Environmental Pollution 190 (2014) 1–9

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Mechanisms of pollution induced community tolerance in a soil microbial community exposed to Cu

Steven Wakelin [a,*], Emily Gerard [a], Amanda Black [b,c], Kelly Hamonts [c,d], Leo Condron [c], Tong Yuan [e], Joy van Nostrand [e], Jizhong Zhou [e], Maureen O'Callaghan [a]

[a] AgResearch Ltd, Lincoln Science Centre, Private Bag 4749, Christchurch, New Zealand
[b] Bio-Protection Research Centre, P.O. Box 85084, Lincoln University, Lincoln 7647, Christchurch, New Zealand
[c] Faculty of Agriculture and Life Sciences, PO Box 85084, Lincoln University, Lincoln 7647, Christchurch, New Zealand
[d] CSIRO Plant Industry, Canberra ACT 2601, Australia
[e] Institute for Environmental Genomics, University of Oklahoma, 101 David L Boren Blvd, OK 73019, USA

ARTICLE INFO

Article history:
Received 22 September 2013
Received in revised form
27 February 2014
Accepted 7 March 2014
Available online 29 March 2014

Keywords:
Co-tolerance
Pollution induced community tolerance
PICT
Soil ecotoxicology

ABSTRACT

Pollution induced community tolerance (PICT) to Cu^{2+}, and co-tolerance to nanoparticulate Cu, ionic silver (Ag^{+}), and vancomycin were measured in field soils treated with Cu^{2+} 15 years previously. EC_{50} values were determined using substrate induced respiration and correlations made against soil physicochemical properties, microbial community structure, physiological status (qCO_2; metabolic quotient), and abundances of genes associated with metal and antibiotic resistance. Previous level of exposure to copper was directly ($P < 0.05$) associated with tolerance to addition of new Cu^{2+}, and also of nanoparticle Cu. However, Cu-exposed communities had no co-tolerance to Ag^{+} and had increased susceptibly to vancomycin. Increased tolerance to both Cu correlated ($P < 0.05$) with increased metabolic quotient, potentially indicating that the community directed more energy towards cellular maintenance rather than biomass production. Neither bacterial or fungal community composition nor changes in the abundance of genes involved with metal resistance were related to PICT or co-tolerance mechanisms.

1. Introduction

Pollution induced community tolerance (PICT) is a phenomenon whereby exposure of a community to a toxic compound results in a selection towards increased tolerance in the community to that compound (Blanck et al., 1988). PICT has been demonstrated across a range of ecosystems, from marine sediment, periphyton, and microalgae communities (e.g. Blanck and Dahl, 1996; Blanck and Wangberg, 1988; Gustavson et al., 1999; Ogilvie and Grant, 2008) to soil invertebrate communities (e.g. Salminen et al., 2001).

PICT has also been demonstrated widely in soil microbial communities and for a wide range of pollutants. These include heavy metals (e.g. Dìaz-Raviña and Bååth, 1996; Stefanowicz et al., 2009; Soler-Rovira et al., 2013), organic contaminants (Gong et al., 2000; Kaufmann et al., 2006; Demoling and Bååth, 2008), pesticides (Zabaloy et al., 2010), and antibiotics (Demoling and Bååth, 2008; Schmitt et al., 2004). In some instances, development of PICT to one pollutant has been found to confer co- or cross-tolerance in the community to other pollutants. For example, previous exposure to Cu, has been shown to confer tolerance to a range of other metals (Dìaz-Raviña et al., 1994), and also to antibiotics such as tetracycline and vancomycin (Berg et al., 2010; Fernández-Calviño and Bååth, 2013). The co-selection of antibiotic resistance is of particular concern with regards to development of environmental reservoirs of antibiotic resistant bacteria that might have plant, animal, or human health implications (Seiler and Berendonk, 2012).

The development of tolerance and co-tolerance to pollutants in microbial communities can occur through a variety of mechanisms. These include processes intrinsic to classic ecological theory, such as species selection and replacement resulting in shifts in overall community composition (Rutgers, 2008; Wakelin et al., 2010a). Communities may also develop physiological adaptations that confer higher tolerance to stress; this can manifest as a shift in metabolic quotient (qCO_2; Ohtonen, 1994), a measure of the steady-state (basal) energy required to maintain the microbial biomass (CO_2—C respired per unit of microbial biomass), which is analogous to maintenance energy requirements (Pirt, 1965). Under conditions of elevated stress, as occurs with chronic metal pollution, a higher

* Corresponding author.
E-mail address: steve.wakelin@agresearch.co.nz (S. Wakelin).

http://dx.doi.org/10.1016/j.envpol.2014.03.008
0269-7491/

amount of energy entering the soil ecosystem (usually plant derived C inputs) is required to support processes associated with maintenance such as metal transport and detoxification, and less energy is available for incorporation into the biomass itself. Under these conditions the qCO_2 is expected to increase. Similar results have been observed when measuring the amount of CO_2–C mineralised from substrate (glucose) added to soil in proportion to the microbial biomass size (Dahlin and Witter, 1998). As such, physiological assessment of the soil microbial community may be a sensitive indicator of ecosystem stress caused by metal or other contaminants (Giller et al., 1998).

The development of pollution tolerance within the soil microbial community may also be facilitated through increased abundance of genes conferring tolerance traits in the community metagenome. Genes associated with microbial resistance to metals such as copper (*cop*-genes; Cooksey, 1994), mercury (*mer*-genes; Mirsa, 1992), and many others have been described (see Bruins et al., 2000; Silver and Phung, 1996). The abundances of these genes may increase through selection of species holding these traits (vertical transfer/cell reproduction), or transfer of the genes laterally between species through processes such as conjugation and plasmid transfer (Coombs and Barkay, 2005).

While the general processes that may contribute to tolerance and co-tolerance to pollutants are understood, there remains a large gap in knowledge about how these processes operate at a community level (Eijsackers et al., 2008). In particular, few studies have examined the development of co-tolerance. Most studies on PICT have used defined functional endpoints such rate of nitrification or C-mineralisation (Blanck et al., 1988), and much of this work relates back to relationships between ecosystem structure (i.e. phylogenetic and functional) and eco-physiology (Rutgers, 2008). Given the importance of the ecosystem as a whole in the development of PICT, further insights into the mechanistic basis of this phenomenon can be gained through application of community level approaches (Breure et al., 2008).

Copper is one of the most common metal contaminants in agricultural production systems (Seiler and Berendonk, 2012). Because of its antimicrobial properties, it is used in broad-spectrum bacterial and fungicidal agricultural pesticides, as a nutritional supplement for animals (e.g. feed supplements), and in fertilisers (Alloway, 2008). At the field site from which our experimental soil was obtained, Cu had added to soil in Cu-spiked biosolid, in 1997 as part of the trial examining the long term fate of biosolids applied to pasture (McLaren and Clucas, 2001). While much of the organic content of the biosolids has been mineralised, Cu persists in the soil and provides a chronic, long-term stress on the soil microbial community. The aim of this work is determine if PICT was present in field soil exposed to Cu 15 years earlier and to determine if this extended to other pollutants. Furthermore, we aimed to identify possible mechanisms related to tolerance and co-tolerance by assessing changes in the community physiological status, species composition, and accumulation of genes associated with metal and antibiotic resistance.

2. Materials and methods

2.1. Site description, soil sampling, and soil physicochemical properties

Samples were collected from a trial established in 1997 at the Lincoln University Dairy Farm (LUDF), South Island New Zealand to assess the impacts of metal-spiked biosolids on selected soil properties and metal uptake by pasture herbage (*Lolium perenne* L). Soil was sampled from control plots, plots amended with biosolids only, and plots amended with Cu spiked biosolids at rates equivalent to 50, 100, 150, 200 mg kg^1 of Cu (hereafter referred to as Cu 1–4). Full details of the trial, incorporation of Cu into the biosolid material, and biosolid application to the trial were described in McLaren and Clucas (2001).

