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Development of a Real-Time PCR Assay for Detection and Quantification of *Rhizobium leguminosarum* Bacteria and Discrimination between Different Biovars in Zinc-Contaminated Soil[▽]

Catriona A. Macdonald,* Ian M. Clark, Penny R. Hirsch, Fang-Jie Zhao, and Steve P. McGrath

Department of Soil Science, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

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Primers were designed to target 16S rRNA and *nodD* genes of *Rhizobium leguminosarum* from DNA extracted from two different soil types contaminated with Zn applied in sewage sludge. Numbers of rhizobia estimated using 16S rRNA gene copy number showed higher abundance than those estimated by both *nodD* and the most-probable-number (MPN) enumeration method using a plant trap host. Both 16S rRNA gene copies and the MPN rhizobia declined with increased levels of Zn contamination, as did the abundance of the functional gene *nodD*, providing compelling evidence of a toxic effect of Zn on *R. leguminosarum* populations in the soil. Regression analysis suggested the total Zn concentration in soil as a better predictor of rhizobial numbers than both NH_4NO_3-extractable and soil solution Zn. *R. leguminosarum* bv. viciae *nodD* gene copies were generally less sensitive to Zn than *R. leguminosarum* bv. trifolii *nodD*. The latter were generally below detection limits at Zn levels of >250 mg kg^{-1}. Although there were differences in the actual numbers estimated by each approach, the response to Zn was broadly similar across all methods. These differences were likely to result from the fact that the molecular approaches assess the potential for nodulation while the MPN approach assesses actual nodulation. The results demonstrate that the use of targeted gene probes for assessing environmental perturbations of indigenous soil rhizobial populations may be more sensitive than the conventional plant bioassay and MPN methods.

Rhizobia are agronomically important as the nitrogen-fixing symbionts of legumes. *Rhizobium leguminosarum* bv. trifolii is an important species because of its symbiosis with white clover (*Trifolium repens*), the most common in-field legume contributing to N_2 fixation in the 11 million hectares of farmland currently under pasture in the United Kingdom (18). Numerous studies have demonstrated the negative effects of metals associated with sewage sludge application to land on the population size of *Rhizobium* in agricultural soils (e.g., see references 3, 15, 22, 24, and 30) and on rates of N_2 fixation (31). Zinc derived from sewage sludge has been shown to have an adverse effect on *R. leguminosarum* bv. trifolii (e.g., see references 4, 7, 8, and 9), as well as other *Rhizobium* species, including the closely related *R. leguminosarum* bv. viciae (6, 27), which is the symbiont of several legume species, including pea (*Pisum sativum*), field bean (*Vicia faba*), and hairy vetch (*Vicia hirsuta*), both at Zn levels in soil near the upper EU limit of 300 mg kg^{-1} (5). This is of concern if sewage sludge is to be used on land as a sustainable management practice.

Most studies to date have investigated the number of symbiotically competent rhizobia using the conventional trap plant nodulation bioassay, which estimates the most probable number of nodule-forming bacteria in soil (MPN). Although laborious and time-consuming, the trap plant MPN approach is still the most commonly used method for assessing the effects of perturbation on rhizobial populations. However, the ability of rhizobia to form symbiotic associations with host plants depends on the presence of nodulation (Nod) genes, which are not always present in the species, are often located on plasmids or other mobile genetic elements, and can transfer between cells. Thus, rhizobial communities in the environment comprise both free-living cells lacking symbiotic genes (Nod^-) that can reproduce actively in soil but do not form root nodules (16, 19) and those capable of symbiotic association with roots. This Nod^- component of the population is not assessed by conventional plant nodulation assays. This raises the question of whether Zn associated with sludge forces a decline in rhizobial populations or whether rhizobia lose their ability to nodulate under stress due to loss of Nod genes but still remain as free-living organisms in the soil.

In this work, quantitative real-time PCR was used with DNA extracted directly from soil to determine the relative abundance of Nod genes specific for either *R. leguminosarum* bv. viciae or *R. leguminosarum* bv. trifolii, compared to use of the 16S rRNA gene, which identifies the species *R. leguminosarum*, to determine whether Zn associated with sludge has a toxic effect on endogenous *R. leguminosarum* populations or whether it selects for variants that lack Nod genes but survive as free-living cells in the soil.

