Environmental Pollution 194 (2014) 78–85

Contents lists available at ScienceDirect

Environmental Pollution

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

Evaluation of the leucine incorporation technique for detection of pollution-induced community tolerance to copper in a long-term agricultural field trial with urban waste fertilizers

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ARTICLE INFO

Article history:
Received 6 April 2014
Received in revised form
10 July 2014
Accepted 12 July 2014
Available online

Keywords:
Bacterial community
Bioreporter
Cu-ion selective electrode
Metal speciation
Metal tolerance

ABSTRACT

Copper (Cu) is known to accumulate in agricultural soils receiving urban waste products as fertilizers. We here report the use of the leucine incorporation technique to determine pollution-induced community tolerance (Leu-PICT) to Cu in a long-term agricultural field trial. A significantly increased bacterial community tolerance to Cu was observed for soils amended with organic waste fertilizers and was positively correlated with total soil Cu. However, metal speciation and whole-cell bacterial biosensor analysis demonstrated that the observed PICT responses could be explained entirely by Cu speciation and bioavailability artifacts during Leu-PICT detection. Hence, the agricultural application of urban wastes (sewage sludge or composted municipal waste) simulating more than 100 years of use did not result in sufficient accumulation of Cu to select for Cu resistance. Our findings also have implications for previously published PICT field studies and demonstrate that stringent PICT detection criteria are needed for field identification of specific toxicants.

1. Introduction

Copper (Cu) is currently accumulating in many agricultural soils around the world due to the widespread use of Cu-based pesticides (Komárek et al., 2010), use of Cu as a growth promoter in livestock production (Gräber et al., 2005), and recycling of urban waste products (McBride, 1995; Smith, 2009). Cu accumulation may compromise soil quality, but it is challenging to elucidate adverse field effects caused specifically by Cu toxicity in agricultural soils receiving organic waste fertilizers due to various confounding soil factors (e.g. soil pH, organic matter content, texture etc.) that prevent establishment of specific cause–effect relationships.

The pollution-induced community tolerance (PICT) approach offers a potential solution allowing researchers to infer causal relationships between toxicant exposure and toxic effects under field conditions provided that potential PICT detection artifacts can be excluded (Blanck, 2002). Any PICT investigation comprises two phases. In the initial PICT selection phase the toxicant exerts a selection pressure causing elimination of the most sensitive community members and proliferation of resistant members (Blanck, 2002). During the subsequent PICT detection phase community tolerance to the toxicant is quantified in a short-term activity assay where the community is again exposed to the toxicant (Blanck, 2002). In general, community tolerance to a specific toxicant will develop only if the toxicant exerts toxicity to a significant fraction of the targeted biotic community. This is exemplified by PICT field studies carried out in Cu contaminated soils originating from various vineyard sites in Spain (Díaz-Raviña et al., 2007; Fernández-Calviño et al., 2011). These studies indicated a specific role of soil Cu for shaping bacterial communities, which could not be easily determined by analysis of microbial community structure due to confounding factors (Fernández-Calviño et al., 2010).

Soil bacteria are generally perceived to be more vulnerable towards Cu contaminations as compared to other soil organisms (Giller et al., 1998; Rajapaksha et al., 2004), and even quite low, frequently encountered concentrations of soil Cu have been linked to PICT development in bacterial communities (Pennanen et al., 1996; Dahlin et al., 1997; Bååth et al., 1998; Brandt et al., 2010; Berg et al., 2012). In all these studies, PICT detection was based on the [^{3}H]thymidine (TdR) or [^{3}H]leucine (Leu) incorporation

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http://dx.doi.org/10.1016/j.envpol.2014.07.013
0269-7491/

methods originally developed for measurement of bacterial growth rates in soil (Bååth, 1990, 1994) and later adopted for determination of PICT (TdR-PICT or Leu-PICT) in soil bacterial communities (Bååth, 1992; Bååth et al., 2001). The Leu-PICT assay probably represents the best candidate for a standardized PICT detection approach for soil bacterial communities as it has very broad community coverage and seems to be more sensitive than competing PICT detection assays such as flow cytometry-based fluorescence probing (Brandt et al., 2009) and PICT detection in Biolog plates (Demoling et al., 2009).