Soil samples were collected to 10 cm depth from 40 × 40 cm area central in each of the 6 plots. Plant (grasses), root and other material was removed by sieving through a 2 mm mesh and the bag shaken to form a homogenous samples. Soil was stored in sealed plastic bags at 4 °C until use (up to 4 weeks). Total Cu was determined using 1 g of soil left overnight in 5 mL of HNO_3 (Kovács et al., 2000). The following day, 5 mL of 50% H_2O_2 was added and the sample digested at 120 °C for 4.5 h. Calcium nitrate extractable Cu was determined using a solution of 0.05 M $Ca(NO_3)_2$, whereby 30 mL of extractant was added to 5 g of soil, the sample shaken for 2 h, then centrifuged at 15,000 rpm for 10 min (Gray et al., 1999). Extracts produced from the procedures outlined above were analysed for metal concentrations using an inductively-coupled plasma optical emission spectrophotometer (ICP-OES; Varian 720-ES). The broader physiochemical properties of soil from each treatment were further characterised by RJ Hill laboratories (Christchurch, N.Z.): Olsen phosphorus was measured in a $NaHCO_3$ extract with molybdenum blue colorimetry; sulphate sulphur (SO_4–S) was extracted in 0.02 M K_2HPO_4 and determined with ion chromatography (Blakemore et al., 1987); pH was measured in a 1:2 (v/v) soil:water slurry followed by potentiometric determination; anion storage capacity was measured using ICP-ES after equilibration of the soil with a 0.02 M potassium phosphate solution; anaerobically mineralisable N (AMN) was determined by anaerobic incubation followed by extraction in 2 M KCl and Berthelot colorimetry; total C was determined by Dumas combustion; Fe, Mn, and Zn were measured in an EDTA-soil extract using ICP-ES; cation exchange capacity was calculated as the total extractable cations and extractable acidity; volume weight was measured on a ratio of dried and ground soil. The units of measurement for each of the soil properties are given in Table 1.

2.2. PICT testing: Cu^{2+}, nanoparticle Cu, Ag^{+}, and vancomycin

In each of the soils previously exposed to Cu, the EC_{50} of newly applied Cu^{2+} ($CuSO_4 \cdot 5H_2O$; Sigma–Aldrich), nanoparticle Cu (Cu_{np}, 25 nm; SkySpring Nanomaterials Inc, TX, USA), and Ag^{+} (Ag_2SO_4; Sigma–Aldrich) on soil respiration were evaluated. The assay system was based on the MicroResp™ whole-soil, substrate-induced respiration (carbon mineralisation) assay (Campbell et al., 2003). The assay has been recently modified for use as an ecotoxicology tool for quantifying the impact of pollutants on microbial function in soils (Wakelin et al., 2013a), and this method was closely followed. In preliminary experiments, the moisture content that gave the optimum rate of microbial activity was empirically tested; a soil θ_d of 66% was used for subsequent PICT assays. Each of the test metals was mixed into soil at a maximum rate and sequential, 0.5× serial dilutions were made with un-amended soil to form a dilution range (12 levels of each metal). The highest rates of metal addition to soils were 13,328 mg°Cu°kg^{-1} soil for $CuSO_4$, 65,331 mg°Cu°kg^{-1} soil for Cu_{np}, and 36,326 mg silver (Ag) kg^{-1} soil for Ag_2SO_4. For each metal, the 12 soil samples spanning the dilution range were loaded each into columns of 8 (replicate) wells across a 96 well plate. Water with glucose was then added to each well to adjust the soil to the θ_d of 66% and also supply the equivalent of 20 mg°glucose°g^{-1}°dry°soil. The MicroResp test plates were attached to colorimetric gel-traps using a rubber seal (Campbell et al., 2003). The gel traps contain a pH sensitive dye (cresol red) that changes colour in response to head-space CO_2, allowing for measurement of carbon mineralisation (soil microbial respiration). Assays were conducted for 5 h at 20 °C and change in absorbance at 590 nm (ΔA_{590}) was measured on a plate scanner (Omega; BMG Labtech). The relationship between headspace $\%CO_2$ and ΔA_{590} becomes non-linear as the gel becomes saturated (Campbell et al., 2003). As such, data collection were timed to ensure that ΔA_{590} were <0.3, i.e. well within the linear range of the gel-trap colour response.

The effect of vancomycin on soil respiration was similarly assayed using the MicroResp system. Stocks of vancomycin (hydrochloride salt; Sigma) were made at 100 mg°vancomycin°mL^{-1} water and pre-testing was used to determine the approximate range of toxicity of the antibiotic when added to soil. A dose-range was established that delivered 0–34 mg°vancomycin°g^{-1} soil. This was delivered in 120 μL of water per well (to reach θ_d of 66%) and with glucose to supply 20 mg°g^{-1}°dry°soil. The MicroResp plates were assembled, incubated and assessed as described above.

From each sample, the EC_{50} of newly applied pollutants was calculated by fitting non-linear regression to the data. C-mineralisation (ΔABS_{590} values) was used as the response variable which was compared over the $\log_{10}$ of the values for the pollutant in the soils. Outlying data were automatically excluded using the ROUT method (Motulsky and Brown, 2006). For some data sets, primarily those with metal salts as the pollutant, hormesis effects were evident and, for these, biphasic equations were used. For other data, best fits were made using sigmoidal dose–response curves. From these, EC_{50} values were determined which corresponded to the level of pollutant that resulted in 50% loss of soil respiration activity, and when possible the corresponding 95% confidence interval for each EC_{50} value. Curve fitting and calculation of EC_{50} values and confidence were performed in GraphPad Prism v6 (GraphPad Software, USA).

2.3. Metabolic quotient

Microbial activity per unit of community size (physiological status) was measured using the MicroResp system. For each treatment, four replicate subsamples of soil were loaded into wells in a deep well plate. Water and glucose was added to each sample (as before) and the amount of CO_2 respired (microbial activity) measured as change in absorbance of the gel trap after 4 h incubation

Table 1
Cu levels and physicochemical properties of the test soils from the Lincoln University Dairy Farm metal trial site.

Sample	Cu_{ext}	Cu_{total}	pH	Olsen phosphorus	SO_4–S	Anion storage capacity	Anaerobically mineralisable nitrogen	C_{total}	Fe	Mn	Zn	Cation exchange capacity	Volume weight	CO_2[a]
	mg kg^{-1}	mg kg^{-1}		mg L^{-1}	mg kg^{-1}	ASC %	kg Ha^{-1}	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	me 100 g^{-1}	g mL^{-1}	ΔA_{590}
Control	0.007	3.79	5.8	6	8	31	79	3.6	178	68	2.1	15	0.89	0.11 ± 0.03
Biosolids	0.008	5.26	5.8	8	4	35	117	3.9	276	124	3.2	16	0.82	0.20 ± 0.06
Cu 1	0.086	27.42	5.4	12	17	43	117	4.3	991	45	3.5	18	0.83	0.20 ± 0.06
Cu 2	0.089	42.81	6.1	6	4	40	108	3.7	244	71	3.3	19	0.9	0.22 ± 0.05
Cu 3	0.123	79.93	6.2	6	4	36	116	3.5	298	131	3.7	16	0.93	0.23 ± 0.07
Cu 4	0.151	86.21	6.7	13	16	47	120	4.2	722	49	2.2	19	0.85	0.26 ± 0.06

[a] Difference in absorbance (ΔA_{590}) of a CO_2-responsive colorimetric gel trap after 5 h incubation over glucose spiked soil. The ΔA_{590} provides a measure of amount of CO_2 respired from the soil due to microbial activity. Values are averages ± SEM.

(ΔA_{590} glucose). Microbial community size was inferred from the AMN content measured in each soil sample, and the ratio between ΔA_{590} glucose and AMN was used as an indicator of physiological status, which we also refer to as a measure of metabolic quotient or qCO_2. Correlations were made between the metabolic quotient and soil physicochemical properties using Pearsons method (2-tailed).

2.4. Abundance and composition of bacterial and fungal communities

DNA was extracted from field soil immediately after collection using the MoBio PowerSoil DNA extraction kit. Extractions were conducted on triplicate 0.25 g samples of soil, and the DNA was pooled together into a final sample (300 μL volume) for each of the six treatments. DNA was quantified using spectrophotometric analysis (NanoDrop; ThermoFisher Inc.).

