; ANOVA, analysis of variance).

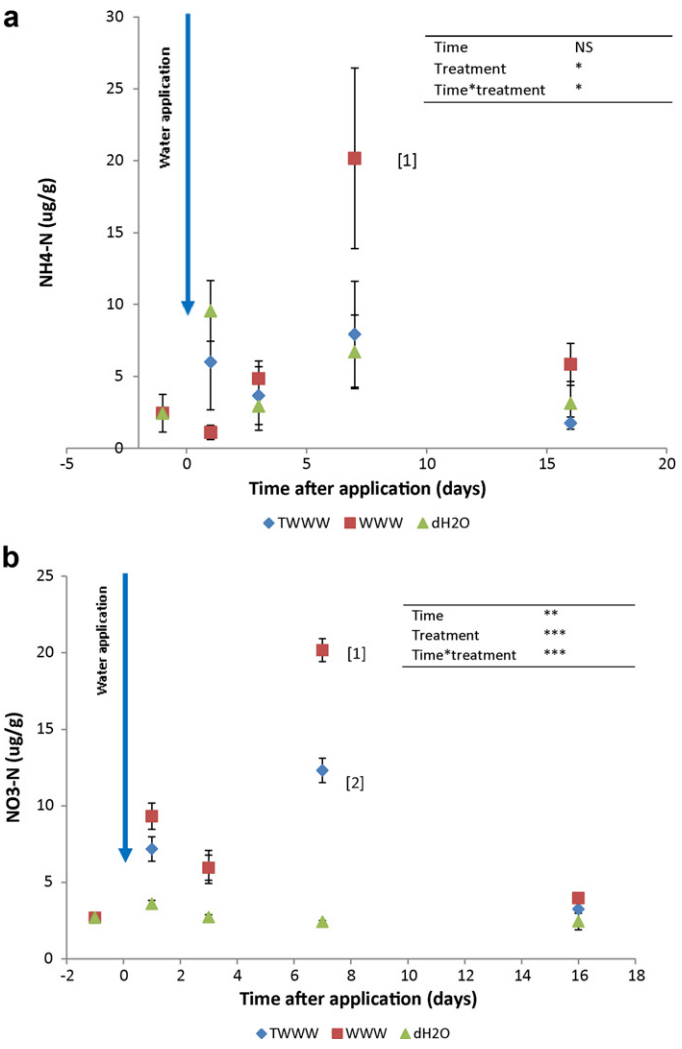

Fig. 2. Effect of winery wastewater application on soil (a) ammonium, and (b) nitrate levels in acclimatised vineyard soils.
Error bars represent standard error ($n = 4$). In 1a, [1] is significantly different from all other means. In 1b, [1] mean is significantly different from all other means except for [2]; [2] mean is not significantly different from any other.

[42]. Concentrations of Zn, Mn, Fe and Cu all exceed guideline values in the acc. soils (0–5 cm), and due the fact that heavy metals typically accumulate in soils, are only likely to further exceed guidelines in the future unless specific crops are grown to facilitate phytoextraction [43]. Low pH at both sites is potentially problematic, due to the capacity for release of soluble Al, which can restrict plant growth [42]. The n. acc. site was more acidic and contained higher levels of soluble Al, which suggests that the application of winery wastes has been beneficial in neutralising an acidic soil to a certain degree, and thus reducing soluble Al. Increased EC values, associated with increased soil Na and K concentrations in the acclimatised soils, may also be of concern, due to the potential for these cations to increase susceptibility to soil dispersion [42]. Increase in these cations is consistent with their presence in winery wastes as a result of Na and K based cleaners [8]. Given the relatively small increase over a 30-year period, compared with the high concentrations of salts typically present in WWW, it appears that the majority of these salts have been leached from the soil profile.

Both soils contained relatively high levels of organic carbon and nitrogen. A calculation of total C stored in the whole 0–30 cm profile, using measured bulk densities for each soil depth increment,

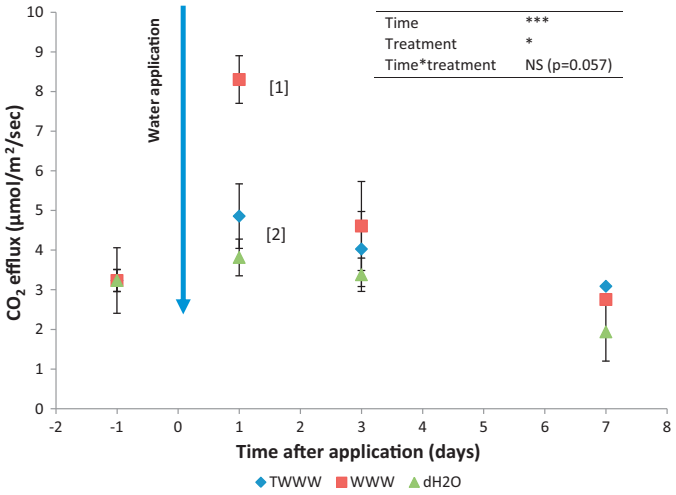

Fig. 3. Effects of short-term winery wastewater application to vineyard soils on CO_2 efflux.
Error bars represent standard error ($n = 4$). ANOVA table presents significance from mixed model testing. [1] Mean is significantly different from all other means except for [2]; [2] mean is not significantly different from any other.

