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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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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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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.

3. Results

3.1. PICT towards Cu in soils amended with organic waste products (PICT experiment 1)

Bacterial Cu tolerance was significantly affected by the different qualities and quantities of organic fertilizers used in the 'CRUCIAL' field trial (Fig. 1; $P < 0.0001$; linear model with only main effects included). Compared to the unfertilized control (U) significantly higher tolerance levels were observed in four of the 'CRUCIAL' field treatments (SA, CH, CMA and in particular CHA). When combining the data across all field treatments, the measured Cu tolerance of the soil bacterial community was positively correlated ($r^2 = 0.482$; $P = 0.0002$) with Cu_{TOT} (Fig. 2A). However, the accumulated soil Cu concentrations were generally quite low (<30 mg kg^{-1}) and the bacterial Cu tolerance was not correlated with [Cu_{bio}] (Fig. 2B). [Cu_{bio}] was thus not affected by any of the organic amendments with the SA treatment as a notable exception. The soil treatments in the 'CRUCIAL' field trial not only affect the content of Cu in the soils but also affect the soil pH and SOM status. Soil pH (H_2O) varied between 6.6 and 7.6, whereas the pH in the soil bacterial suspensions used for PICT detection fell within the 6.6–7.0 range implying that the used MOPS pH 7.1 buffer (0.2 mM final concentration) was too weak to ensure stable pH conditions during PICT detection. We therefore investigated if the measured Cu tolerance (Fig. 1; 16 μM added Cu) was correlated with independent values for soil pH and the total carbon content of the soils (Fig. 2C and D). Bacterial Cu tolerance was not significantly correlated with soil pH, but there was a tendency towards a positive relationship between the two parameters ($r^2 = 0.160$, $P = 0.0529$, Fig. 2C). There was a strong positive relationship between community tolerance and SOM content ($r^2 = 0.781$, $P < 0.0001$, Fig. 2D). The same overall conclusions were derived when PICT detection data for 8 μM added Cu were analyzed (data not shown).

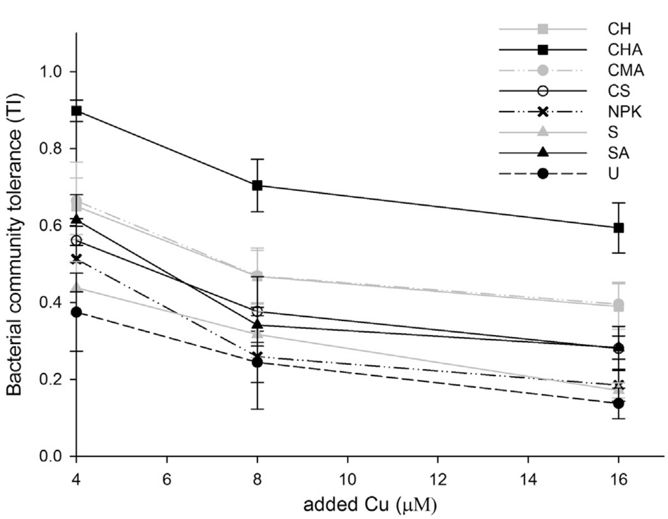

Fig. 1. Bacterial community-level Cu tolerance measured in weakly buffered soil bacterial suspensions (PICT experiment 1). Tolerance indices (TI) were calculated as [^{3}H]leucine incorporation rates in $CuSO_4$ amended suspensions (4, 8 or 16 μM) divided by [^{3}H]leucine incorporation rates in corresponding control suspensions. Abbreviations for field treatments: unfertilized control (U), inorganic fertilizer (NPK), cattle slurry (CS), cattle manure – accelerated rate (CMA), sewage sludge (S), sewage sludge – accelerated rate (SA), composted municipal household waste (CH), composted municipal household waste – accelerated rate (CHA). Means ± standard errors ($n = 3$) are shown.

3.2. Evaluation of possible PICT detection artifacts (PICT experiments 2 and 3)

To check for pH artifacts during PICT detection we carried out a second series of Cu tolerance determinations (PICT experiment 2), where the PICT detection assay pH was tightly controlled. In contrast to the results obtained in PICT experiment 1 (Fig. 1), increased Cu tolerance was only observed for the CHA treatment (Fig. 3; $P < 0.0001$; Tukey's test, model with only main effects included on data obtained for added Cu levels of 2–16 μM). Hence, by extracting soil bacteria in 10 mM MES buffer and thereby also controlling the pH in the PICT detection assay more tightly we were able to eliminate some of the measured differences in bacterial Cu tolerance between soils observed in PICT experiment 1. PICT experiment 2 was repeated for the treatments U and CHA, and the significant difference in Cu tolerance between these two treatments was confirmed (Fig. S1; $P = 0.00794$; model with only main effects included) showing that the results obtained with the method were highly reproducible. Hence, the bacterial community originating from the CHA treatment consistently showed higher tolerance towards Cu compared to the other treatments and this could not be explained solely by pH artifacts.