Our aims were to investigate if application of sewage sludge and composted municipal household waste allows Cu to accumulate to toxic levels in soil using the Leu-PICT method and to critically evaluate the Leu-PICT approach for inferring toxic effects of copper in field soils with contrasting soil organic matter (SOM) status. To this end, we took advantage of a long-term agricultural field trial ('CRUCIAL') that simulate more than 100 years of organic waste disposal according to current Danish agricultural practice (Magid et al., 2006b). Only low levels of Cu contamination were expected to occur during the 'CRUCIAL' field trial and we therefore hypothesized that Cu inputs via organic amendments were insufficient to increase the level of bacterial Cu tolerance. However, we also hypothesized that contrasting SOM status of the studied soils could lead to differences of solution chemistry (eg. pH, dissolved organic matter etc.) in the soil bacterial suspensions used for Leu-PICT detection. This could potentially lead to bioavailability artifacts due to differential Cu speciation and toxicity during PICT detection (Blanck, 2002). Hence, we thoroughly evaluated possible Cu speciation and bioavailability artifacts during PICT detection in order to provide operational recommendations for future studies relying on the leu-PICT approach.

2. Materials and methods

2.1. Description of field site and soil sampling

The CRUCIAL field site was established in 2002 using a randomized block design with three replicate blocks (Magid et al., 2006b). Each plot covers 891 m^2 and the soil is characterized as a sandy loam (clay content 12–19%, pH_{H_2O} 6.6–7.5, %C 1.1–3.2). Soil was sampled from the following 'CRUCIAL' treatments: unfertilized control (U), mineral fertilizer control (NPK), cattle slurry at a rate corresponding to Danish agricultural practice (CS), sewage sludge (S), sewage sludge at an accelerated rate (SA), cattle manure at an accelerated rate (CMA), composted municipal sorted household waste (CH) and composted municipal sorted household waste at an accelerated rate (CHA). Soil for PICT detection was sampled at two occasions: in January 2011 and October 2011. Representative soil samples were collected by pooling at least 20 sub-samples from each plot to a depth of approximately 20 cm using a soil auger. Soil was air-dried to a soil moisture of 10% (wt/dry wt), sieved (<8 mm), and stored in closed polyethylene (PE) bags at 4 °C until further analyses. Total soil Cu content (Cu_{TOT}) was assessed in soil sampled in January 2011.

2.2. Chemical soil analysis

Total Cu content was determined in finely grinded soil (0.25 g) subjected to a microwave assisted digestion using 9 mL 70% HNO_3, 1 mL 30% H_2O_2, 2 mL 30% HCl and 3 mL 40% HF according to the USEPA 3052 method (USEPA, 1996). Cu was quantified by graphite furnace atomic absorption spectroscopy (GFAAS, Perkin Elmer 5100, Zeeman 5100, PE Applied Biosystems, Foster City, CA). Soil pH was measured in water (soil:water ratio of 1:2.5). Dissolved organic carbon (DOC) in soil bacterial suspensions was measured using a Shimadzu TOC-V CPN total organic carbon analyzer after dilution (1:1) in Milli-Q water. Soil C content was analyzed by isotope-ratio mass spectrometry (IR-MS) on a Europa Scientific ANCA NT System.

2.3. Biosensor analysis of bioavailable Cu

A Cu-specific, *Pseudomonas fluorescens* DF57-Cu15 biosensor strain expressing *luxAB* reporter genes (Tom-Petersen et al., 2001) was used to estimate the amount of bioavailable Cu in soil–water extracts as described previously (Brandt et al., 2008) except that a final concentration of 0.01% Tween 20 non-ionic surfactant was added to the biosensor cell suspension. In brief, exponential phase biosensor cells were harvested by centrifugation and resuspended in a strongly buffered (pH 7.2) minimal medium with a low capacity for Cu complexation (Nybroe et al., 2008). Subsequently, biosensor aliquots (100 μL) were mixed with an equal volume of soil–water extracts (samples). Biosensor activity (bioluminescence) was quantified in a FluoStar Optima plate reader after 1.5 h of incubation (BMG Labtech, Offenburg, Germany) (Nybroe et al., 2008). The amount of bioavailable Cu per mass of dry soil (Cu_{bio}) was calculated assuming zero bioavailability of particle-associated Cu (Brandt et al., 2006).