The size of the bacterial and fungal communities was measured using real-time PCR (qPCR). For bacteria, the 16S rRNA gene was targeted for quantification using primers Eub338 and Eub518 (Lane, 1991; Muyzer et al., 1993), and for fungi the rRNA ITS gene region using primers 5.8S and ITS1f (Gardes and Bruns, 1993; Vilgalys and Hester, 1990). Gene quantification was conducted using SYBR-based reaction chemistry (SensiMix; Bioline) on a Qiagen RotorGene 6000 machine. Each PCR was conducted in a 25 μL volume, containing either 2 μL of template (at 5 ng°DNA°uL^{-1} concentration), known gene copy number DNA (standard curve), or no-template controls. Primers were used at 0.2 μM each. Thermocycling included hot-start enzyme activation at 95 °C for 10 min, then amplification across 40 cycles of 95 °C for 30 s, 53 °C for 30 s, then 72 °C for 30°s. A dissociation curve was run after amplification. Standard curves for both genes were created by cloning PCR product into the pGEMT vector (Promega). The DNA was quantified (NanoDrop) and from this the numbers of gene copies per ng template were calculated (Lee et al., 2006; Whelan et al., 2003). Abundance of bacterial and fungal rRNA genes from the soil DNA was calculated against a standard curve generated by serial dilution of the stock plasmids. Gene copies were back-calculated to copies g dry $soil^{-1}$ basis.

The structures of the bacterial and fungal communities were assessed using denaturing gradient gel electrophoresis (DGGE). For the bacterial community, the primers F968-GC and R1378 (Heuer and Smalla, 1997; Lane, 1991) were used to amplify the V6–V8 region of the 16S rRNA gene from the soil DNA. For the fungal community, the primers ITS1* and ITS4 (Gardes and Bruns, 1993; Wakelin et al., 2007) were used that specifically amplify the ITS1-5.8S-ITS2 region. The rRNA fragments were PCR amplified in 25 μl volumes using 2 μl of template DNA (10 ng), 1°×° Bioline MyTaq reaction buffer (containing 1 mM dNTPs, 3 mM $MgCl_2$, stabilizers and enhancers), 0.2 μM each primer and 1 U MyTaqTM DNA Polymerase (Bioline Pty. Ltd, Australia). DGGE was carried out using a DGGE mutation detection system (CBS Scientific Company, Inc.). PCR fragments (5 μl aliquots) were separated in 7% polyacrylamide gels (acrylamide/bisacrylamide; 37.5:1) with denaturing gradients of 35–65% (where 100% denaturant contains 7 M urea and 40% formamide). The gels were run in 0.5 × TAE buffer (Tris-acetate-EDTA, pH 8.0) at 60 °C for 17 h at 85 V. Following electrophoresis, gels were stained in SYBR Gold (Molecular Probes) for 30 min and visualised on a DarkReader (Clare Chemical Research, U.S.). Gel images were captured with an Olympus E410 camera and band location and intensity data collected using TotalLab TL120 (Nonlinear Dynamics, U.K.).

Similarity in community composition between treatments was calculated using the Bray–Curtis similarity co-efficient (Bray and Curtis, 1957) on square-root transformed abundance data as described by Clarke and Warwick (2001). The resulting similarity matrix was compared against those generated from each of the soil physicochemical properties (Euclidean distances on normalised data; Clarke and Warwick, 2001) using the RELATE test (Clarke, 1993) based on Spearman rank correlation and permutation-generated (999×) null-distribution to provide both goodness of fit (Spearmans Rho; ρ) and statistical confidence (P_{perm}). Statistical testing was conducted in Primer6 (Primer-E Ltd, U.K.)

2.5. GeoChip analysis

The abundance of genes involved in metal resistance, antibiotic resistance, and stress physiology (among others) were assessed using the GeoChip functional gene array system (Lu et al., 2012). GeoChip 4.0 contains approximately 84,000 50-mer oligonucleotide probes covering over 150,000 genes from 410 gene categories. Sample processing and hybridization methods are described in Lu et al. (2012).

Briefly, 50 ng DNA was amplified using whole community genome amplification (Wu et al., 2006). All amplified DNA (~2°ug) was then labelled with cyanine 3 using random priming. Labelled DNA was purified and then dried down before hybridization. Just prior to hybridization, DNA was rehydrated with 2.68 μL sample tracking control (Nimblegen; Madison, WI, USA) and then 7.32 μL hybridization buffer containing 40% formamide. Samples (6.8 μL) were then loaded onto the array and hybridized for 16 h with mixing on a MAUI hybridization station (BioMicro Systems, Salt Lake City, UT, USA).

Arrays were scanned with a laser power and photomultiplier tube settings of 100% (MS 200 Microarray Scanner, Nimblegen). Poor quality spots were removed as described previously (He et al., 2010). Spots were considered positive if the signal-to-noise ratio (SNR) was ≥2.0 and the CV of the background was <0.8. Genes that were detected in only one sample were removed.

The array signal intensity for Cu (*cop*A, *cue*O, *cus*A, *cus*C, and *cus*F), Ag (*sil*A, *sil*C, and *sil*P), and vancomycin resistance (*van*) genes were each used as individual variables when determining relationships with toxicological data. In addition, the combined signal intensities for all metal resistance genes (Cu, Zn, Cd), and also for antibiotic resistance genes were assessed. In each case, the signal data for each gene, originating from a standard amount of DNA probed to each array, was multiplied by the ng DNA g^{-1} from each soil to provide a measure of gene abundance per unit soil. These data were then log-transformed before analysis.

2.6. Relating PICT and co-tolerance to soil edaphic and biological properties

The EC_{50} values of each pollutant across the soils previously exposed to Cu were compared against the soil physicochemical properties using linear regression. Relationships between bacterial and fungal community structure and EC_{50} values was tested using the RELATE test as described previously.

3. Results

3.1. Soil physicochemical properties

The physicochemical properties of the soils are given in Table 1. There was a strong relationship between total and extractable Cu ($R^2 = 0.91$; $P = 0.003$); these two variables were not related to any other soil properties ($P > 0.05$).

3.2. Community tolerance to Cu^{2+}, Cu_{np}, Ag^{+}, and vancomycin

Field soils previously exposed to increasing concentrations of Cu^{2+} had a concomitant increase in tolerance to recently applied Cu^{2+} when challenged 15 y later (Fig. 1). In soil not previously exposed to Cu, the EC_{50} for recently applied Cu^{2+} was low (1.38 g°Cu°kg°$soil^{-1}$), but increased strongly following the lowest level of Cu addition treatment, i.e. the non-amended biosolid material (Fig 1a). Thereafter, the tolerance to Cu^{2+} increased in a more linear fashion, with the highest tolerance of 7 g°Cu°kg°$soil^{-1}$ in the mostly highly pre-exposed soil (Cu 4).

Community tolerance to nanoparticle Cu was much greater than for ionic Cu (Fig 1b). In untreated soil, the EC_{50} of Cu_{np} was 74.6 g°Cu°kg°$soil^{-1}$. Tolerance to Cu_{np} did not increase monotonically with rate of previous exposure to Cu, rather two 'plateaus'