Based on the strong correlation between SOM content and Cu tolerance (Fig. 2D) and the results from PICT experiment 2 we hypothesized that the higher tolerance measured in soil from the CHA treatment could be explained by Cu-DOM speciation artifacts during PICT detection. We therefore repeated the [^{3}H]leucine incorporation assay for PICT detection with soil from three of the 'CRUCIAL' treatments (U, SA & CHA) using either MES buffer or Milli-Q water as extractants (PICT experiment 3) and correlated the obtained Cu tolerance indices to Cu toxicity (*Pseudomonas fluorescens* DF57-40E7 biosensor assay; inhibition of bioluminescence), free Cu^{2+} ion activity (electrode) and dissolved organic carbon (DOC) in the soil bacterial suspensions used for PICT detection.

Bacterial community-level Cu tolerance (measured at 16 μM added Cu) was again higher for the CHA treatment than observed for the other two field treatments regardless of extractant used (Fig. 4A, $P = 0.001$, Tukey's test). This difference could, however, clearly be attributed to Cu bioavailability artifacts during PICT detection. Hence, the toxicity of the added Cu to the *P. fluorescens* biosensor (measured at 8 μM added Cu) was significantly lower in soil bacterial suspensions derived from the CHA soil treatment than in suspensions derived from the other two soil treatments (Fig. 4B, $P < 0.001$, Tukey's test). Moreover, bacterial community-level Cu tolerance was strongly correlated to Cu toxicity in *P. fluorescens* (Fig. 5A; $P < 0.0001$; non-linear regression). The differential Cu toxicity in the analyzed soil bacterial suspensions could be linked to significantly increased concentrations of DOC (Fig. 4C, $P < 0.01$) and lower free Cu^{2+} ion activities (Fig. 4D, $P < 0.0001$). This was further confirmed by significant linear correlations of bacterial community tolerance to Cu versus DOC concentration (Fig. 5B, $P = 0.001$; non-linear regression) and the log-transformed activity of the free Cu^{2+} ion, respectively (Fig. 5C; all measured at 16 μM total Cu; $P < 0.0001$; non-linear regression). Finally, using the Cu^{2+} activity data at 16 μM of added Cu for the treatments U, SA and CHA we plotted Cu tolerance indices derived from PICT experiment 2 against estimated Cu^{2+} activities assuming that the same proportion of added Cu existed as the free Cu^{2+} ion at all levels of added Cu (Fig. S2). The bacterial community from the CHA soil treatment was not more tolerant to the estimated Cu^{2+} ion activity as compared to communities from the other two soil treatments (U and SA; Fig. S2). In conclusion, our data strongly suggest that the apparent PICT response observed for the CHA treatment could be explained entirely by a changed Cu speciation leading to bioavailability artifacts (i.e. differential toxicity of added Cu) during PICT detection.