MATERIALS AND METHODS

Experimental field sites and treatments. Two experimental field sites were used in the study, Woburn, a sandy loam (pH 6.6), and Watlington, a loam (pH 6.6). These experimental sites formed part of a larger study on the long-term

* Corresponding author. Present address: Hawkesbury Institute for the Environment, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 2751, Australia. Phone: 61 (0) 245701332. Fax: 61 (0) 245701314. E-mail: c.macdonald@uws.edu.au.

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TABLE 1. Soil chemical properties of Woburn and Watlington soils exposed to increasing levels of Zn-contaminated sewage sludge[a]

Parameter	Soil	Value with sludge exposure (target Zn concn, mg kg^{-1})				
		No sludge (43–45[b])	Sludge (43–45[b])	Low Zn (200–250)	Medium Zn (300–350)	High Zn (400–450)
Soil pH	Woburn	6.63 (0.09)	6.57 (0.03)	6.57 (0.12)	6.50 (0.00)	6.57 (0.09)
	Watlington	6.57 (0.09)	6.57 (0.14)	6.53 (0.10)	6.50 (0.01)	6.63 (0.01)
Solution pH	Woburn	5.40 (0.38) b	6.11 (0.20) a	6.26 (0.04) a	5.94 (0.07) a	6.18 (0.04) a
	Watlington	6.79 (0.05) a	6.36 (0.19) abc	6.29 (0.16) bc	6.50 (0.06) ab	6.00 (0.21) c
DOC	Woburn	81.00 (3.77)	66.75 (13.06)	87.65 (4.39)	85.55 (4.10)	86.05 (3.32)
	Watlington	146.55 (3.50) b	165.00 (4.82) ab	145.15 (24.76) b	158.50 (4.36) b	195.00 (24.56) a
Total Zn	Woburn	34.45 (1.37) d	48.57 (2.5) d	149.17 (12.08) c	200.19 (15.14) b	274.72 (19.05) a
	Watlington	41.04 (5.50) d	54.75 (1.96) d	169.57 (2.88) c	239.59 (33.40) b	300.33 (15.01) a
Soil Solution Zn	Woburn	0.11 (0.02) c	0.01 (0.00) c	0.20 (0.14) c	1.12 (0.20) a	0.67 (0.07) b
	Watlington	0.02 (0.01) bc	0.01 (0.01) c	0.18 (0.02) a	0.23 (0.05) a	0.32 (0.09) a
Extractable Zn	Woburn	0.01 (0.00) c	0.01 (0.00) c	0.08 (0.02) c	0.37 (0.07) a	0.24 (0.06) b
	Watlington	0.00 (0.00) d	0.01 (0.00) d	0.09 (0.02) c	0.13 (0.01) b	0.20 (0.03) a
% C	Woburn	1.15 (0.06) b	2.01 (0.09) a	1.91 (0.02) a	2.01 (0.02) a	1.95 (0.06) a
	Watlington	1.55 (0.05) c	2.42 (0.03) b	2.56 (0.09) ab	2.63 (0.04) ab	2.66 (0.15) a
% N	Woburn	0.10 (0.00) c	0.20 (0.01) a	0.18 (0.00) b	0.18 (0.00) b	0.17 (0.00) b
	Watlington	0.15 (0.00) b	0.25 (0.00) a	0.25 (0.01) a	0.25 (0.00) a	0.25 (0.01) a

[a] Values are means (± SE) ($n = 3$). Lowercase letters indicate values that are significantly different. Values sharing the same letter are not significantly different ($P > 0.05$).

[b] Values represent natural background metal levels at the two sites.

effects of metals in sewage sludge encompassing nine experimental field sites throughout the United Kingdom (21). Full details of the field experiment were described previously (9), and the treatments and soil properties are described in Table 1. Each treatment was done in triplicate in a randomized block design. In this study only the Zn-treated and associated control plots were used. Plots had been under arable wheat rotation since 2005, prior to which they had been under arable wheat/ryegrass rotation since 1997.