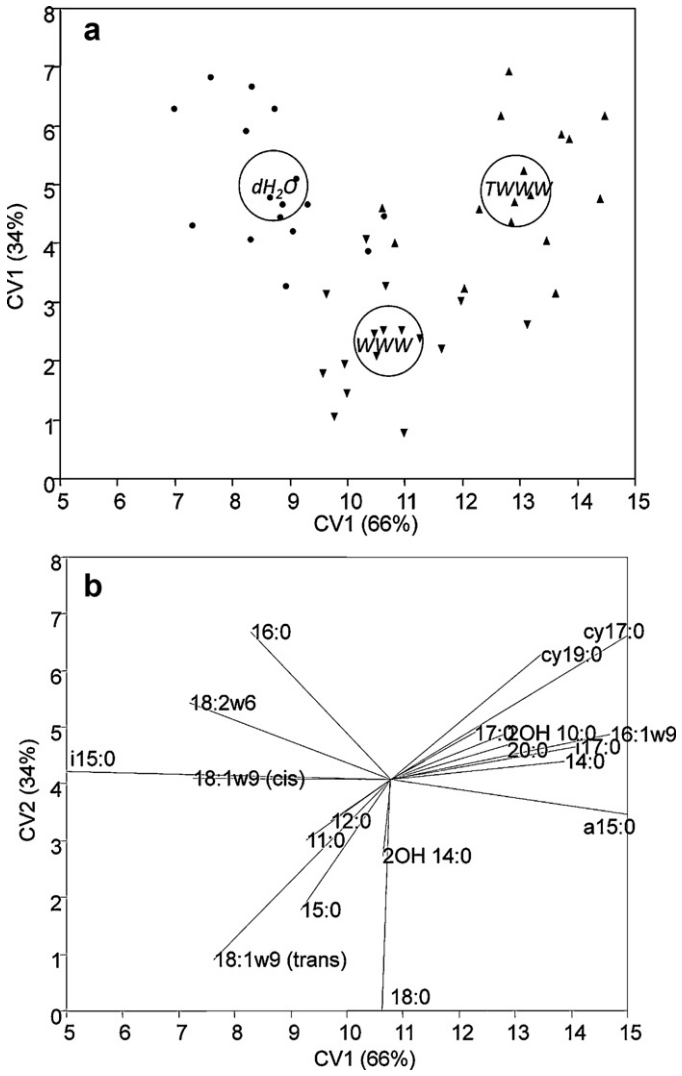

Fig. 4. Canonical variate analysis of PLFA profiles based on treatment of acc soils with WWW, TWWW and dH2O waters. Score plot is presented in (a), and the loading plot in (b). Note that, in (b) biplot rays clustered around the origin have been removed to facilitate ease of interpretation.
Ellipses represent a 95% confidence interval; overlapping ellipses indicate no significant differences between treatments ($n = 4$).

indicated that the acc. soil contained 43 g of carbon per kg of soil compared with 33 g of carbon per kg of soil at the n. acc. site, thus suggesting that a considerable amount of the soluble organic matter applied in the waste material has been retained within the soil profile. Soil organic matter is a dynamic component of soil, and it is difficult to quantify recommended values, due to the different nature of carbon molecules that may be present. There were no clear differences observed in the nature of the organic matter present in the two sites via NMR studies. The long-term additions of WWW provides a substantial source of additional organic matter and the nature of the SOM may also be influenced by differences in microbial populations caused by WWW application, and by differences in vegetation cover between the two sites [44]. It is, therefore, somewhat surprising that differences in organic matter composition were not evident in the NMR spectra. Clearly, the composition of the additional 33% organic carbon present at the acc. site is quite similar to that of the remaining pre-existing C. This suggests that there is substantial microbial conversion of the additional C added in the WWW.