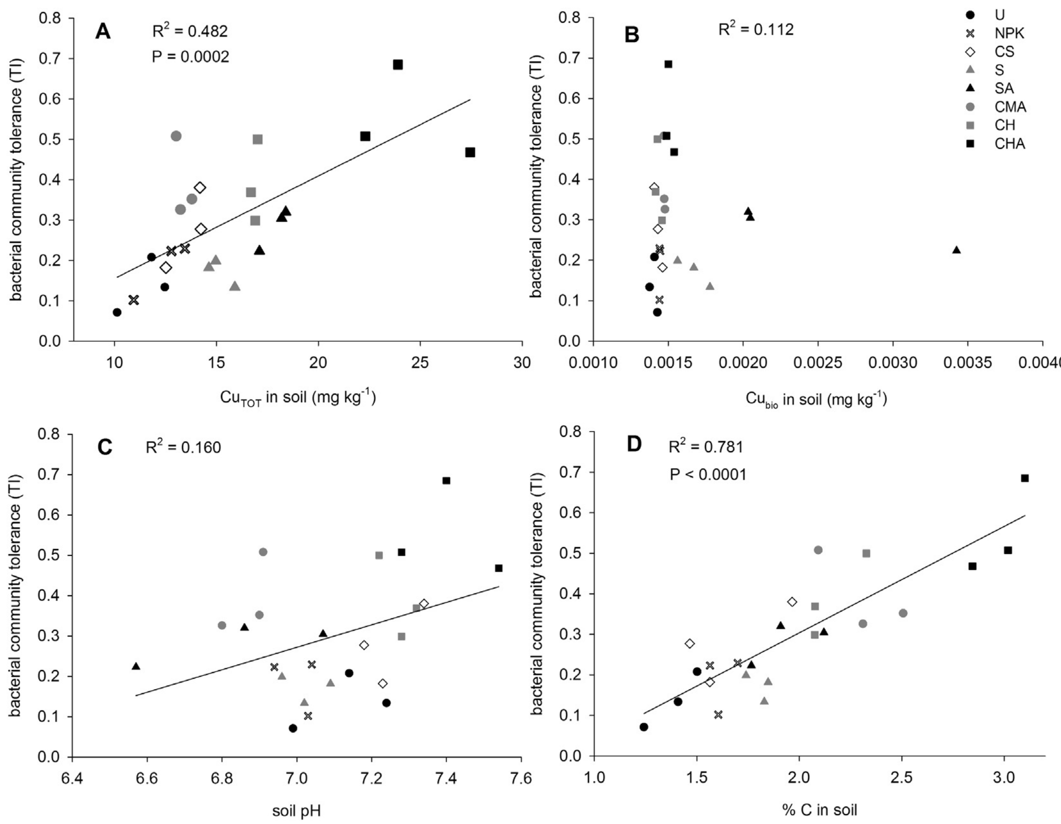

Fig. 2. Bacterial community-level Cu tolerance (PICT experiment 1) as a function of different soil characteristics: **A**) total soil Cu content (Cu_{TOT}), **B**) bioavailable Cu (Cu_{bio}), **C**) soil pH_{water}, and **D**) soil organic matter content. Tolerance indices (TI) were calculated as [^{3}H]leucine incorporation rates in $CuSO_4$ amended suspensions (16 μM) divided by [^{3}H]leucine incorporation rates in corresponding control suspensions. Abbreviations for field treatments: unfertilized control (U), inorganic fertilizer (NPK), cattle slurry (CS), cattle manure – accelerated rate (CMA), sewage sludge (S), sewage sludge – accelerated rate (SA), composted municipal household waste (CH), composted municipal household waste – accelerated rate (CHA).

Measured bacterial Cu tolerance was significantly lower (i.e. higher Cu toxicity to soil bacteria) when bacteria were extracted in MES buffer rather than in water (Fig. 4A; $P < 0.001$, paired *t*-test). Likewise, the Cu toxicity to *P. fluorescens* was significantly higher in soil bacterial suspensions extracted with MES buffer than in suspensions extracted with water (Fig. 4B, $P < 0.001$, paired *t*-test). The higher Cu toxicity in MES extracted soil bacterial suspensions was reflected in a significantly higher free Cu^{2+} ion activity (Fig. 4D; $P = 0.001$, paired *t*-test). In conclusion, our results strongly suggest that fewer Cu-complexing ligands were co-extracted in MES buffer than in water although we were unable to experimentally prove this due to the dominant presence of MES in the DOC pool in buffer extracted suspensions.