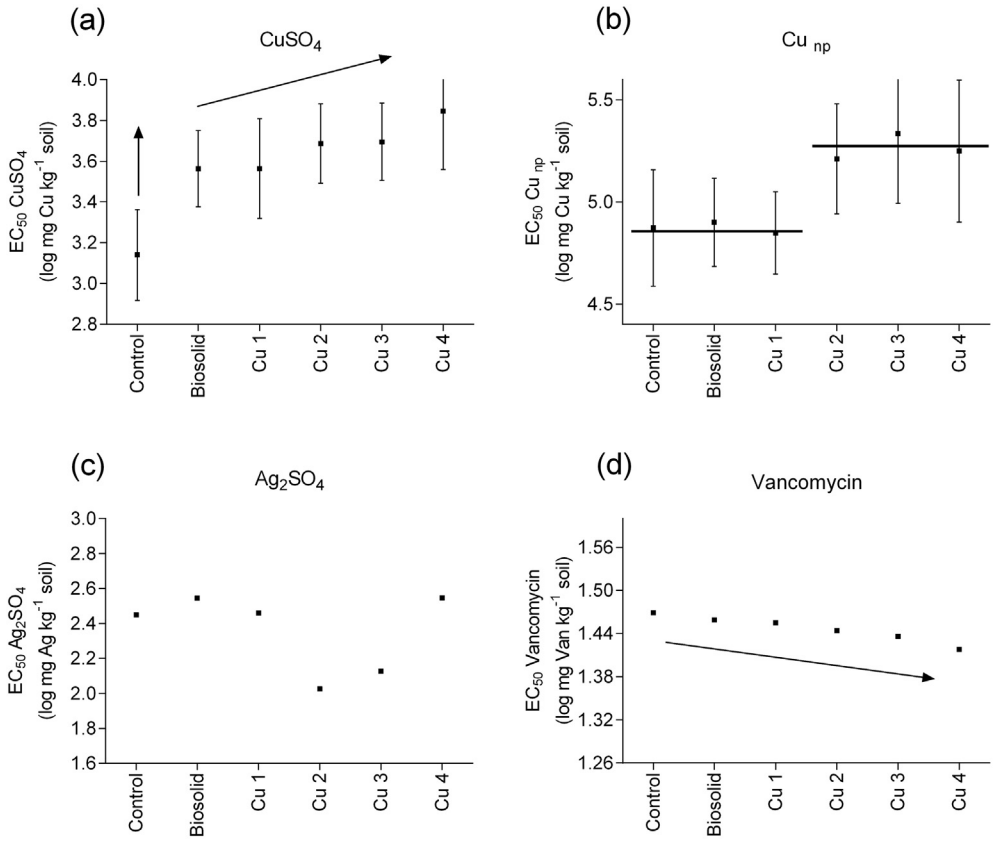

Fig. 1. EC_{50} of soil glucose respiration (MicroResp measured Δ_{590} values) in soils previously exposed to Cu contamination when newly exposed to (a) Cu^{2+}, (b) Cu_{np}, (c) Ag^{+}, and (d) vancomycin. Error bars indicate 95% confidence interval in the calculated EC_{50} value; these could not be reliably determined for Ag^{+} and vancomycin data.

of low and high tolerance were detected (Fig 1b), and these are supported by the 95% confidence intervals calculated for each EC_{50}. At the high tolerance level (treatments Cu 2–Cu 4) the EC_{50} for Cu_{np} on soil respiration was approximately 185 g Cu kg^{-1} soil.

Ag^{+} had greater impact on soil respiration than Cu^{2+}, with EC_{50} values ranging from 0.1 g°Ag°kg°$soil^{-1}$ (Cu 2 soil treatment) to 0.35 g°Ag°kg°$soil^{-1}$ (biosolid only and Cu 4 soil treatments) (Fig. 1c). No relationship was evident between previous soil exposure to Cu, and co-tolerance to Ag (Fig 1c).

The relationship between pre-exposure to Cu and tolerance to vancomycin was negative (Fig 1d), demonstrating increased susceptibility to vancomycin for microbial communities pre-exposed to higher rates of Cu. However, the total range in susceptibility was narrow, with EC_{50} values ranging from 26.2 to 29.4 mg vancomycin Kg soil. In this study, vancomycin was the most toxic compound affecting soil respiration.

The 95% confidence intervals calculated around EC_{50} for both the Ag^{+} and vancomycin data were very wide, and in many cases could not be fitted. For these data sets, therefore, considerable variation exists around the EC_{50} data and the results should be interpreted in light of this.

3.3. Soil metabolic quotient, microbial community size and structure, and links to soil properties

With increasing soil Cu concentrations, the amount of CO_2 respired after glucose addition increased relative to 'per unit' of soil microbial community (Fig 2a). Correlation of the metabolic quotient with soil physicochemical properties found significant, positive associations with both extractable and total Cu, and AMN levels (Table 2).

Neither the abundance of bacteria or fungi in the soils had observable trends across the Cu-treated soils (Fig. 2b and c). Furthermore, there were no significant correlations between the abundances of these groups and soil physicochemical properties ($P > 0.05$). In contrast, the ratio of bacteria to fungi showed a strong response to soil Cu addition (Fig 2d). In the un-treated soil, the ratio was the narrowest (5.13) and increased with Cu-added; i.e. bacteria became more dominant. There was a significant correlation between the ratio of bacteria to fungi and soil AMN (Table 2).

The structure (species composition) of the bacterial community was most closely correlated with soil SO_4, Fe, and pH levels (Table 3), but had no association with soil Cu concentration. Changes in fungal community composition were primarily associated with AMN (Table 3), a measure of organic matter content in soil. The fungal community structure was also correlated with other biological properties of the soil, including the bacteria:fungal ratio ($\rho = 0.698$), bacterial abundance ($\rho = 0.686$), and metabolic quotient ($\rho = 0.698$; all $P < 0.05$; Table 3).

3.4. Abundances of metal and antibiotic resistance genes

Genes associated with Cu-resistance, Ag-resistance, or all metals (i.e. total of all metal resistance genes from GeoChip) did not vary statistically in abundance across the Cu-treated soils (Fig 3a, b, c). With the exception of *cus*A ($r = -8.13$ with SO_4), there was no correlation between any metal resistance genes and any of the soil physicochemical properties measured. This trend was similar for abundance of genes for resistance to vancomycin, and when calculated using the total antibiotic resistance genes (Fig 3b,c).

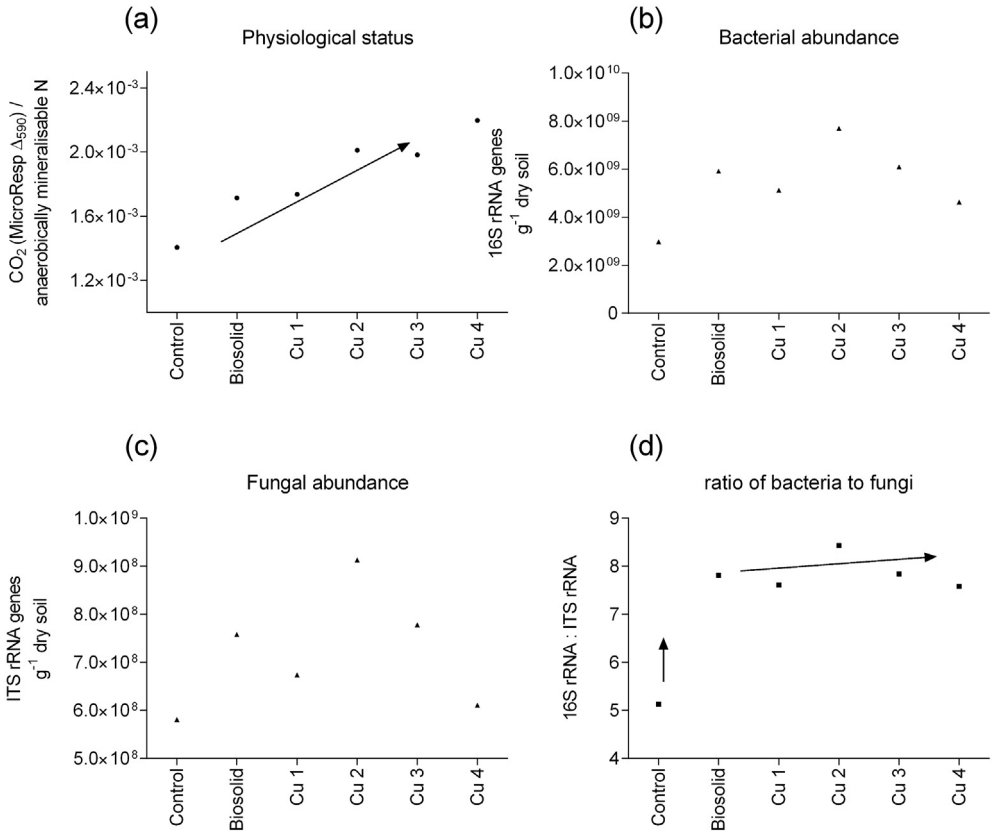

Fig. 2. Relationship between increasing previous exposure to Cu and the soil microbial community (a) physiological status, (b) bacterial abundance, (c) fungal abundance, and (d) ratio of bacteria to fungi.

Table 2
Significant correlations (Pearsons) between metabolic quotient, and soil bacteria:fungal ratio with soil physicochemical properties.

	Soil property	*r*	*P*
Metabolic quotient	Total Cu (mg°kg^{-1}°soil)	0.884	0.019
	Extractable Cu (mg°kg^{-1}°soil)	0.879	0.021
	AMN[a] (kg°N°Ha^{-1})	0.849	0.032
Bacteria:fungi ratio[b]	AMN	0.864	0.027

[a] Anaerobically mineralisable nitrogen.
[b] Calculated by 16S (bacterial) to ITS (fungal) rRNA gene copy numbers.

Table 3
Significant correlations (Spearman rank) between fungal and bacterial community structures (similarities assessed between samples using the Bray–Curtis index) with soil physicochemical properties, EC_{50} values of the pollutants, metabolic quotient and microbial abundances.