Soil sampling and chemical analysis. Soils were sampled in May 2009. Fifteen to twenty soil cores (1.5 cm in diameter and 15 cm in depth) were collected from each plot. Cores from within a plot were bulked, sieved (<2 mm), and split into three subsamples that were either stored at 4°C (for 2 months), air-dried for chemical analysis, or stored at −20°C for molecular analyses. A subsample of the air-dried soil was ground (<0.5 mm) and used for determination of total metal concentrations using aqua regia digestion (29). Pore water metal was determined, following the extraction method of Kinniburgh and Miles (25), and NH_4NO_3-extractable metal was determined as previously described (32). Soil pH was measured on fresh soil (in water, 1:2.5 [wt/vol]). Dissolved organic C (DOC) was determined in soil pore water using a Thermalox total carbon/TN analyzer (Analytical Sciences, Cambridge, United Kingdom). Total C and N were determined by combustion (Leco CNS 2000 combustion analyzer).

MPN enumeration of indigenous *R. leguminosarum* bv. trifolii and bv. viceae. The most-probable-number (MPN) method was used to estimate the number of indigenous rhizobia from each plot using a 10-fold dilution series (34) as previously described (9). *Trifolium repens* cv. Menna was used as the trap host for *R. leguminosarum* bv. trifolii. For Woburn soils, *R. leguminosarum* bv. viceae was enumerated using *Vicia hirsuta* as the trap host. Seedlings were grown for 4 weeks in a controlled environment growth cabinet with 14 h days at 20°C, light intensity 350 μm m^2 s^{-1}, and 16°C nights, after which tubes were scored (+ or −) for nodulation and the most probable number of rhizobia was calculated using the MPNES computer program (35).

Determination of indigenous *R. leguminosarum* bv. trifolii and bv. viceae 16S rRNA and Nod gene copy numbers. DNA was extracted from soil samples (0.500 g), and DNA was isolated (0.5 ml overnight culture) using the MoBio PowerSoil DNA extraction kit, following the manufacturer's instructions with slight modifications, whereby the 15 min of shaking on a flat bed vortex was replaced by a 30-s bead beading step (5.5 m s^{-1}; Fastprep). Primers that would amplify 16S rRNA gene sequences of *R. leguminosarum* from soil samples were designed using sequences (285 bp in length) from the Ribosomal Database Project II (RDPII, release 10; http://rdp.cme.msu.edu/ [12]) and the NCBI website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using MUSCLE alignment software (http://www.ebi.ac.uk/Tools/muscle/index.html [20]), and regions potentially specific for the detection of *R. leguminosarum* were identified using BioEdit (23). Oligonucleotides designed for this region were assessed for their specificity by checking them against the Ribosome Database Project (RDP II) using the probe matching facility (Table 2). Neither the forward nor the reverse primers were 100% specific to *Rhizobium leguminosarum*, but both proportionally amplified a greater number of *Rhizobium leguminosarum* bacteria than other species. For both forward (F979) and reverse (R1264) primers, a very small number of matches were found with sequences not within the *Rhizobium* genus (Table 2). Finally, candidate oligonucleotide pairs were evaluated using the Primer Express v.3 software program (Applied Biosystems) to ensure that there were no dimers or secondary structure generated with the chosen primers.

To determine the effect of zinc on nodulation, primers were designed to target genes involved in nodulation. Two genes (*nodC* and *nodD*) were initially targeted. All available *nodC* and *nodD* sequences were extracted from the NCBI database, multiple sequence alignments were performed, and regions potentially specific for the detection of *R. leguminosarum* bv. trifolii and bv. viciae identified as before. For *nodC*, it was not possible to design probes that were specific to *R. leguminosarum* and could also discriminate between bv. trifolii and bv. viciae (data not shown). Four *nodD* probes (Table 3) that were specific to *R. leguminosarum* and could discriminate between bv. trifolii and bv. viciae were designed. Primers were then blasted against the NCBI nucleotide database using the megablast software program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn) and found to be highly specific for each biovar (Table 3), with the exception of a match with two *Rhizobium fabae* isolates (accession no. EU430078 and EU430079) for the *nodD* viciae primer set.

