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Letter to the Editor

Long-term effects of metal contamination on *Rhizobium*

The paper "*Rhizobium* in soils contaminated with copper and zinc following long-term application of sewage sludge and other organic wastes" by S.R. Smith (Soil Biology & Biochemistry 29(9/10) 1475–1489, 1997) is fundamentally flawed in the application of the methodology used, and makes wild speculations about one previously reported experimental site that do not stand up to scrutiny.

Publications by Smith's group (Smith, 1991, 1992, 1997; Smith and Giller, 1992) do not include a proper count of rhizobia. The 'presence or absence' he reports is not statistically or microbiologically correct, because although the standard method he quotes (Vincent, 1970) relies on the results of a dilution series, he in fact only looked at nodulation at one dilution. Yet, others (e.g. Evans, 1998) are wrongly led to assume that this paper contradicts the previous findings that Zn or other metals reduce the population size of *Rhizobium leguminosarum* biovar *trifolii,* and also its diversity (McGrath et al., 1988; Giller et al., 1989, 1998; Angle et al., 1993; Chaudri et al., 1993; Hirsch et al., 1993).

In brief, the basis of the most probable number (MPN) test is that a dilution series of the soil is made and inoculated onto sterile clover seedlings under axenic conditions. However, when discussing the limitations in the use of plant infection counts, Vincent (1970, p. 72) stated: "When the sample contains a large enough population of other organisms which affect the invasibility of the seedling, this may seriously interfere with the expected quantitative relationships between the number of rhizobia and the formation of nodules".

What this means in practice is that when relatively large amounts of soil are present, such as in the single 100-fold dilution used by Smith (1997), one often gets unhealthy plants that do not nodulate because of the presence and proliferation of other organisms that are pathogenic. Vincent states explicitly that negatives at low dilution should be set as positives. It is the extinction of positive scores at high dilution that in fact determines the statistical calculation of the MPN cells. Depending on the rhizobial population in the soil, we normally perform 7–8 dilutions in triplicate for each sample to accurately perform the MPN test as specified in Vincent (1970). Problems in nodulation at low dilution are therefore an artefact, which will vary according to other soil characteristics that affect the numbers of other organisms present. This apparent variability between soil is in fact shown in Smith's own paper. However, he did not quantify the pathogen populations in the different soils.

This artefact calls all results obtained using only 100-fold dilution into doubt. Therefore, because proper MPN tests were not performed, the erroneous results obtained negate all of the statistical models used by Smith (1997) to explain effects of 'presence' and 'absence' against environmental variables. Put basically, the method does not indicate 'absence' of rhizobia in soil at all. In any case, because the approach does not yield quantitative results about the effects of metals, it is a poor variable to use in statistical models to test against the other soil data.

Smith (1997, p. 1484) also says: "One suggestion is that these characteristics (ineffective N_2-fixation) of the Woburn strain were acquired by mutation as heavy metals accumulated in the soil as a result of the application of sewage sludge (Hirsch et al., 1993)". Again he misrepresents previously published work. Hirsch et al. did not say that mutation caused ineffectiveness. There may be a misreading by Smith, as Hirsch et al., did say that *metal resistance* might have been acquired as a mutation. However, there is no evidence that this is linked in any way with ineffectiveness.

Finally, Smith (1997) follows this with the assertion that "it is possible that this ineffective strain was selected by some past influence of soil conditions prevailing at Woburn, such as low soil pH, which is likely... and perhaps occurred even before the site was managed by Rothamsted". We are shocked that such inaccurate, pure speculation was published in a reputable refereed journal. This is really a diversionary tactic, because Hirsch et al. (1993) showed that what actually occurred was a change, *compared with control soils*, in diversity of the *R. leguminosarum* biovar *trifolii*. There seems to be what can only be described as an obsession of Smith with ineffectiveness, thus missing the main point about the quantitative change in whole

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populations of rhizobia that had taken place. In the original papers, its ineffectiveness on white and red clover were scientifically reported, but their main emphasis was the death of the large and diverse *R. leguminosarum* biovar *trifolii* population that still existed in the low metal control plots and should also exist on the sludge-treated plots.