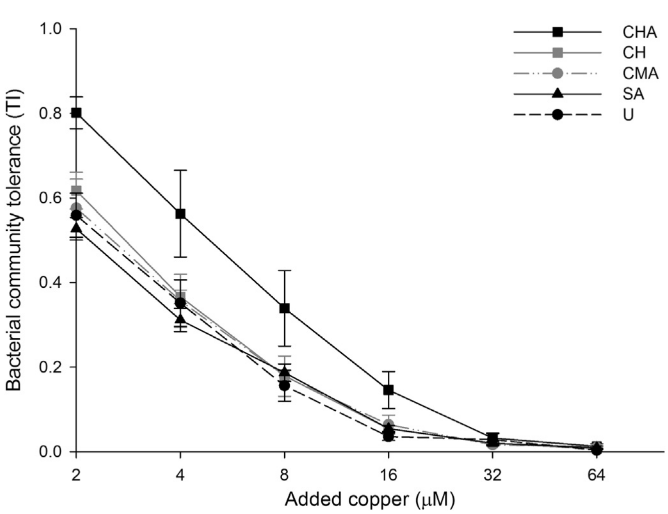

Fig. 3. Bacterial community-level Cu tolerance measured in strongly buffered soil bacterial suspensions (PICT experiment 2). Tolerance indices (TI) were calculated at six different levels of added Cu as [^{3}H]leucine incorporation rates in $CuSO_4$ amended suspensions divided by [^{3}H]leucine incorporation rates in corresponding control suspensions without Cu. Abbreviations for field treatments: unfertilized control (U), cattle manure – accelerated rate (CMA), sewage sludge – accelerated rate (SA), composted municipal household waste (CH), composted municipal household waste – accelerated rate (CHA). Means ± standard errors ($n = 3$) are shown.

4. Discussion

4.1. Urban wastes as fertilizers for sustainable agriculture

Collectively, our results demonstrate that Cu did not select for Cu resistance in any of the analyzed soil bacterial communities from the 'CRUCIAL' field trial with estimated total Cu inputs (January 2011) of 40 kg ha^{-1} and 75 kg ha^{-1} for the SA and CHA treatments, respectively. This estimate is based on the amount of organic waste applied during the experimental period, the measured content of P in organic wastes and a yearly application rate of 30 kg P ha^{-1} which is the maximal application rate following current Danish legislation. Hence, urban waste application did not cause Cu to build up to toxic levels. This conclusion was supported by the fact that most treatments did not increase [Cu_{bio}] (Fig. 2B). A slight increase of [Cu_{bio}] was only observed for the SA treatment which possibly could be due to the presence of bioavailable Cu-DOM complexes in sludge-amended soil. Cu-citrate complexes have for instance been suggested to be present in soil–sludge

