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Bacterial pollution induced community tolerance (PICT) to Cu and interactions with pH in long-term polluted vineyard soils

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Pollution induced community tolerance (PICT) has been suggested as an end-point measurement less affected by confounding environmental factors compared to standard methods of microbial growth, activity and community composition. We evaluated the use of PICT to determine Cu toxicity in vineyard soils polluted with Cu based fungicides (25–1120 mg Cu kg^{-1}). These soils also varied in pH (4.3–7.3), organic C (0.31–6.91%) and texture (14–56% silt). PICT was estimated as bacterial community tolerance to Cu measured by the [^{3}H]leucine incorporation method. Bacterial tolerance to Cu increased 9 times in the most polluted compared to the unpolluted soils. Cu tolerance was also affected to a minor degree by pH, organic C and soil texture. Lower bacterial tolerance was found in soils with high pH and organic C, probably due to Cu becoming less bioavailable in soils with high pH and organic C content. The silt content appeared to increase bacterial tolerance, probably due to fine soil particles decreasing Cu bioavailability during the PICT detection phase. Despite the effects of other environmental factors, the main determinant of increased bacterial community tolerance to Cu was the pollution level. PICT measured with the leucine incorporation technique thus appears to be a sensitive and stable concept to evaluate toxic impacts, unless soils with very different pH, organic C or texture are studied.

1. Introduction

The long-term use of copper-based fungicides (such as the Bordeaux mixture) in viticulture has resulted in increased Cu concentration in soils used for wine production all over the world (Komarek et al., 2010). High Cu concentrations have also been found in Cu fungicide-treated soils devoted to other crops such as hop (Schramel et al., 2000), avocado (Van Zwieten et al., 2004), coffee (Loland and Sing, 2004) or cocoa (Josanidia, 1994). There are, however, few studies on the effect of Cu on microbial communities in these type of agroecosystems (Díaz-Raviña et al., 2007; Miguéns et al., 2007; Dell'Amico et al., 2008; Viti et al., 2008; Fernández-Calviño et al., 2010a, 2010b). Furthermore, field studies of metal effects are problematic since other environmental factors, like pH, can confound the results. In vineyards soils with acid parent materials it is very common to use lime to increase the soil pH in order to improve crop yields. Vineyard soils in areas with a significant presence of acid rocks as parent materials thus can have a significant variability in both pH and Cu concentrations. For example, in vineyard soils developed mainly on granites, schists and slates in NW Iberian Peninsula, pH ranged from 4.0 to 7.9, and total soil Cu from 25 to 1120 mg kg^{-1} (Fernández-Calviño et al., 2009a, 2010a). pH was found to be more important than the Cu content in determining the microbial community structure using phospholipid fatty acid (PLFA) pattern in these vineyard soils (Fernández-Calviño et al., 2010a), thus being a confounding factor when trying to elucidate heavy metal toxicity.

It is fairly easy to detect and determine toxic effects of Cu in laboratory experiments (Rajapaksha et al., 2004). However, even under controlled conditions confounding effects, for example due to the addition of Cu salts changing the soil pH, are common (Speir et al., 1999; Ginocchio et al., 2009; Brandt et al., 2010). Moreover, soil pH is very important for Cu^{2+} availability (Fernández-Calviño et al., 2009b). In a field situation the presence of other environmental factors confounding effects of increasing Cu concentration in soil is even more problematic, especially if soils with different texture, pH and organic matter concentrations are included. Environmental factors like these will affect most end-points used to detect heavy metal effects, such as biomass, activity or community structure-based measurements. One way to avoid this would be to

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use end-points that are specifically related to the toxicant studied. The determination of pollution induced community tolerance (PICT, (Blanck, 2002)) could be used as such an indicator of toxic effects, since it is assumed that if toxicity has affected the microbial community, species more tolerant to the toxicant will be selected for and thus the total community will become more tolerant to the toxicant. This technique has successfully been used to detect toxicity both in laboratory spiked and field polluted soils (Almås et al., 2005; Demoling and Bååth, 2008; Demoling et al., 2009; Stefanowicz et al., 2009; Brandt et al., 2010; Macdonald et al., 2010). Brandt et al. (2010) also stated that PICT constituted the most sensitive ecotoxicological end-point for determining Cu impacts. However, Díaz-Raviña et al. (2007) estimated PICT in some Cu polluted vineyard soils. Although the bacterial community appeared more tolerant in the high Cu polluted soils, an increased bacterial tolerance was observed in some soils with low Cu concentration. This suggests that PICT determination may be affected by other factors in soil. Confounding environmental factors on bacterial PICT to Zn was also found in a study in different river systems in Europe (Blanck et al., 2003).

In the present study we attempted to differentiate between effects of Cu and pH in vineyard soils with a range of different pH and different history of Cu pollution. This was done by measuring the bacterial community tolerance (PICT) to Cu using bacterial growth (estimated with leucine (Leu) incorporation), to indicate changes in tolerance (Bååth, 1992; Díaz-Raviña et al., 1994), and statistical techniques to assess the degree of interference of pH in the measurements. Leu-PICT has recently been suggested as a future standard method for PICT detection in soil (Brandt et al., 2009; Demoling et al., 2009). It is also well know that the main factors governing Cu^{2+} availability in soils besides pH are the presence of organic and/or inorganic colloids (McBride et al., 1997). We therefore included other potential confounding factors, like soil texture and soil C content, to study if these factors affect PICT determinations. Finally, we compared the effect of Cu and pH in determining bacterial and fungal growth in soil, since both low pH and high Cu concentrations have been shown to favour fungal over bacterial growth in soil (Rajapaksha et al., 2004; Rousk et al., 2009).