Smith knows very well that other sludge-treated sites (Chaudri et al., 1993; Dahlin et al., 1997) never showed the presence of ineffective strains, and so this can be viewed as a rare chance event and not much emphasis should be placed on it. Certainly, to justify its occurrence by invoking acidity is misleading, as shown below. It is simply a single surviving strain of *Rhizobium* which is metal tolerant (Chaudri et al., 1992), with an effective host range that does not include red and white clover (Hirsch et al., 1993). If it were not for this strain, the net result would have been as observed in experimental plots at Braunschweig in Germany, where no surviving *R. leguminosarum* biovar *trifolii* and no nodulation occurs at high metal concentrations (Chaudri et al., 1993).

The soil pH of the Woburn long-term sludge experiment was around 6 in the 1940s and was maintained at 6.5 to 7 thereafter (McGrath, 1984). Furthermore, the records of Woburn experimental farm have been consulted and, although no soil pH measurements exist before 1940, it is recorded that this field was used for a series of experiments including a long term experiment started in 1896 on green manures that gave good wheat yields. A rotation of swede, barley, peas and wheat grew 'healthy and strong' back as far as 1881, even though these, especially barley, are sensitive to acidity. In the later years this included barley and clover (red or white). Various other short experiments on the field at that time also included crops such as barley and root crops, again with good yields. Importantly, the green manure experiment included the legume *Vicia hirsuta*, which grew well from 1896–1936, and other short experiments on the field included varieties of clover and lucerne grown without rhizobial inoculation over all these years without any problems.

One can only conclude that healthy populations of many rhizobial species were present in the soil for 60 y or more *before* the sludge was applied, *and that this diverse community was present on what were to become either high metal or low metal plots, across the whole gradient of metal concentrations that were eventually created in the experiment*. This community still exists after the treatments on the low metal plots, but has been wiped out on the high metal plots. This point is ignored by Smith. The same thing happened on the plots at Braunschweig at the highest rates of sludge (McGrath, 1997), even with non metal amended (plain ex-treatment works) sewage sludge treatments, after only 10 y of application (Chaudri et al., 1993). Mention of this fact is also omitted from Smith (1997).

Rhizobia, especially *R. leguminosarum* biovar *trifolii*, are particularly sensitive indicators of metals in soil that could be used as a 'sentinel' species. This is no different to other environmental indicators that have been identified by researchers looking at the effects of pollutants on other microbial properties of soils (e.g. Eijsackers, 1983; Zonneveld, 1983; Lorenz et al., 1992; Brookes, 1993; Korthals et al., 1996). An independent scientific committee (MAFF, 1993) saw this sentinel strain as being useful particularly because increasing metal concentrations in soil resulted in *quantitative* decreases in its population, ultimately to zero. However, complete kill of whole populations of this agronomically important symbiont is not a desirable end point. Ignoring for a moment the flawed nature of the work, Smith's method additionally misses the inherent value of properly conducted quantitative population studies of sentinel species. The Smith (1997) analysis of heavily sludge-treated sites for presence or absence of native *R. leguminosarum* biovar t*rifolii* did not show any effects of Cd, but their own data indicated that Zn could be having an effect. More studies are clearly needed, and this whole area of disagreement underlines the difficulties in interpretation and lack of understanding that exists about real-world multimetal contaminated sites (Giller et al., 1998). It also adds weight to the need for new long-term sludge experiments.

References

Angle, J.S., McGrath, S.P., Chaudri, A.M., Chaney, R.L., Giller, K.E., 1993. Inoculation effects on legumes grown in soil previously contaminated with sewage sludge. Soil Biology & Biochemistry 25, 575–580.

Brookes, P.C., 1993. The potential of microbiological properties as indicators in soil pollution monitoring. In: Soil Monitoring, Monte Veritá. Birkhäuser Verlag, Basel, pp. 229–254.