	Soil property	ρ	P_{perm}
Bacterial community	SO_4 (mg°kg^{-1}°soil)	0.891	0.009
	Fe (mg°kg^{-1}°soil)	0.839	0.012
	pH	0.643	0.019
Fungal community	AMN[a]	0.771	0.008
	EC_{50} Cu^{2+}	0.725	0.019
	qCO_2[b]	0.718	0.011
	Bacteria:fungi ratio[c]	0.698	0.031
	Bacterial abundance	0.686	0.03
	EC_{50} Van	0.679	0.027

[a] Anaerobically mineralisable nitrogen.
[b] Ratio between ΔA_{590} glucose (glucose induced C-respiration using MicroResp) and AMN (microbial biomass).
[c] Calculated by 16S (bacterial) to ITS (fungal) rRNA gene copy numbers.

3.5. Relating PICT and co-tolerance of metals and vancomycin to soil edaphic and biological properties

PICT to Cu^{2+} was most strongly correlated with soil metabolic quotient (qCO_2), with a very high coefficient of determination ($R^2 = 0.927$; Table 4). Significant positive relationships were also present with AMN, bacteria:fungal ratio, and the extractable Cu concentration of the soils ($P < 0.05$). Variation in Cu^{2+} EC_{50} across the soils was significantly correlated to change in fungal community composition (Table 3). Tolerance to nanoparticle Cu was also correlated to the metabolic quotient (qCO_2), although not as strongly as for tolerance to Cu^{2+} (Table 4). Tolerance to Cu_{np} was also associated with total soil Cu concentration, and soil pH (Table 4).

Tolerance to vancomycin was related to soil metabolic quotient, soil Cu concentration (total or $Ca(NO_3)_2$-extractable), and soil pH (Table 4). However, all these were inversely related – i.e. as soil Cu concentration increased, community tolerance to vancomycin decreased. A relationship was also found between tolerance to vancomycin and the fungal community composition (Table 3).

Community tolerance to Ag^+ was inversely related to *cus*C gene abundance, but not to other soil edaphic or biological properties (Table 4).

3.6. Use of AMN to interpret soil microbial biomass

The AMN content of soil is tightly correlated with soil organic matter content and forms a suitable surrogate measure of the microbial biomass size (Wakelin et al., 2013a,b). As the microbial biomass C can decrease as a proportion of the total organic C in

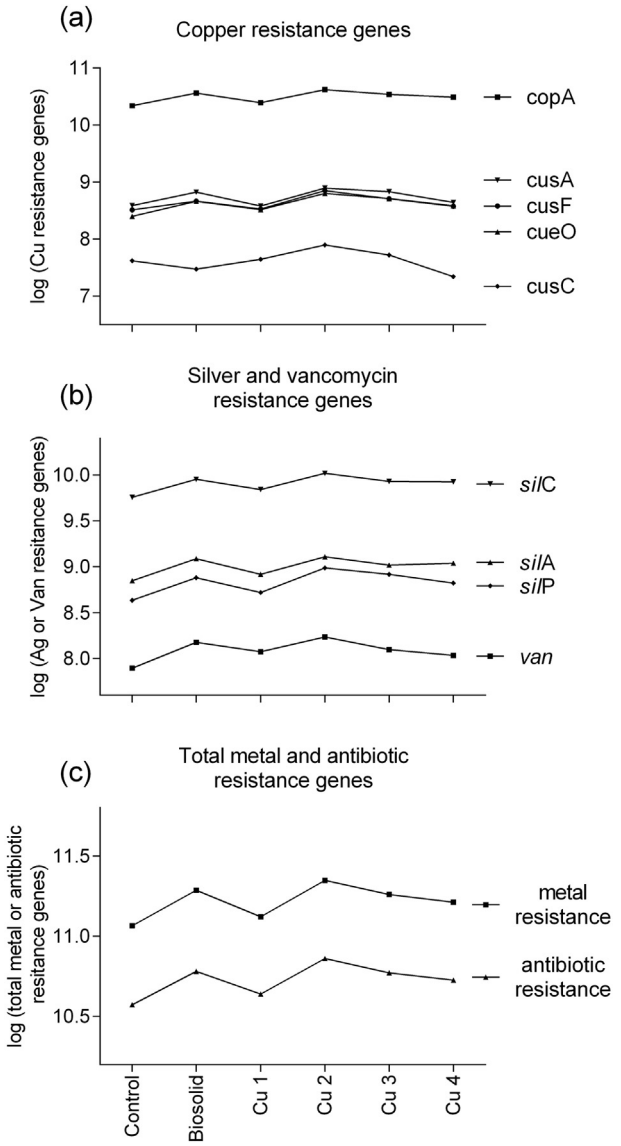

Fig. 3. Relationship between increasing previous exposure to Cu and functional genes in the soil metagenome associated with (a) copper resistance, (b) silver and vancomycin resistance, and (c) total metal and antibiotic resistance genes. Gene intensity data is log values of microarray probe-label intensity data, i.e. the amount of DNA present in the soil-extract that hybridised to a complimentary target spot on the GeoChip array.

metal-enriched soils (Dahlin and Witter, 1998), our use of AMN as a surrogate for microbial biomass may have affected the qCO_2 statistic. To test this, we created an alternative metabolic quotient based on CO_2 respired (following glucose addition) and soil DNA concentration and found the same positive relationship existed across the Cu gradient and with PICT.

4. Discussion

Pollution induced community tolerance to Cu^{2+} was evident in a grassland field soil ecosystem 15 years after the original Cu-spiked biosolids application. The level of PICT within the microbial community had a very close relationship with previous exposure levels and, as such, provides a sensitive estimate of environmental exposure to Cu^{2+}. This has been shown in a number of other studies where the principal factor related to increased PICT to Cu in soil

Table 4
Significant correlations (Pearsons) between PICT to Cu^{2+}, and co- or cross-tolerance to Cu_{np}, Ag^{+}, and vancomycin, with metabolic quotient (qCO_2), soil bacteria:fungal ratio, metal resistance genes, and soil physicochemical properties.

	Variable (X)	Equation	R^2	P
EC_{50} Cu^{2+}	qCO_2[a]	$Y = 0.001125*X - 0.002187$	0.927	0.002
	AMN[b]	$Y = 57.31*X - 95.77$	0.791	0.018
	Bacteria:fungi[c]	$Y = 4.143*X - 7.439$	0.745	0.027
	Exc Cu (mg kg^{-1} soil)	$Y = 0.2008*X - 0.6420$	0.666	0.048
EC_{50} Cu_{np}	Total Cu (mg kg^{-1} soil)	$Y = 146.5*X - 702.0$	0.802	0.016
	qCO_2	$Y = 0.001071*X - 0.003587$	0.697	0.039
	pH	$Y = 1.667*X - 2.451$	0.679	0.044
EC_{50} Ag^{+}	*cus*C gene	$Y = -0.7614*X + 9.412$	0.787	0.018
EC_{50} Van	qCO_2	$Y = -0.01466*X + 0.02305$	0.907	0.003
	Total Cu (mg kg^{-1} soil)	$Y = -1865*X + 2739$	0.902	0.004
	Ext Cu (mg kg^{-1} soil)	$Y = -2.997*X + 4.414$	0.855	0.008
	pH	$Y = -20.69*X + 35.93$	0.726	0.031

[a] Ratio between ΔA_{590} glucose (glucose induced C-respiration using MicroResp) and AMN (microbial biomass).
[b] Anaerobically mineralisable nitrogen.
[c] Calculated by 16S (bacterial) to ITS (fungal) rRNA gene copy numbers.

microbial communities is extent of pre-exposure (Dìaz-Raviña and Bååth, 1996; Fernández-Calviño et al., 2011), a property which makes PICT a sensitive measure for levels of exposure to toxicants in the environment (Blanck et al., 1988).

Tolerance within the microbial community was strongly correlated with a measure of the metabolic quotient (qCO_2). This finding indicates that physiological attributes associated with the microbial community represent a primary factor underpinning PICT in this ecosystem. In the field soil, we found that increased PICT was strongly correlated with an increase in qCO_2 (substrate added). However, different studies have found system-specific variation in the response of qCO_2 and metal contamination. For example, Stefanowicz et al. (2010) found an increase in qCO_2 with metal contamination in grassland soil, but not in samples collected from under pine forest. Prasad et al. (2013) described variation in increasing qCO_2 with arsenic toxicity across three different soil types, and Khan and Scullion (2000) described positive or negative relationships depending on level of pollution. Furthermore, declines in qCO_2 have been found in some metal-contaminated systems (e.g. Khan and Scullion, 2002; Killham, 1985; Liu et al., 2012). As such, a high qCO_2 in itself is not proof of high-maintenance energy requirements or physiological adaptation in metal contaminated soil (Giller et al., 1998).