2.4. PICT detection

2.4.1. PICT experiment 1

Bacteria were extracted from soil by shaking 10 g (fresh weight) of soil with 100 mL of Milli-Q water on a horizontal shaker (250 rpm, 15 min, 22 °C) followed by centrifugation (1000 g, 10 min, 22 °C). Soil bacterial suspensions (supernatants) were amended with 6.4 mM MOPS (3-(N-morpholino) propanesulfonic acid, CAS no: 1132-61-2, Sigma–Aldrich, pH 7.1) to give a final concentration of 0.2 mM MOPS. Subsamples (1.5 mL) were pre-incubated (30 min, 22 °C) in 2 mL micro-tubes with different concentrations of $CuSO_4$ (see Results). [^{3}H]leucine incorporations were initiated by adding 50 μL of a mixture of [^{3}H]leucine (2.59 TBq $mmol^{-1}$, 37 MBq mL^{-1}, Amersham, Hillerød, Denmark) and unlabeled L-leucine to give 6 kBq per microtube and 200 nM L-leucine. Negative controls were amended with 160 μL of 50% trichloroacetic acid (TCA) before adding [^{3}H]leucine. The incubations were stopped after 3 h by the addition of 160 μL ice cold 50% TCA. The incorporation of [^{3}H]leucine into precipitated proteins was separated from non-incorporated [^{3}H] leucine through a series of washing and centrifugation steps (Bååth et al., 2001) and quantified by scintillation counting (Brandt et al., 2004). Bacterial community tolerance was quantified as a tolerance index (TI) (Brandt et al., 2009): $TI = L_{inc\_Cu}/L_{inc\_control}$, where L_{inc_Cu} equals the average amount of incorporated [^{3}H]leucine (dpm mL^{-1}) in replicated samples ($n = 3$) amended with Cu and $L_{inc\_control}$ equals the average amount of incorporated [^{3}H]leucine (dpm mL^{-1}) in corresponding replicated control samples ($n = 3$) not amended with Cu.

2.4.2. PICT experiment 2

We had experienced an insufficient pH control in PICT experiment 1 (data not shown) and hypothesized that pH-dependent Cu speciation artifacts could potentially bias PICT detection. In a subsequent PICT experiment we therefore improved pH control during PICT detection by increasing buffer concentration, but we experienced problems with precipitates in Cu standards at pH 7.1. The assay pH for PICT detection was therefore lowered to 6.4 and MOPS was replaced by a MES (4-morpholineethanesulfonic acid, CAS no: 4432-31-9, Sigma–Aldrich) buffer (final concentration in assay: 10 mM, pH 6.4). MES buffer was directly used for extraction of soil bacteria, but all other procedures were performed as described for PICT experiment 1.

2.4.3. PICT experiment 3

During PICT experiment 2 we realized that the soil bacterial suspensions made using MES as an extraction medium were less colored. We therefore hypothesized that less dissolved organic carbon was extracted when using MES as an extraction medium compared to Milli-Q water. A final PICT experiment was therefore carried out as described for PICT experiment 2 except that buffer (10.66 mM, pH 6.4) was compared with Milli-Q water as extraction medium for bacteria prior to PICT detection. Soil bacterial suspensions prepared with water as extractant were amended with 320 mM MES buffer (pH 6.4) in order to carry out all [^{3}H]leucine incorporation assays in the same buffer solution (pH 6.4 MES buffer, 10 mM as final concentration).

2.5. Cu toxicity and speciation in spiked soil bacterial suspensions used for PICT detection

A bioluminescent *P. fluorescens* DF57-40E7 biosensor strain constitutively expressing the *luxAB* genes was used to estimate Cu toxicity (i.e. inhibition of bioluminescence emission) in the spiked soil bacterial suspensions used for PICT detection. Soil bacterial suspensions to be analyzed for Cu toxicity were spiked with either 0, 4, 8, or 16 μM $CuSO_4$. Following pre-incubation (2 h, 22 °C), soil bacterial suspensions (100 μL) and external Cu standards (100 μL) were mixed with biosensor cell suspensions (100 μL) and bioluminescence was recorded 90 min later as described above (Section 2.2).

Free Cu^{2+} ion activities were quantified in soil bacterial suspensions spiked with 16 μM $CuSO_4$ by an ion selective electrode (ISE_{Cu}, Cu-electrode ISE25Cu, Radiometer, Copenhagen, Denmark) coupled with a double-junction (KCl-saturated inner reservoir) reference electrode (Reference electrode 251, Radiometer) as described previously (Brandt et al., 2008). The protocol was based on Rachou et al. (2007).

2.6. Data analyses

SigmaPlot Version 12 (Systat Software, Point Richmond, CA) was used for regression analyses. Significance testing of treatment effects was performed by ANOVA or paired *t*-test using the [stats] package in R version 2.15.2 (R Core Team, 2012). Models were reduced to only include main effects if the interaction terms were not significant. All pair wise multiple comparisons were performed using Tukey's test.