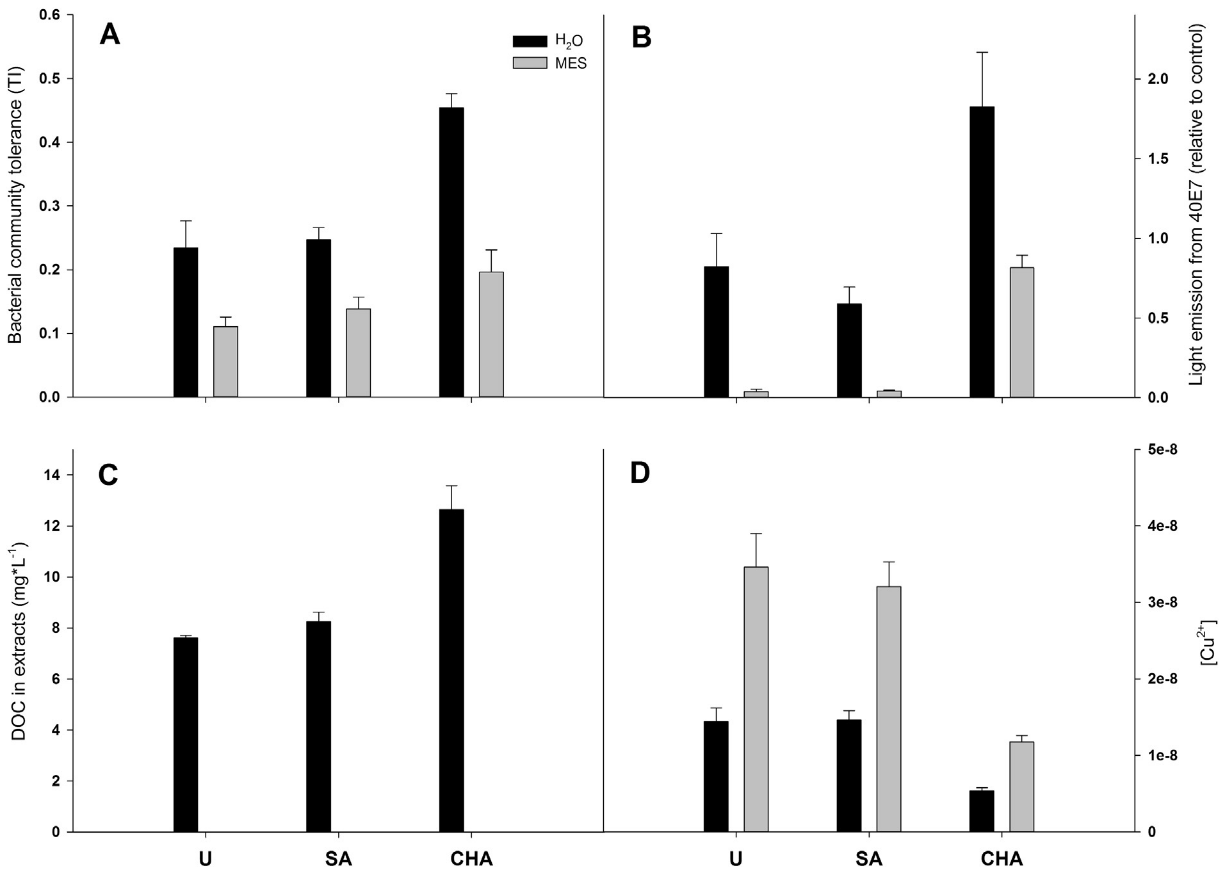

Fig. 4. Effects of field treatments and extractant (MES buffer or Milli-Q water) used for preparation of soil bacterial suspensions on (**A**) bacterial community-level Cu tolerance (tolerance index, TI), (**B**) bioluminescence (normalized values) emitted by *Pseudomonas fluorescens* DF57-40E7 in soil bacterial suspensions amended with 8 μM $CuSO_4$ (bioluminescence inhibition indicates Cu toxicity), (**C**) dissolved organic carbon (DOC) in soil bacterial suspensions, (**D**) chemical activity of the free Cu^{2+} ion ($[Cu^{2+}]$) in soil bacterial suspensions amended with 16 μM $CuSO_4$. Tolerance indices (TI) were calculated as [^{3}H]leucine incorporation rates in $CuSO_4$ amended samples (16 μM) divided by [^{3}H]leucine incorporation rates in corresponding control suspensions. Means ± standard errors of the means (n = 3) are shown.

mixtures (Vulkan et al., 2002) and these complexes are able to induce the used Cu biosensor (Nybroe et al., 2008).

The used bacterial PICT approach provides a rather sensitive measure of Cu toxicity in soil as compared to ecotoxicological endpoints targeting microbial community structure and function (Brandt et al., 2010; Berg et al., 2012) and the accelerated rate of metal accumulation in the studied soils represents a worst-case scenario as compared to the much slower Cu accumulation occurring in agricultural soils (Magid et al., 2006b). Further assuming that soil bacteria are more or at least equally sensitive to Cu than fungi (Rajapaksha et al., 2004), our study thus predicts that soil microbial communities will remain unaffected by Cu toxicity even following approximately 105 years and 169 years of repeated field application, according to the current Danish agricultural practice, of household compost and municipal sewage sludge, respectively. Our results are consistent with recent studies of the CRUCIAL field trial showing negligible field treatment effects on bacterial and archaeal community composition and on bacterial community-level physiological profiles (Poulsen et al., 2013a, 2013b).

Studies addressing specific impacts of Cu on microbial communities in soils amended with 'real-world' organic waste products are rare, as most studies have relied on Cu-spiked organic waste materials. To the best of our knowledge only one previous study has demonstrated a significant Cu PICT response in soils amended with non-spiked urban waste products (Dahlin et al., 1997). This study reported a difference in Cu tolerance (TdR-PICT) between control soil and the highest application of sewage sludge in a field trial during the period from 1966 to 1989 (4 t dry matter ha^{-1} yr^{-1}). However, the Cu threshold concentration for PICT development was higher (66 mg kg^{-1}) than the Cu concentrations measured in soils from the CRUCIAL field trial (<30 mg kg^{-1}). In conclusion, PICT responses have been observed previously in soils amended with urban waste products, but only in more Cu contaminated soils and mainly in studies applying Cu-spiked organic waste products (Bååth et al., 1998; Chaudri et al., 2008; Macdonald et al., 2010; Witter et al., 2000).

4.2. Limitations in the Leu-PICT approach

In theory, PICT should allow for sensitive detection of toxicants that shape bacterial communities by toxicant-induced succession even in contrasting soil types under field conditions. However, our study showcases the potential for PICT detection artifacts when measuring bacterial community tolerance to metals in suspensions derived from soils with contrasting soil properties. Hence, DOM, inorganic ions like H^+, Ca^{2+} and K^+ competing with Cu^{2+} for biotic binding sites, and suspended solids may be co-extracted along with bacteria, thereby causing changes in metal speciation, bioavailability and toxicity of added Cu during the PICT detection phase. In the present study a high concentration of DOM in soil bacterial extracts was specifically observed to decrease the toxicity of added Cu in the Leu-PICT detection assay. The quality of DOM may also be important here since some labile Cu-DOM complexes may be bioavailable (Nybroe et al., 2008) and contribute to Cu toxicity