In soil with increasing amount of Cu^{+2} contamination the soil microbial community differed, even 15 years post Cu-application, with a shift towards bacterial dominance. The data plots show that changes in the bacteria:fungal ratio (Fig 2d) matched very closely with those of PICT to Cu^{2+} (Fig 1a) and soil physiological status (Fig 2a) across the soils. However, it is possible that increasing bacterial abundance may underpin the observed PICT; i.e. the mechanism is not related to broad physiological adaptation (stress-induced qCO_2 across the ecosystem) *per se*, but reflects a shift in dominant taxa which innately have different qCO_2. In support of this hypothesis, Six et al. (2006) showed that C use efficiency (CUE) varied between fungi and bacteria, with fungi (on average) allocating a greater amount of C-metabolised to biomass. This may explain the altered qCO_2 observed in this study. The relative susceptibly of soil bacteria and fungi to Cu is an area of on-going research, however various studies give conflicting results. There have been reviews of this work previously (e.g. El-Ghamry et al., 2000).

Witter et al. (2000) measured microbial respiration and community compositional changes (phospholipid fatty acid profiles) in soils exposed to metal-amended sewage sludge. Their findings indicated that change in growth characteristics of metal-exposed microorganisms (e.g. lag-time for exponential growth following substrate addition) was unlikely to be due to inherent changes in physiological status of the microorganisms *per se*, but rather alteration of the microbial community or substrate utilisation patterns. These findings lend support to our hypothesis that community structural change may be a primary factor underpinning PICT in soil ecosystems. This can be assessed by conducting more detailed analysis of the microbial community composition, assessment of substrate utilisation patterns, and qualitative assessment of soil C fractions (assuming long-term alteration of substrate utilisation may leave a signature in the soil C pool). These analyses will be conducted on the set of Cu-exposed LUDF soils used in the current study.

A number of genetic mechanisms have been associated with Cu resistance in bacteria, the best well known being the *cop* genes that were originally described in *Pseudomonas syringae* (Mellano and Cooksey, 1988) but have subsequently been found in a diverse range of microorganisms. The *cop* system encodes a P-type ATPase that pumps excess Cu out of the cytoplasm (Rensing et al., 2000). In studies that have recovered Cu-resistant bacteria from contaminated soils, the development of Cu-adaption has been linked to the presence of *cop*A and Lejon et al. (2007) provided evidence that *cop*A gene diversity is linked to soil physicochemical properties. Given these ecosystem properties control the wider structure of the bacterial community (Lauber et al., 2008; Wakelin et al., 2008), and the broad distribution of *cop*A across soil taxa, this might be expected. Furthermore, on application of $CuCl_2$ to soils, shifts in the banding patterns of *cop*A were detected supporting that a change in community diversity had occurred, but relating this directly to *cop*A mediated selection remains tenuous. The GeoChip array has >750 probes for *cop*A genotypes spanning the major bacteria Phyla (*Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and others), and also Archaeal and fungal *cop*A genotypes. Accordingly, the array represents a novel tool whereby functions, such as metal resistance, can be assessed within diverse communities holding reservoirs of wide genotypic diversity for any particular process. We found the abundance of total *cop*A genes was not related to an increase in the community tolerance to Cu^{2+}. Thus, shifts in *cop*A genotypes observed in other studies may simply be due to more general trends in total community compositional changes. Similarly, the abundances of *cus* and *cue* genes were not related to Cu^{2+} PICT. The *cus*CFBA operon encodes a copper transporting efflux system and confers resistance to both Cu and Ag (Munson et al., 2000), while the *cue* genes protect enzymes from Cu damage (Grass and Rensing, 2001). Although it remains possible that tolerance to Cu^{+2} may be linked to the abundance of Cu-resistance genes not covered on the array, for example *pco* genes (Brown et al., 1995), we found no evidence to support the hypothesis that accumulation of metal tolerance genes provides the mechanistic basis of PICT in long term (15 y) Cu exposed soils.

While there was no evidence for co-tolerance to Ag^{+} in soils previously treated with Cu spiked biosolids, community tolerance to Ag^{+} was inversely correlated to the *cus*A gene signal intensity in the soils ($r = -0.787$; $P = 0.018$). This relationship was even stronger when the *cus*AFC genes were combined (0.835; $P = 0.011$), indicating that the relationship was genuine. As the *cus*ABCF operon confers tolerance to both Cu and Ag, it would be anticipated that the relationship would be positive. Further research will be required to understand this inconsistency.

Co-tolerance to Cu_{np} was evident, but only in soils previously exposed to the highest doses of Cu^{2+}. In some microorganisms, such as yeasts, Cu nanoparticles can induce toxicity by mechanisms that are different to those related to soluble Cu^{2+} (Kasemets et al., 2009); in these instances, mechanisms directly associated with Cu^{2+} resistance may not confer co—or cross-tolerance to Cu_{np}. However, it is generally found that the cause of toxicity is soluble Cu^{2+} which has been 'chemically speciated' from the Cu_{np} (Rousk et al., 2012). In these cases, soil and environmental factors affecting rate of dissolution of Cu^{2+} from the Cu_{np} will control the extent of toxicity to the microbial community.

Exposure of soils to Cu has previously been shown to result in co-selection of resistance to a range of antibiotics, including vancomycin, tetracycline, tylosin, chloramphenicol and others (Berg et al., 2010; Fernández-Calviño and Bååth, 2013; Seiler and Berendonk, 2012). At a community level, this is thought to occur through increased co-selection for antibiotic resistance genes (Knapp et al., 2011) which may, for example, be present on multi-resistance plasmids, and thus be inadvertently selected for. In this study, we found no increase in vancomycin resistance with increased Cu, nor was there any indication of an enrichment of genes encoding resistance to vancomycin or other antibiotics in the soil metagenome. Thus, the phenomenon of metal-selected antibiotic cross resistance appears to be ecosystem specific. This is supported by study by Brandt et al. (2012) who examined antibiotic selection across 18 Cu-contaminated soils and found that while development of tetracycline resistance was frequent, resistance to vancomycin, streptomycin and other antibiotics occurred infrequently. These findings further demonstrate the variation in co- and cross-tolerance phenomena between different microbial ecosystems.

While microbial community structure provides a sensitive measure of Cu-stress in the environment (Berg et al., 2012; Macdonald et al., 2011; Wakelin et al., 2010b), changes in species composition within bacterial or fungal communities may not directly underpin PICT and co-tolerance mechanisms. In particular, changes in the composition of bacterial species across the field samples were only related to soil physicochemical properties (pH, SO_4, and Fe) and had no relationship to soil Cu level, PICT to Cu^{2+} or tolerance to other pollutants tested. While the fungal community composition was found to co-vary with PICT to Cu and tolerance to vancomycin, it also had relationships to qCO_2 and the bacteria:fungal ratio. As such, we propose that fungal community composition is not causally linked to the PICT and tolerance mechanisms measured, but is a secondary indicator of these. However, species selection and replacement appears to be an important process occurring in the soil ecosystem, particularly for the fungal community in response to soil Cu^{2+} level. These compositional changes are important to support function in Cu contaminated soils, such as degradation of plant residues (Wakelin et al., 2010b) and N cycling (Mertens et al., 2010), and are therefore likely to be involved in PICT. Our inability to detect this in the LUDF soils may either be the result of relatively low resolution of the molecular tools used (even DGGE can only reasonably assess hundreds of species which is several orders-of-magnitude lower than the total richness; Curtis et al., 2002), or is due to the relatively low level of samples available from the field site for analysis. Ideally the field trial design would have included a higher number of plots spanning a greater Cu-range, thus providing for improved statistical power. However, this study is largely exploratory of the potential community level processes associated with PICT and co-tolerance with the goal of identifying the most appropriate areas for future investigation.