PCR conditions were optimized on a thermal cycler and tested against a range of bacterial isolates, including *Rhizobium leguminosarum* (RSM 2004), *Rhizobium leguminosarum* bv. trifolii (RCR221), *Rhizobium leguminosarum* bv. viciae (VP39), *Sinorhizobium meliloti* (RCR 2001), *Mesorhizobium* sp., *Agrobacterium rhizogenes* (8/96), *Agrobacterium rhizogenes* (LBA9402), *Pseudomonas fluorescens* (PCM 2004), and *Pasteuria penetrans* (16S 51102). Positive PCR products derived from 16S rRNA *R. leguminosarum* primers were detected only for DNA extracted from *R. leguminosarum* isolates, and T *nodD* and V *nodD* primer sets amplified only PCR product from *R. leguminosarum* bv. trifolii and *R. legumi-*

TABLE 2. Specificities of 16S rRNA *Rhizobium* forward and reverse primers to sequences within the Ribosomal Database Project[a]

Primer and category[b]	Sequence or specificity	No. of hits	Total hits
F979	CCCGGCTACYTGCAGAGATG		
Domain	*Bacteria*	450	600,316
Class	*Alphaproteobacteria*	450	171,211
Order	*Rhizobiales*	450	12,491
Family	*Rhizobiaceae*	450	2,734
Genus	*Rhizobium*	450	2,129
Species	*Rhizobium leguminosarum*	209	269
	Rhizobium sp.	149	1,126
	Rhizobium etli	35	73
	Rhizobium mesosinicum	11	11
	Rhizobium sullae	9	12
	Rhizobium gallicum	8	42
	Rhizobium indigoferae	4	5
	Rhizobium alamii	2	2
	Rhizobium fabae	2	2
	Rhizobium genospecies	1	5
	Rhizobium mongolense	1	28
	Rhizobium phaseoli	1	17
	Rhizobium pisi	1	3
	Uncultured *Rhizobium* sp.	11	85
	Mesorhizobium sp.	3	697
	Sinorhizobium sp.	2	397
	Arthrobacter viscosus	1	1
R1264	TAGCTCACACTCGCGTGCTC		
Domain	Bacteria	1,359	6,000,316
Class	*Alphaproteobacteria*	1,358	35,398
Order	*Sphingomonadales*	19	4,965
	Unclassified *Rhizobiales*	3	1,220
	Rhizobiales	1,339	12,491
Family	*Aurantimonadaceae*	16	206
	Brucellaceae	540	700
	Phyllobacteriaceae	6	1,148
	Rhizobiaceae	774	2,734
Genus	Unclassified *Rhizobiaceae*	4	12
	Rhizobium	770	2,129
Species	*Rhizobium leguminosarum*	205	269
	Rhizobium sp.	302	1,126
	Rhizobium etli	51	73
	Rhizobium tropici	56	74
	Rhizobium rhizogenes	38	57
	Rhizobium gallicum	27	42
	Rhizobium mongolense	16	28
	Rhizobium multihospitium	11	24
	Rhizobium lusitanum	5	5
	Rhizobium genospecies	4	5
	Rhizobium indigoferae	4	5
	Rhizobium loessense	4	4
	Rhizobium radiobacter	3	234
	Rhizobium sullae	3	12
	Rhizobium fabae	2	12
	Rhizobium galegae	2	33
	Rhizobium miluonense	2	2
	Rhizobium rubi	2	23
	Rhizobium yanglingense	2	3
	Rhizobium cnuense	1	1
	Rhizobium hainanense	1	2
	Rhizobium huautlense	1	11
	Rhizobium phaseoli	1	17
	Rhizobium pisi	1	3
	Rhizobium taeanense	1	1
	Mesorhizobium sp.	2	697
	Sinorhizobium sp.	1	397
	Agrobacterium sp.	5	161
	Burkholderia sp.	2	1,073

[a] Release 10 (http://rdp.cme.msu.edu/).
[b] F979 and R1264 are forward and reverse primers, respectively.

nosarum bv. viciae, respectively (Table 4). Compared to other primer combinations, the *nodD* primer pair F88 and R443, designed to target *Rhizobium leguminosarum* bv. viciae, gave reproducible PCR products when tested against isolate VP39 and with soil DNA extracts. This primer pair was chosen over other primer pairs for *Rhizobium leguminosarum* bv. viciae *nodD* gene quantification.