Chaudri, A.M., McGrath, S.P., Giller, K.E., 1992. Metal tolerance of isolates of *Rhizobium leguminosarum* biovar *trifolii* from soil contaminated by past applications of sewage sludge. Soil Biology & Biochemistry 24, 83–88.

Chaudri, A.M., McGrath, S.P., Giller, K.E., Rietz, E., Sauerbeck, D., 1993. Enumeration of indigenous *Rhizobium leguminosarum* biovar *trifolii* in soils previously treated with metal-contaminated sewage sludge. Soil Biology & Biochemistry 25, 301–309.

Dahlin, S., Witter, E., Mårtensson, A.M., Turner, A., Bååth, E., 1997. Where's the limit? Changes in the microbiological properties of agricultural soils at low levels of metal contamination. Soil Biology & Biochemistry 29, 1405–1415.

Eijsackers, H., 1983. Soil fauna and soil microflora as possible indicators of soil pollution. Environmental Monitoring and Assessment 3, 307–316.

Evans, T., 1998. Agricultural use of biosolids (sewage sludge). In: Proceedings of the Fertiliser Society, No. 409. The Fertiliser Society, York, UK.

Giller, K.E., McGrath, S.P., Hirsch, P.R., 1989. Absence of nitrogen

fixation in clover grown on soil subject to long term contamination with heavy metals is due to survival of only ineffective *Rhizobium*. Soil Biology & Biochemistry 21, 841–848.

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

Hirsch, P.R., Jones, M.J., McGrath, S.P., Giller, K.E., 1993. Heavy-metals from past applications of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum* biovar *trifolii* populations. Soil Biology & Biochemistry 25, 1485–1490.

Korthals, G.W., Vandeende, A., Vanmegen, H., Lexmond, T.M., Kammenga, J.E., Bongers, T., 1996. Short-term effects of cadmium, copper, nickel and zinc on soil nematodes from different feeding and life-history strategy groups. Applied Soil Ecology 4, 107–117.

Lorenz, S., McGrath, S.P., Giller, K.E., 1992. Assessment of free living nitrogen fixation activity as a biological indicator system for heavy metal toxicity in soil. Soil Biology & Biochemistry 24, 601–606.

McGrath, S.P., 1984. Metal concentrations in sludges and soil from a long-term sludge trial. Journal of Agricultural Science 103, 25–35.

McGrath, S.P., 1997. Behaviour of trace elements in terrestrial ecosystems. In: Prost, R. (Ed.), Contaminated Soils. Editions INRA, Les Colloques No. 85, Paris, pp. 35–54.

McGrath, S.P., Brookes, P.C., Giller, K.E., 1988. Effects of potentially toxic metals in soil derived from past applications of sewage sludge on nitrogen fixation by *Trifolium repens* L. Soil Biology & Biochemistry 20, 415–424.

MAFF (Ministry of Agriculture, Fisheries and Food), 1993. Review of the rules for sewage sludge application to agricultural land. Soil Fertility Aspects of Potentially Toxic Elements. MAFF Publications, London MAFF PB 1561.

Smith, S.R., 1991. Effects of sewage sludge application on soil microbial processes and soil fertility. Advances in Soil Science 16, 191–212.

Smith, S.R., 1992. Sites with a long history of sludge disposal: phase II (EHA9019). Report to UK Department of the Environment, WRc plc, Medmenham, UK.

Smith, S.R., 1997. Rhizobium in soils contaminated with copper and zinc following long-term application of sewage sludge and other organic wastes. Soil Biology & Biochemistry 29, 1475–1489.

Smith, S.R., Giller, K.E., 1992. Effective *Rhizobium leguminosarum* biovar *trifolii* present in five soils contaminated with heavy metals from long-term applications of sewage sludge or metal mine spoil. Soil Biology & Biochemistry 24, 781–788.

Vincent, J.M., 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell, Oxford.

Zonneveld, I.S., 1983. Principles of bio-indication. I. Some concepts. Environmental Monitoring and Assessment 3, 207–217.

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