5. Conclusion

We demonstrated that PICT to Cu^{2+} was present in the soil microbial ecosystem exposed to Cu-spiked biosolids since 1997. The

primary factor associated with PICT was alteration in the physiological status of the soil microbial community, as measured by qCO_2, an indication of either increasing overall tolerance stress, or shift in the community composition towards bacterial dominance. In these soils, the development of neither PICT to Cu^{+2} nor co-tolerance to Ag^{+} or vancomycin was underpinned by changes in the numbers of genes associated with metal or antibiotic resistance. The abundances of these genes may be important for development of community-level tolerance immediately after exposure, but appear to have no long-term role in underpinning tolerance in Cu-exposed field soils. Further work on short and long-term contaminated soils is required to determine how these tolerance and adaptation mechanisms evolve in communities over time and then operate when stable systems are reached.

Acknowledgements

This work was supported by the New Zealand Ministry of Science and Innovation, contract C08X0802. The authors would like to acknowledge the Institute of Environmental Science and Research for access to the field trials. A draft of this manuscript was kindly reviewed by Dr Maria Tourna, AgResearch Ltd. We especially acknowledge an anonymous journal reviewer who provided excellent and useful professional comment on this manuscript.

References

Alloway, B.J., 2008. Copper and zinc in soils: too much or too much?. In: New Zealand Trace Elements Group Conference, 13–15 Feb. University of Waikato, Hamilton, New Zealand, 10 pp.

Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O., Brandt, K.K., 2010. Cu exposure under field conditions co-selects for antibiotic resistance as determined by novel cultivation-independent bacterial community tolerance assay. Environ. Sci. Technol. 44, 8724–8728.

Berg, J., Brandt, K.K., Al-Soud, W.A., Holm, P.E., Hansen, L.H., Sørensen, S.J., Nybroe, O., 2012. Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term cu exposure. Appl. Environ. Microbiol. 78, 7438–7446.

Blakemore, L.C., Searle, P.L., Daly, B.K., 1987. Methods for chemical analysis of soils. N. Z. Soil Bur. Sci. Rep. 80, 31–45.

Blanck, H., Dahl, B., 1996. Pollution-induced community tolerance (PICT) in marine periphyton in a gradient of tri-*n*-butyltin (TBT) contamination. Aquat. Toxicol. 35, 59–77.

Blanck, H., Wangberg, S.A., 1988. Induced community tolerance in marine periphyton established under arsenate stress. Can. J. Aquat. Sci. 45, 1816–1819.

Blanck, H., Wangberg, S.A., Molander, S., 1988. Pollution-induced community tolerance. A new ecotoxicological tool. In: Cairns, J.J., Pratt, J.R. (Eds.), Functional Testing of Aquatic Biota for Estimating Hazards of Chemicals. ASTM STP 988. American Society for Testing and Materials, Philadelphia, pp. 219–230.

Brandt, K.K., Berg, J., Hangler, M., Modrzynski, J., Eckstein, C., Nicolaisen, M.H., Anskjær, G.G., Magid, J., Holm, E., Sørensen, J., Nybroe, O., 2012. Antibiotic resistance in soil bacterial communities: comparison of metals and antibiotic residues as selecting agents and spatial heterogeneity of co-selected resistance patterns. In: 14th International symposium on Microbial Ecology, 19–24 August 2012. Denmark, Copenhagen.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 325–349.

Brown, N.L., Barrett, S.R., Camakaris, J., Lee, B.T.O., Rouch, D.A., 1995. Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Mol. Microbiol. 17, 1153–1166.

Breure, A.M., Groot, M., Eijsackers, H.J.P., 2008. System-oriented ecotoxicological research: which way to go? Sci. Total Environ. 406, 530–536.

Bruins, M.R., Kapil, S., Oehme, F.W., 2000. Microbial resistance to metals in the environment. Ecotoxicol. Environ. Saf. 45, 198–207.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microbiol. 69, 3593–3599.

Clarke, K.R., 1993. Non-parametric multivariate analysis of changes in community structure. Aust. J. Ecol. 18, 117–143.

Clarke, K.R., Warwick, R.M., 2001. Change in Marine Communities: an Approach to Statistical Analysis and Interpretation, second ed. PRIMER-E Ltd, Plymouth, U.K.

Cooksey, D.A., 1994. Molecular mechanisms of copper resistance and accumulation in bacteria. FEMS Microbiol. Rev. 14, 381–386.

Coombs, J.M., Barkay, T., 2005. New findings on evolution of metal homeostasis genes: evidence from comparative genome analysis of bacteria and archaea. Appl. Environ. Microbiol. 71, 7083–7091.

Curtis, T.P., Sloan, W.T., Scannell, J.W., 2002. Estimating prokaryotic diversity and its limits. In: Proceedings of the National Academy of Sciences USA, vol. 99, pp. 10494–10499.

Dahlin, S., Witter, E., 1998. Can the low microbial biomass C-to-organic C ratios in an acid and a metal contaminated soil be explained by differences in growth characteristics, substrate utilisation efficiency or maintenance requirements? Soil Biol. Biochem. 30, 633–641.

Demoling, L.A., Bååth, E., 2008. Use of pollution-induced community tolerance of the bacterial community to detect phenol toxicity in soil. Environ. Toxicol. Chem. 27, 334–340.

Dìaz-Raviña, M., Bååth, E., 1996. Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. Appl. Environ. Microbiol. 62, 2970–2977.

Dìaz-Raviña, M., Bååth, E., Frostegård, Å., 1994. Multiple heavy-metal tolerance of soil bacterial communities and its measurement by a thiamine incorporation technique. Appl. Environ. Microbiol. 60, 2238–2247.

Eijsackers, H.J.P., Groot, M., Breure, A.M., 2008. Upgrading system-oriented ecotoxicological research. Sci. Total Environ. 406, 373–384.

El-Ghamry, A.M., Subhani, A., Moh'd, W., Changyong, H., Zhengmiao, X., 2000. Effects of copper toxicity on soil microbial biomass. Pak. J. Biol. Sci. 3, 907–910.

Fernández-Calviño, D., Estévez, M., Díaz-Raviña, M., Bååth, E., 2011. Bacterial pollution-induced community tolerance (PICT) to Cu and interactions with pH in long-term polluted vineyard soils. Soil Biol. Biochem. 43, 2324–2331.

Fernández-Calviño, D., Bååth, E., 2013. Co-selection for antibiotic tolerance in Cu-polluted soil is detected at higher Cu-concentrations than Cu-tolerance. Soil Biol. Biochem. 57, 953–956.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol. Biochem. 30, 1389–1414.

Gong, P., Gasparrini, P., Rho, D., Hawari, J., Thiboutot, S., Ampleman, G., Sunahara, G.I., 2000. An *in situ* respirometric technique to measure pollution-induced microbial community tolerance in soils contaminated with 2,4,5-trinitrotoluene. Ecotoxicol. Environ. Saf. 47, 96–103.

Grass, G., Rensing, C., 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. Biochem. Biophys. Res. Commun. 286, 902–908.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M., 1999. The effect of long-term phosphatic fertiliser applications on the amounts and forms of cadmium in soils under pasture in New Zealand. Nutr. Cycl. Agroecosystems 54, 267–277.

Gustavson, K., Petersen, S., Pedersen, B., Stuer-Lauridsen, F., Pedersen, S., Wängberg, S.-Å., 1999. Pollution-induced community tolerance (PICT) in coastal phytoplankton communities exposure to copper. Hydrobiologia 416, 125–138.

He, Z., Deng, Y., Van Nostrand, J.D., Tu, Q., Xu, M., Hemme, C.L., Li, X., Wu, L., Gentry, T.J., Yin, Y., Leibich, J., Hazen, T.C., Zhou, J., 2010. GeoChip 3.0 as a high-throughput tool for analyzing microbial community composition, structure and functional activity. ISME J. 4, 1167–1179.

Heuer, H.K., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: van Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology. Marcel Dekker, New York, pp. 353–373.

Kasemets, K., Ivask, A., Dubourguier, H.C., Kahru, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol. In Vitro 23, 1116–1122.

Kaufmann, K., Chapman, S.J., Campbell, C.D., Harms, H., Höhener, P., 2006. Miniaturized test system for soil respiration induced by volatile pollutants. Environ. Pollut. 140, 269–278.

Khan, M., Scullion, J., 2000. Effect of soil on microbial responses to metal contamination. Environ. Pollut. 110, 115–125.

Khan, M., Scullion, J., 2002. Effects of metal (Cd, Cu, Ni, Pb or Zn) enrichment of sewage-sludge on soil micro-organisms and their activities. Appl. Soil Ecol. 20, 145–155.