Quantitative PCR was performed using a Stratagene Mx3000P QPCR thermal cycler (Agilent Technologies) in a 25 μl reaction mixture consisting of 20 ng DNA template 2× QuantiTect SYBR green master mix (Qiagen), 300 nM for each primer. Standards were generated from PCR products that had been generated from soil DNA extracts, gel purified, and quantified using the Quant-iT Pico Green ds DNA assay kit (Invitrogen) and diluted accordingly. The number of copies of each gene was calculated using the following equation: gene copy number = (ng × number/mol)/(base pairs × ng/g × g mol base pairs) (http://www.uri.edu/research/gsc/resources/cndna.html). Standards were diluted accordingly to give a concentration range from 0 to 10^5 gene copies μl^{-1}. PCR amplifications were performed in triplicate for all standards and soil samples with a 15-min denaturing step at 95°C, followed by 42 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. Melt curve analysis was performed between 55°C and 95°C, and products were run on a 1.5% ethidium bromide-stained agarose gel to ensure a single correct product was obtained and to check for primer dimers. To determine the potential effect of PCR inhibition on quantification of gene copy numbers, each soil DNA extract was spiked with 10^5 gene copies. In spiked samples, following subtraction of the spiked 10^5 gene copies, for all DNA extracts, values were found to fall within the range for the three replicate non-spiked samples (data not shown), and we therefore considered there to be no PCR inhibition in our samples. Data were expressed as numbers of cells g^{-1} dry weight (dw) soil, assuming that the 16S rRNA gene is present in three copies per genome and *nodD* is present in one copy per genome as revealed in the *R. leguminosarum* genomes sequenced to date (http://ribosome.mmg.msu.edu/rrndb/search.php).

Statistical analysis. An analysis-of-variance generalized linear model (ANOVA-GLM) was used to test the effect of sludge and metal treatment on rhizobial MPN and quantitative PCR (qPCR) data. Data that did not fit the assumptions of the model were $\log_{10}$ transformed prior to analysis. Pearson's correlation was used to determine the relationship between MPN and qPCR estimates of *R. leguminosarum* number. Linear regression was used to determine the statistical relationship between soil chemical factors and rhizobial MPN and qPCR numbers. All analyses were done using the Genstat v.12 software program (VSN International Ltd., Hemel Hempstead, United Kingdom).

RESULTS

Effect on indigenous populations of *R. leguminosarum* bv. trifolii and bv. viciae. MPN estimates of both *R. leguminosarum* bv. trifolii and bv. viciae were in the region of 10^4 cells g^{-1} (95% confidence interval [CI] = $\pm 7.8^3$ to 1.6^4) soil in no-sludge and low-metal sludge-treated soils, which is consistent with previous reports for these soils (9). In Zn-sludge-treated soils, numbers of *R. leguminosarum* bv. trifolii fell significantly, to around 10 cells g^{-1} (95% CI = ± 2.0 to 1.3^1) in both Woburn (Fig. 1a; $P < 0.001$) and Watlington (Fig. 1b; $P < 0.001$) soils irrespective of the Zn dose. Zn had a significant negative effect on *R. leguminosarum* bv. viciae in Woburn soils, and cells were detected in only one of the three block replicates at 250 mg Zn kg^{-1} soil and could not be detected in soils with Zn levels of >250 mg kg^{-1} (Fig. 1c, $P < 0.001$).