The lack of differences in NMR spectra in this study contrast with previous findings. It has been reported that changes in solid-state ^{13}C NMR spectral features can reflect differences in microbial communities [45]. It has been suggested that the changes in the relative content of alkyl-C as well as carbonyl-C can be used as an indicator of microbial community changes [39]. However, overlapping signals in the spectral regions that include C-alkyl, O-alkyl, N-alkyl-, alkyl- and carbonyl-C can be associated with lipids, proteins and nucleotides from both microbial and plant sources [46,47]. The same applies to carbonyl-C which can originate from proteins and humic substances. Furthermore, microorganisms are protein-rich, however the relationship between proteins and microbial communities may not be readily observable by NMR, hence the use of PLFA analysis in this study.

4.2. Short-term impacts of WWW application on soil microbiology

4.2.1. Respiration

Application of all water types resulted in an increase in soil respiration, with the greatest amount of soil respiration occurring in the WWW treatment. This increase in soil respiration is consistent with the different amounts of organic carbon present in each water type (WWW > TWWW > dH_2O), with greater amounts of carbon resulting in greater microbial activity and therefore higher respiration rates. The pulse occurred quite quickly, suggesting that the organic matter present in the WWW is readily available to the microbial population, and does not appear to be toxic. In this study, wetting alone (dH_2O treatment) did not have any significant impact upon microbial respiration, which suggests that the soil moisture content was already adequate for microbial function, and therefore there was no pulse as has been previously reported for soils in drier initial condition [48]. Effects of wastewater application on soil respiration rates can be quite variable, with both increases [49–51] and decreases [52] reported. Such variability is not unexpected, due to the extreme variability in the composition of wastewaters, in terms of labile carbon concentrations, availability of key nutrients such as nitrogen, and the presence or absence of various toxic compounds such as heavy metals.

Whilst the peak of microbial activity occurred quite rapidly, peak concentrations of ammonium and nitrate were detected several days later. Given that ammonium and nitrate are both indicators of the nitrogen cycling activities of the microbial population

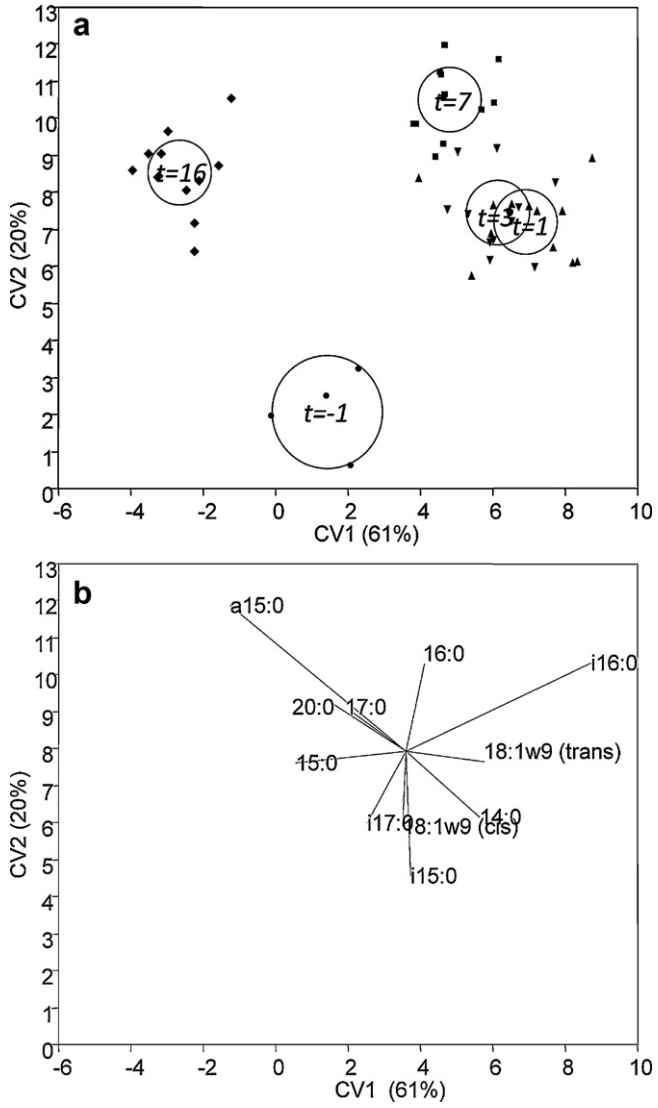

Fig. 5. Canonical variates analysis of PLFA profiles based on time (days) after water application to acc soils. Score plot is presented in (a), and the loading plot in (b). Note that, in (b) biplot rays clustered around the origin have been removed to facilitate ease of interpretation. Ellipses represent a 95% confidence interval; overlapping ellipses indicate no significant differences between treatments ($n = 4$).

this suggests that the organisms involved in nitrate and ammonium production were slower to respond to the water application than the increase in overall microbial activity, or that the ammonium oxidisers are generally less active. This delayed response to nitrogen cycling is consistent with the fact that nitrifying bacteria are typically slow growing, and hence there is a lag between the application of nutrients in WWW and their subsequent cycling. Ammonia oxidising bacteria (AOB) have previously been shown to be affected by application of other types of wastewater [53–56], and changes to the AOB population following WWW application would be consistent with the increased concentrations of nitrate detected.