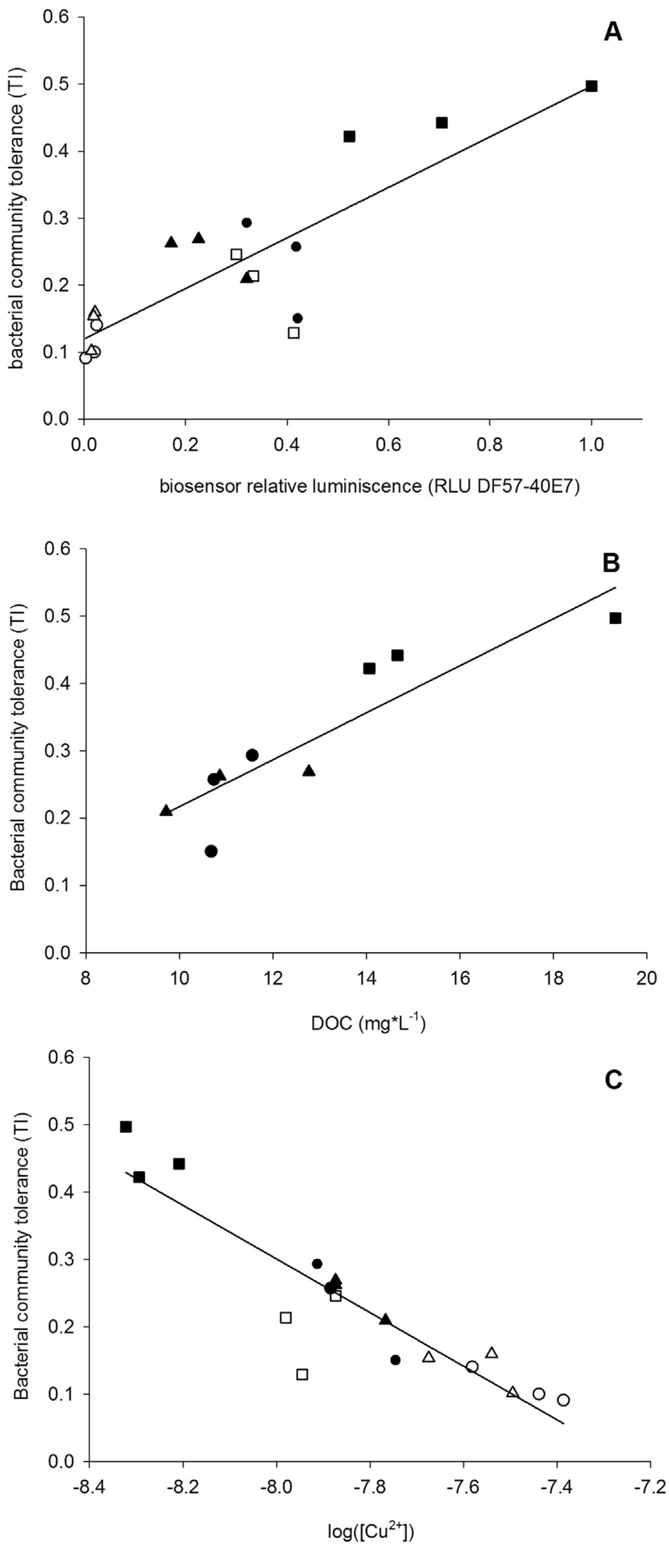

Fig. 5. Bacterial community-level Cu tolerance (PICT experiment 3) as a function of: (**A**) bioluminescence (normalized values) emitted by *Pseudomonas fluorescens* DF57-40E7 in soil bacterial suspensions amended with 8 μM $CuSO_4$ (bioluminescence inhibition indicates Cu toxicity), (**B**) dissolved organic carbon (DOC) in soil bacterial suspensions, (**C**) the chemical activity of the free Cu^{2+} ion ($[Cu^{2+}]$) in soil bacterial suspensions amended with 16 μM $CuSO_4$. Tolerance indices (TI) were calculated as [^{3}H]leucine incorporation rates in $CuSO_4$ amended samples (16 μM) divided by [^{3}H]leucine incorporation rates in corresponding control suspensions. Nonlinear regression yielded significant relationships between the variables (see *P* values in each graph panel). Abbreviations and symbols used for field treatments: unfertilized control (U, circles), sewage sludge – accelerated rate (SA, triangles) and composted municipal household waste – accelerated rate (CHA, squares). Soils were extracted in either water (closed symbols) or 10 mM pH 6.4 MES buffer (open symbols).