The qPCR results showed a similar trend, whereby addition of Zn-enriched sludge had a negative impact on the number of cells (Fig. 2). Using the 16S rRNA gene and the knowledge that each cell contains three copies, the numbers of *Rhizobium* bacteria in unsludged soils were in the region of 1.7×10^5 cells g^{-1} dw soil (95% CI = $\pm 4.1^4$) at Woburn (Fig. 2a) and 1.0×10^5 cells g^{-1} dw soil (95% CI = $\pm 1.2^5$) at Watlington (Fig. 2b). In low-metal sludge-treated soils, numbers were lower (3.4×10^4 cells g^{-1} dw soil [95% CI = $\pm 4.9^4$] at Woburn and 2.9×10^4 cells g^{-1} dw soil [95% CI = $\pm 1.0^4$] at Watlington) but not significantly so ($P > 0.05$). However, numbers did fall signifi-

TABLE 4. PCR amplification of bacterial isolates using *Rhizobium leguminosarum*-specific 16S and *nodD* primers

Bacterial isolate	Amplification by primer set		
	16S *R. leguminosarum*	T *nodD*	V *nodD*
Rhizobium leguminosarum (RSM 2004)	+	−	+
Rhizobium leguminosarum bv. trifolii (RCR221)	+	+	−
Rhizobium leguminosarum bv. viciae (VP39)	+	−	+
Sinorhizobium meliloti (RCR 2001)	−	−	−
Mesorhizobium sp.	−	−	−
Agrobacterium rhizogenes (8/96)	−	−	−
Agrobacterium rhizogenes (LBA9402)	−	−	−
Pseudomonas fluorescens (PCM4002)	−	−	−
Pasteuria penetrans (16S 51102)	−	−	−

cantly at both Woburn and Watlington upon addition of Zn-contaminated sludge. At the highest rate of contamination, *R. leguminosarum* numbers as estimated by the 16S rRNA gene were 1.4×10^4 cells g^{-1} dw soil (95% CI = $\pm 9.2^3$) at Woburn and 1.3×10^4 cells g^{-1} dw soil (95% CI = $\pm 2.3^4$) at Watlington, which was significantly lower than in the low-metal and no-sludge soils at both sites ($P < 0.001$).

For *R. leguminosarum* bv. trifolii *nodD*, the number of rhizobia in no-sludge and low-metal sludge-treated soils was in the region of 7.8×10^3 cells g^{-1} dw soil (95% CI = $\pm 3.9^3$) at Woburn (Fig. 2c) and 7.5×10^4 cells g^{-1} dw soil (95% CI = $\pm 2.2^3$) at Watlington (Fig. 2d). Numbers fell with increasing soil Zn level and were below the detection limit (130 copies g^{-1} dw soil) in the highest level of Zn contamination at Woburn (Fig. 2c) and at all levels of Zn contamination in Watlington soils (Fig. 2d). For *R. leguminosarum* bv. viciae *nodD*, the copy number in uncontaminated and low-metal sludge-treated soils was in the region of 3.8×10^3 cells g^{-1} dw soil (95% CI = $\pm 8.2^2$ to 3.0^3) at Woburn (Fig. 2e) and 2.0×10^3 copies g^{-1} dw soil (95% CI = $\pm 1.6^3$ to 4.2) at Watlington (Fig. 2f) and fell significantly with increasing soil Zn at both Woburn and Watlington. A high level of correlation was found between MPN and qPCR estimates of *R. leguminosarum* number at Woburn and Watlington (Table 5).

Relationship between *Rhizobium leguminosarum* MPN and gene copy numbers with soil physical and chemical properties. Generally, the total Zn concentration was a better predictor of the negative impact of Zn on rhizobial cell number than was either soil solution Zn or NH_4NO_3-extractable Zn, consistently explaining a larger proportion of the variance (Table 6) for MPN and molecular approaches. The percentage variance explained by total Zn for all MPN and gene copy measures was greater for Watlington soils than for Woburn soils (Table 6). There was a stronger negative impact of Zn on MPN *R. leguminosarum* bv. viciae numbers than on *R. leguminosarum* bv. trifolii MPN numbers in Woburn soils (Table 6) but a similar level of effect on *nodD* trifolii and *nodD* viciae gene copies, explaining 56 and 59% of the variance, respectively. For Watlington soils, total Zn explained a larger proportion of the variance for *nodD* trifolii (85%) than for *nodD* viciae (53%). For both Woburn and Watlington soils, a lower percentage of variance was explained by total Zn for the *R. leguminosarum* 16S rRNA gene (38 and 50%, respectively) than was explained by total Zn for *nodD* (Table 6).