2. Materials and methods

2.1. Soil samples

For this study 53 vineyard soils and 7 soils devoted to other crops without the use of copper-based fungicides were sampled from the A horizon (0–20 cm). All soils came from the NW of the Iberian Peninsula, and are predominantly Cambisols and Regosols (FAO, 1998). They were earlier used in a study on the combined effect of Cu and pH on the microbial community structure of vineyards soils determined using phospholipid fatty acids (PLFAs) analysis (Fernández-Calviño et al., 2010a), where more details on the sampling procedure and physico-chemical analyses are found.

Physical and chemical properties of the soils are summarized in Table 1. The non-vineyard soils were similar to the vineyard ones in texture, but were in the lower pH range of vineyard soils. Non-vineyard soils generally had higher total C and N contents, and much lower Cu concentrations than the vineyard soils.

The soils used were mainly classified as sandy loam in texture (65% of the samples), 15% were loam soils and 11% were sandy clay loams. The soil pH was very variable; pH in water (pH_W) ranged from 4.3 to 7.3 and pH_{KCl} from 3.5 to 6.8. Total organic carbon ranged from 0.31 to 6.91% and total nitrogen from 0.04 to 0.47%. The total copper concentration, Cu_T (quantified by extraction with a 5:4:1 (v/v/v) mixture of HNO_3, HF and HCl in a microwave oven at 700 kPa), varied over a wide range: 25–1121 mg kg^{-1}. Copper bound to organic matter, Cu_P (pyrophosphate-extracted Cu according to McKeague (1967), varied from 4 to 727 mg kg^{-1}. Cu_P was highly correlated to Cu_T ($r = 0.971$, $p < 0.01$) and accounted for 54% of Cu_T in the soils. Exchangeable Cu content, Cu_{EX} (extracted with 1 M NH_4Ac at pH 7 according to Gupta and Chen (1975)), was relatively low (0.2–30 mg kg^{-1}) and represented as a mean only 3.1% of Cu_T. The concentration of Cu in DPTA (Lindsay and Norwell, 1978) and EDTA (Lakanen and Erviö, 1971) extracts (potentially bioavailable Cu) was 0.8–661 and 0.6–936 mg kg^{-1}, respectively, and represented on average around 35% of Cu_T.

2.2. Microbiological analyses

Fungal growth and bacterial community tolerance to Cu were measured using all 53 of the vineyard soils but only 5 and 3 of the non-vineyard soils for fungal growth and community tolerance, respectively. Bacterial growth was only estimated in 41 of the vineyard soils, but all 7 of the non-vineyard soils. The selected soils were always chosen in order to include the whole range of different environmental factors.

The bacterial growth rate was estimated using leucine (Leu) incorporation (Kirchman et al., 1985) into bacteria extracted from soil with the homogenization and centrifugation technique described by Bååth (1994) and Bååth et al. (2001). Soil samples (2 g, fresh weight) were mixed with 20 ml distilled water using a multivortex shaker at maximum intensity for 3 min. This was followed by low-speed centrifugation at 1000 x g for 10 min to create a bacterial suspension in the supernatant. Aliquots (1.5 ml) of this suspension were transferred to 2-ml microcentrifugation tubes, and 2 µl [^{3}H]Leu (37 MBq ml^{-1} and 5.74 TBq mmol^{-1}; Amersham) was added with nonlabeled Leu to each tube, resulting in 275 nM Leu in the bacterial suspensions. After 2 h of incubation, growth was terminated by adding 75 µl 100% trichloroacetic acid. Washing and subsequent measurement of radioactivity were performed as described by Bååth et al. (2001). The amount of Leu

Table 1
General soil characteristics for vineyard and non-vineyard soils (summarized from Fernández-Calviño et al. (2009a)).

		Sand (%)	Silt (%)	Clay (%)	pH_W	pH_{KCl}	C (%)	N (%)	Cu_{DTPA} (mg kg^{-1})	Cu_{EDTA} (mg kg^{-1})	Cu_{EX} (mg kg^{-1})	Cu_P (mg kg^{-1})	Cu_T (mg kg^{-1})
Vineyard Soils ($n = 53$)	Mean	57	25	18	5.5	4.6	2.54	0.21	90	104	7.3	141	250
	SD	12	8	6	0.7	0.8	1.35	0.10	103	136	6.6	123	194
	Maximum	77	56	40	7.3	6.8	6.91	0.47	661	936	30	727	1121
	Minimum	23	14	7	4.3	3.4	0.31	0.04	1.0	1.2	0.1	3.7	25
Non-vineyard soils ($n = 7$)	Mean	53	29	18	4.6	4.1	4.71	0.51	2.9	3.7	0.4	15	46
	SD	20	13	7	0.3	0.4	2.87	0.30	2.1	2.6	0.2	10	42
	Maximum	74	48	28	5.2	4.7	10.50	1.07	6.3	7.4	0.6	31	110
	Minimum	24	16	10	4.2	3.6	2.60	0.19	0.8	0.6	0.2	3.9	2.0

pH_W is pH in water; pH_{KCl} is pH in 0.1M potassium chloride; C is total organic carbon; N is total nitrogen; Cu_{DTPA} is DTPA extractable Cu; Cu_{EDTA} is EDTA extractable Cu; Cu_{EX} is ammonium acetate extractable Cu; Cu_P is sodium pyrophosphate-extractable Cu; and Cu_T is total Cu.