(Brandt et al., 2010). The risk of false PICT responses due to bioavailability artifacts during PICT detection has been described previously (Blanck, 2002), but has not received much experimental attention in previous Leu-PICT studies addressing soil bacterial communities. The used community tolerance metrics for PICT detection constitutes another important consideration. In the present study we adopted the tolerance index (TI) as a measure of the level of tolerance in the bacterial community. Although TI has previously been shown to be strongly correlated with IC_{50} for Leu/TdR-PICT detection (Díaz-Raviña et al., 2007), it must be stressed that IC_{50} is preferable when comparing bacterial communities with very different levels of tolerance to Cu. However, IC_{50} is a fitted value and statistical confidence intervals tend to be quite wide. This makes it difficult to detect small differences in community tolerance as expected in our study and we therefore chose to directly measure community tolerance using the tolerance index approach.

4.3. Reevaluation of PICT detection artifacts in previous field studies

The large majority of previous Cu PICT studies did not use a buffer to control pH during PICT detection (Díaz-Raviña and Bååth, 1996; Pennanen et al., 1996; Dahlin et al., 1997; Bååth et al., 1998; Kiikkilä et al., 2000; Witter et al., 2000; Díaz-Raviña et al., 2007; Aaen et al., 2011; Fernández-Calviño et al., 2011, 2012; see Table S1 for more experimental details), and consequently there is a risk that some of these studies have led to false conclusions. Differential pH during PICT detection can directly affect the microbial activity or lead to differences in Cu toxicity through changes in chemical Cu speciation (Ore et al., 2010). None of the above cited studies (see Table S1) checked the actual toxicity of Cu added during the PICT detection assay. Apart from pH-derived artifacts, differential Cu toxicity could also have occurred due to differential degrees of Cu-complexation and due to differential Cu sorption to suspended solids when comparing soils with contrasting soil properties such as SOM content and soil texture.

Leu-PICT studies that aim to link chemical soil characteristics to PICT development across many contrasting soil types are the most likely to suffer from the PICT detection artifacts described above. Fernández-Calviño et al. (2012) showed that bacterial community tolerance was better correlated with soil pH than with soil Cu in a study of Spanish vineyard soils. The authors suggested that this result could probably be attributed to a relative narrow span of soil Cu concentrations (Cu_{TOT} 25–272 mg kg^{-1}), but it is also plausible that PICT detection artifacts biased the results thereby introducing analytical noise to the PICT detection assay that made it difficult to assess the true effect of Cu. In a related study, Díaz-Raviña et al. (2007) investigated the bacterial Cu tolerance in a number of vineyard soils with a range of different SOM contents and Cu_{TOT} (35–550 mg kg^{-1}). Higher Cu tolerance was observed in bacterial communities from soils with Cu_{TOT} above 100 mg kg^{-1}, but notably high Cu tolerance was also indicated for some of the low-Cu soils (55–60 mg kg^{-1}) with relatively high SOM levels (95.1 and 101.4 g kg^{-1}). We therefore speculate that some of the Cu tolerance data reported in this study were biased due to bioavailability artifacts during PICT detection as also cautioned by the authors. Fernández-Calviño et al. (2011) also investigated the PICT response towards Cu in a broad range of vineyard soils and correlated EC_{50}-values with relevant soil parameters in order to investigate if other factors than the Cu content of the soils had an effect on the tolerances measured. However, Cu tolerance was also found to be negatively correlated to SOM and soil pH leading the authors to conclude that soil pH and SOM influenced Cu toxicity during the PICT selection phase. Since no attempt was made to evaluate bioavailability artifacts during PICT detection, we speculate that some of the conclusions reached could be biased due to PICT detection artifacts. Based on our study and the preceding discussion, it is evident that care must be taken, when comparing bacterial community tolerance to Cu in contrasting soil types and some of the conclusions from previous PICT studies such as the ones presented in Section 4.1 should be treated with caution. Although we have here focused our discussion of potential PICT detection artifacts on the Leu-PICT and TdR-PICT approaches, it is also important to note that other PICT detection techniques may suffer from similar PICT detection artifacts. PICT detection methods based on measurement of potential respiration rates (Brandt et al., 2003; Mertens et al., 2006; Wakelin et al., 2013) rely on PICT detection in intact soils or soil slurries and are probably even more prone to bioavailability artifacts, as bacteria are not separated from the soil prior to PICT detection. These PICT detection approaches are also more prone to toxicant carry-over from the selection to the detection phase (Blanck, 2002).