TABLE 3. *nodD* primers for specific amplification of *Rhizobium leguminosarum* bv. trifolii and bv. viciae and number of BLAST hits scoring 100% identity and percent identity with top nontarget hit

Primer name	Target	Primer sequence(s)[a]	Length (bp)	T_m (°C)	% GC	No. of 100% BLAST hits		Top nontarget hit, % identity
						Target	Clones or *R. leguminosarum* sp.	
nodD trifolii F235	*R. leguminosarum* bv. trifolii	CSGATTTCATGACGCTBGTATTC	22	59	43	9 (total)	10	
		CcGATTTCATGACGGCTcGTATTC				3	4	91
	Degeneracies for *nodD* trifolii F235 primer	CgGATTTCATGACGCTcGTATTC				1		78
		CgGATTTCATGACGCTtGTATTC				1	1	73
		CcGATTTCATGACGGCTgGTATTC				2	5	78
		CgGATTTCATGACGCTgGTATTC				2		82
nodD trifolii R566	*R. leguminosarum* bv. trifolii	GCCGCTTAAAACCGTGCTC	18	59	58	9	11	89
nodD viciae F88	*R. leguminosarum* bv. viciae	TGCAGAGACGGGAGCTARTTC	21	58	52	85	55	81
nodD viciae R328	*R. leguminosarum* bv. viciae	TCGTCAAGTGGCAGCAACTC	20	59	55	84	62	80
nodD viciae F443	*R. leguminosarum* bv. viciae	GACGCACACCAGTCTCTCTTCG	22	61	59	80	60	77
nodD viciae R662	*R. leguminosarum* bv. viciae	GGGGATGGTTGCTATTCGAT	20	57	60	79	46	90

[a] Lowercase letters indicate site of degeneracy.