Killham, K., 1985. A physiological determination of the impact of environmental stress on the activity of microbial biomass. Environ. Pollut. (Ser. A) 38, 283–294.

Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G., Graham, D.W., 2011. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. PLoS One 6, e27300.

Kovács, B., Prokisch, J., Györi, Z., Kovács, A.B., Palencsár, A.J., 2000. Studies on soil sample preparation for inductively coupled plasma atomic emission spectrometry analysis. Commun. Soil Sci. Plant Anal. 31, 1949–1963.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Chichester, UK, pp. 115–175.

Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 40, 2407–2415.

Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative qPCR quantification of plasmid copy number in Escherichia coli. J. Biotechnol. 123, 273–280.

Lejon, D.P.H., Nowak, V., Bouko, S., Pascault, N., Mougel, C., Martins, J.M.F., Ranjard, L., 2007. Fingerprinting and diversity of bacterial *cop*A genes in

response to soil types, soil organic status and copper contamination. FEMS Microbiol. Ecol. 61, 424–437.
Liu, Y., Zhou, T., Crowley, D., Li, L., Liu, D., Zheng, J., Yu, X., Pan, G., Jussain, Q., Zhang, X., Zheng, J., 2012. Decline in topsoil microbial quotient, fungal abundance and C utilization efficiency of rice paddies under heavy metal pollution across South China. PLoS One 7, e38858.
Lu, Z.-M., Deng, Y., Van Nostrand, J.D., He, Z., Voordeckers, J., Zhou, A., Lee, Y.-J., Mason, O., Dubinsky, E., Chavarria, K., Tom, L., Fortney, J., Lamendella, R., Jansson, J.K., D'hasesleer, P., Hazen, T.C., Zhou, J.-Z., 2012. Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. ISME J. 6, 451–460.
Macdonald, C.A., Clark, I.M., Zhao, F.-J., Hirsch, P.R., Singh, B.K., McGrath, S.P., 2011. Long-term impacts of zinc and copper enriched sewage sludge additions on bacterial, archaeal and fungal communities in arable and grassland soils. Soil Biol. Biochem. 43, 932–941.
McLaren, R.G., Clucas, L.M., 2001. Fractionation of copper, nickel, and zinc in metal-spiked sewage sludge. J. Environ. Qual. 30, 1968–1975.
Mellano, M.A., Cooksey, D.A., 1988. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 170, 2879–2883.
Mertens, J., Wakelin, S.A., Broos, K., McLaughlin, M.J., Smolders, E., 2010. Extent of copper tolerance and consequences for functional stability of the ammonia-oxidizing community in long-term copper-contaminated soils. Environ. Toxicol. Chem. 29, 27–37.
Mirsa, T.K., 1992. Bacterial resistance to inorganic mercury salts and organomercurials. Plasmid 27, 4–16.
Motulsky, H.M., Brown, R.E., 2006. Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinforma. 7, 123.
Munson, G.P., Lam, D.L., Outten, F.W., O'Halloran, T.V., 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J. Bacteriol. 182, 5864–5871.
Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
Ogilvie, L.A., Grant, A., 2008. Linking pollution induced community tolerance (PICT) and microbial community structure in chronically metal polluted estuarine sediments. Mar. Environ. Res. 65, 187–198.
Ohtonen, R., 1994. Accumulation of organic matter along a pollution gradient: application of Odum's theory of ecosystem energetic. Microb. Ecol. 27, 43–55.
Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. In: Proceedings of the Royal Society London Series B, vol. 163, pp. 224–231.
Prasad, P., George, J., Masto, R.E., Rout, T.K., Ram, L.C., Selvi, V.A., 2013. Evaluation of microbial biomass and activity in different soils exposed to increasing level of arsenic pollution: a laboratory study. Soil Sediment. Contam. 22, 483–497.
Rensing, C., Fan, B., Sharma, R., Mitra, B., Rosen, B.P., 2000. CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. In: Proceedings of the National Academy of Sciences USA, vol. 97, pp. 652–656.
Rousk, J., Ackermann, K., Curling, S.F., Jones, D.L., 2012. Comparative toxicity of nanoparticulate CuO and ZnO to soil bacterial communities. PLoS One 7, e34197.
Rutgers, M., 2008. Field effects of pollutants at the community level—experimental challenges and significance of community shifts for ecosystem functioning. Sci. Total Environ. 406, 469–478.
Salminen, J., van Gestel, C.A.M., Oksanen, J., 2001. Pollution-induced community tolerance and functional redundancy in a decomposer food web in metal-stressed soil. Environ. Toxicol. Chem. 20, 2287–2295.
Schmitt, H., van Beelen, P., Tolls, J., van Leeuwen, C.L., 2004. Pollution-induced community tolerance of soil microbial communities caused by the antibiotic sulfachloropyridazine. Environ. Sci. Technol. 38, 1148–1153.
Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3. Article 399.
Silver, S., Phung, L.T., 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. 50, 753–789.
Six, J., Frey, S.D., Batten, K.M., 2006. Bacterial and fungal contributions to carbon sequestration in agroecosystems. Soil Sci. Soc. Am. J. 70, 555–569.
Soler-Rovira, P., Fernández-Calviño, D., Arias-Estévez, M., Plaza, C., Polo, A., 2013. Respiration parameters determined by the ISO-17155 method as potential indicators of copper pollution in vineyard soils after long-term fungicide treatment. Sci. Total Environ. 447, 25–31.
Stefanowicz, A.M., Niklińska, M., Laskowski, R., 2009. Pollution-induced community tolerance of soil bacterial communities in meadow and forest ecosystems polluted with heavy metals. Eur. J. Soil Biol. 45, 363–369.
Stefanowicz, A.M., Niklińska, M., Kapusta, P., Szarek-Lukaszewska, G., 2010. Pine forest and grassland differently influence the response of soil microbial communities to metal contamination. Sci. Total Environ. 408, 6134–6141.
Vilgalys, R., Hester, M., 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172, 4238–4246.
Wakelin, S.A., Colloff, M.J., Harvey, P.R., Marschner, P., Gregg, A.L., Rogers, S.L., 2007. The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundance under irrigated maize. FEMS Microbiol. Ecol. 59, 661–670.
Wakelin, S.A., Macdonald, L.M., Rogers, S.L., Gregg, A.L., Bolger, T.P., Baldock, J.A., 2008. Habitat selective factors influencing the structural composition and functional capacity of microbial communities in agricultural soils. Soil Biol. Biochem. 40, 803–813.
Wakelin, S.A., Chu, G., Broos, K., Clarke, K.R., Liang, Y., McLaughlin, M.J., 2010a. Structural and functional responses of soil microbiota to addition of plant substrate are moderated by soil Cu levels. Biol. Fertil. Soils 46, 333–342.
Wakelin, S.A., Chu, G.X., Lardner, R., Liang, Y.C., McLaughlin, M., 2010b. A single application of Cu to field soil has long-term effects on bacterial community structure, diversity, and soil processes. Pedobiologia 53, 149–158.
Wakelin, S.A., Lombi, E., Donner, E., MacDonald, L., Black, A., O'Callaghan, M., 2013a. Application of MicroResp™ for soil ecotoxicology. Environ. Pollut. 179, 177–184.
Wakelin, S.A., van Koten, C., O'Callaghan, M., Brown, M., 2013b. Physicochemical properties of 50 New Zealand pasture soils: a starting point for assessing and managing soil microbial resources. N. Z. J. Agric. Res. 56, 248–260.
Whelan, J.A., Russell, N.B., Whelan, M.A., 2003. A method for the absolute quantification of cDNA using real-time PCR. J. Immunol. Methods 278, 261–269.
Witter, E., Gong, P., Bååth, E., Marstrop, H., 2000. Study of the structure and metal tolerance of the soil microbial community six years after cessation of sewage sludge applications. Environ. Toxicol. Chem. 19, 1983–1991.
Wu, L., Liu, X., Schadt, C.W., Zhou, J.-Z., 2006. Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. Appl. Environ. Microbiol. 72, 4931–4941.
Zabaloy, M.C., Garland, J.L., Gomez, M.A., 2010. Assessment of the impact of 2,4-dichlorophenoxyacetic acid (2,4-D) on indigenous herbicide-degrading bacteria and microbial community function in an agricultural soil. Appl. Soil Ecol. 46, 240–246.