4.4. Recommendations for future PICT studies in soil

Our study is unique as it allows for specific evaluation of possible PICT detection artifacts by comparing different approaches for Leu-PICT detection. The use of a strongly buffered soil bacterial suspension for PICT detection eliminated most of the observed false PICT responses (Figs. 1 and 3) and we therefore strongly recommend the use of appropriate non-complexing buffers for terrestrial PICT studies whenever possible. Non-toxic Good's buffers such as MES and MOPS have pK_a values of 5.9 and 7.2, show negligible complexation with Cu (Mash et al., 2003), and are therefore suitable buffers, but more studies are needed to compare the performance of different buffers. The above discussion primarily concerns field PICT studies comparing soils with contrasting properties. PICT detection bioavailability artifacts are in general not likely to lead to false conclusions in laboratory PICT studies, where PICT selection is studied in one soil type spiked with different levels of a toxicant. However, we still recommend the use of non-complexing pH buffers to prevent metal-induced acidification during PICT detection. Importantly, it may also be argued that pH buffers should also be used to eliminate bioavailability artifacts when performing PICT experiments for other metals and for organic toxicants showing pH-driven ionic speciation (Tappe et al., 2008; Brandt et al., 2009).

The use of pH buffers may serve to reduce co-extraction of DOM that can bias PICT detection by affecting Cu speciation. Nevertheless, as shown in the present study Cu speciation artifacts due to co-extracted DOM may still remain. In general, we therefore strongly recommend to measure the toxicity of the added toxicant during PICT detection using a reliable single-species bioassay or by chemical speciation analysis. The use of whole-cell bacterial biosensors represents a very attractive approach as it should be applicable for most toxicants, whereas chemical approaches will vary widely depending on the toxicant to be studied. Ruyters et al. calculated the modeled free Cu^{2+} and Zn^{2+} concentrations in soil bacterial suspensions based on chemical equilibrium speciation modeling and expressed the tolerance of nitrifying bacteria as the concentration of Cu^{2+} and Zn^{2+} at which the potential nitrification rate was reduced by 50% as compared to the control treatment (Ruyters et al., 2012). Our results support the use of $[Cu^{2+}]$ to calculate EC_{50} values instead of added Cu_{TOT}. This is in accordance with the conventional view that the free metal ion is the primary toxic metal species (Giller et al., 1998; Ore et al., 2010), but as noted earlier some labile Cu-DOM complexes may also be bioavailable (Nybroe et al., 2008) and contribute to Cu toxicity (Brandt et al., 2010). Furthermore, the toxicity of the free Cu^{2+} ion depend on the ionic composition of the soil bacterial solution used for PICT detection according to the biotic ligand model (Ore et al., 2010). It should be possible to model Cu toxicity in soil bacterial suspensions used for PICT detection provided that detailed chemical data are available and use model predictions to ensure that bacterial communities from different soils are exposed to comparable levels of Cu stress during PICT detection.

5. Concluding remarks

Our study has important implications for several previously published PICT studies in soil and indicates that more stringent PICT detection criteria are needed for future terrestrial PICT studies. Despite the risk of PICT detection artifacts in previous field studies, it is remarkable that the TdR-PICT and Leu-PICT approaches in general compare well to competing approaches that target community structure or function for microbial community-level toxicity testing under field conditions (Pennanen et al., 1996; Bååth et al., 1998; Berg et al., 2012). Nevertheless, adoption of the simple guidelines presented above should allow for even more sensitive and reliable detection of toxicant-induced succession in bacterial communities.

Our study also indicates that Cu is unlikely to reach toxic levels high enough to select for resistant bacterial communities even after 105 and 169 years of repeated household compost and municipal sewage sludge application according to the current Danish agricultural practice. It is technically feasible to extract urban waste resources with very low contamination levels by source separation of both solid and liquid waste streams (Magid et al., 2006a). But considering the substantial decrease in toxic metal loading that has occurred in the European waste streams over the past decades (Eriksen et al., 2009; Smith, 2009) and the results presented here it may be questioned if this is at all necessary in order to prevent adverse effects of Cu on soil quality.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2014.07.013.

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