Nitrogen cycling is an especially important consideration with respect to land application of wastewaters, from the perspectives of both potential benefits, as well as environmental detriment. Mineralisation of organic nitrogen by microorganisms makes it available to plants, and can therefore increase the fertility of soils, resulting in increased agricultural productivity [57]. Excessively high levels of nitrate within the soil, however, often leads to nitrate leaching, which can pollute both surface and groundwater sources. This is of significant concern with land application of wastewaters that contain high levels of nitrogen [58]. WWW does not typically contain especially high concentrations of nitrogen [8], however, the added nutrients in WWW evidently facilitate microbial nitrogen cycling, thereby increasing soil ammonium and nitrate levels. In this instance, the nitrate concentrations returned to pre-application levels within the 16-day period, and are therefore unlikely to result in environmental harm via leaching. In an applied setting, however, repeated applications would be occurring, and therefore the rate of leaching may not be adequate to prevent a build up of nitrate. This indicates that a suitable cropping regime would need to be implemented to absorb the excess nutrients being applied to the system.

4.2.2. Soil microbial community structure

The microbial community (as measured by PLFA composition) in vineyard soil was significantly impacted upon by both treatment and time (Fig. 4), which implies that whilst the changes in soil moisture content have a significant effect on the soil microbial community, the additional chemistry of the WWW and TWWW had an additional significant effect. The different treatments show the most interesting trends with respect to microorganism classes. The control treatment (dH_2O) separated from the other treatments and showed correlation with fungal markers, whilst the TWWW treatment associated with anaerobes, and the WWW treatment with bacterial signature markers. Taken together, this suggests that there are certain components of WWW chemistry that advantage bacteria over fungi, and that are not removed via the treatment process. The association of the TWWW treatment with anaerobes is interesting, as such increases in anaerobes suggests either that the soil environment became waterlogged by TWWW application, thereby encouraging anaerobe proliferation, or that anaerobes were introduced with the TWWW. That soil moisture contents were not significantly different between treatments suggests that waterlogging was not a likely cause. Reports regarding the WWW treatment plant in use on-site suggest that it did become anaerobic on some occasions, which may therefore have resulted in the introduction of certain anaerobic microbes to the soil plots with the TWWW application. In this study, it is important to consider the potential for microorganisms contained in the irrigation water to cause shifts in soil microbial communities. Wastewater is not sterile, and hence its application would also have resulted in the introduction of a certain number of microorganisms to the soil. This is likely to have contributed to a certain amount of the shift observed at $t = 1$. However, the fact that the differences between the soil microbial community structure is negligible between $t = 1$ and $t = 3$ suggests that, if any microorganisms were introduced in the wastewater, they are able to persist in the soil environment. Although the soil microbial community also changed with time, there are no distinct microbial groups that were associated with this separation. Changes in soil microbial communities over time following various forms of treatment have been shown previously [59].

Whilst association of individual PLFA markers with classes of microorganisms is a commonly utilised technique [40,41,60], there are limitations to this approach [61]. This analysis has shown that application of different water treatments causes shifts in the microbial populations, although the exact nature of these shifts cannot be elucidated. Further studies utilising DNA based sequencing techniques may help to provide further insight into these population shifts. However, due to the unknown nature of the majority of soil microorganisms [62], this technique would not be able to provide a complete and detailed measure of the microbial community to the species level.

5. Conclusions

Application of WWW, both long- and short-term was shown to have a number of effects on soil physicochemical and biological properties. In the long-term experiment, the majority of changes related to inorganic species, and the nature of the carbon present appeared to be quite consistent between the acc. and n. acc. sites. In the short-term experiment, a single application of WWW had significant impacts on soil respiration, nitrogen cycling and soil microbiological community profiles. Reassuringly, the fact that TWWW application to land caused no significant differences in nitrogen or carbon cycling to equivalent application of dH_2O suggests that the WWW treatment facility is performing adequately for purposes of WWW reuse. However, the microbial community was impacted upon, which does suggest that there are differences in the TWWW that may be of concern in the longer term. The long-term study suggests that the inorganic material present in the waste materials builds up with time, which is of concern as most WWW treatment processes fail to significantly reduce salt concentrations of wastewater streams.

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