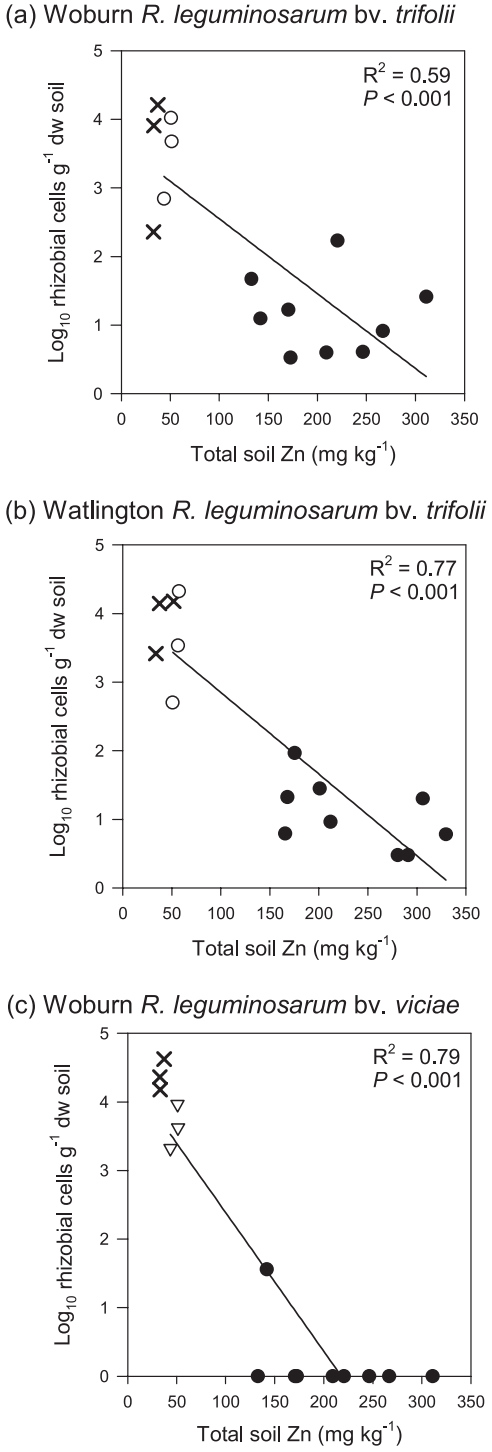

FIG. 1. The effect of Zn-enriched sludge addition on the number of indigenous *Rhizobium leguminosarum* bv. trifolii bacteria estimated by MPN in Woburn (a) or Watlington (b) soils or *R. leguminosarum* bv. viciae in Woburn soils (c). Crosses represent no-sludge control soils, open symbols represent low-metal sludge-treated soils, and closed symbols represent Zn-rich sludge-treated soils.

DISCUSSION

The negative effect of Zn from sewage sludge on *R. leguminosarum* bv. trifolii observed in both Woburn and Watlington soils is in accordance with previous findings for these soils (9) and demonstrates that the negative impact of Zn on these organisms is long lasting (15 years after the initial sludge addition) even at Zn concentrations in soil that are within the current EU guideline limits (300 mg kg^{-1}) (5). For Woburn soil, using the MPN approach, *R. leguminosarum* bv. viciae was more sensitive to Zn contamination than *R. leguminosarum* bv. trifolii, but this was not so for *nodD* genes. The *nodD* viciae genes were sensitive to Zn contamination, exhibiting lower numbers when Zn was present, but could still be detected at appreciable levels in even the highest level of Zn contamination (Fig. 2e), whereas *nodD* trifolii genes were below detection limits in the highest level of Zn contamination (Fig. 2c). The apparent greater sensitivity of *nodD* trifolii to Zn than of *nodD* viciae was also demonstrated for Watlington soils (Fig. 2d and 2f). The reasons for the discrepancy between the greater sensitivity of *R. leguminosarum* bv. viciae than of *R. leguminosarum* bv. trifolii using the MPN approach but not with the molecular approach that targets *nodD* genes are unclear. However, the establishment of nodulation in the host plant requires a complex molecular dialogue between the rhizobial and plant cells, involving many genes (13, 17). It may be that there are rhizobia that carry *nodD* but lack other symbiotic genes in the contaminated soils. It is also possible that there is differential host affinity within *R. leguminosarum* bv. viciae: it is known to form symbioses with several host plants, including pea (*Pisum sativum*), field bean (*Vicia faba*), and hairy vetch (*Vicia hirsuta*). Here we used just one trap plant (*Vicia hirsuta*), and further work is needed to determine whether the results observed here for Woburn soil are repeatable across different host plants. Previous studies have shown *R. leguminosarum* bv. viciae to be sensitive to Zn additions (8) but not more sensitive than *R. leguminosarum* bv. trifolii.

The numbers of *R. leguminosarum* bacteria estimated using the 16S rRNA gene targets were greater than those estimated by *nodD* genes and the MPN estimates. The reasons for this are severalfold. First, the MPN estimate relies on successful nodulation, which involves both a bacterial and plant component. The abundance of 16S rRNA and *nodD* genes is an indicator of the potential for nodulation rather than nodulation *per se*. Thus, the use of soil-extracted DNA with targeted primers may be more sensitive than the MPN approach for determining potential nodulation. It has previously been demonstrated that the plant trapping method does not consider the viable but nonculturable fraction (2), and the larger population detected by the molecular approaches in contaminated soils may reflect this nonculturable component. Further, it has been previously suggested that only a small percentage of the *R. leguminosarum* populations in soil contain symbiotic genes (26), and an *R. leguminosarum* isolate cured of its symbiotic plasmid was shown to have become successfully established 5 years after it was inoculated into a field trial release site (10). *R. leguminosarum* populations which lack symbiotic plasmids may therefore also be reflected in the higher numbers estimated using the 16S rRNA gene probes than with *nodD* genes and the MPN estimates. Second, the 16S rRNA primers could not be designed to be 100% specific to *R. leguminosarum* (Table 2), and it is possible that other *Rhizobium* spp. were amplified with these primers. A more extensive screening of isolated clones is needed to confirm this. Because DNA survives

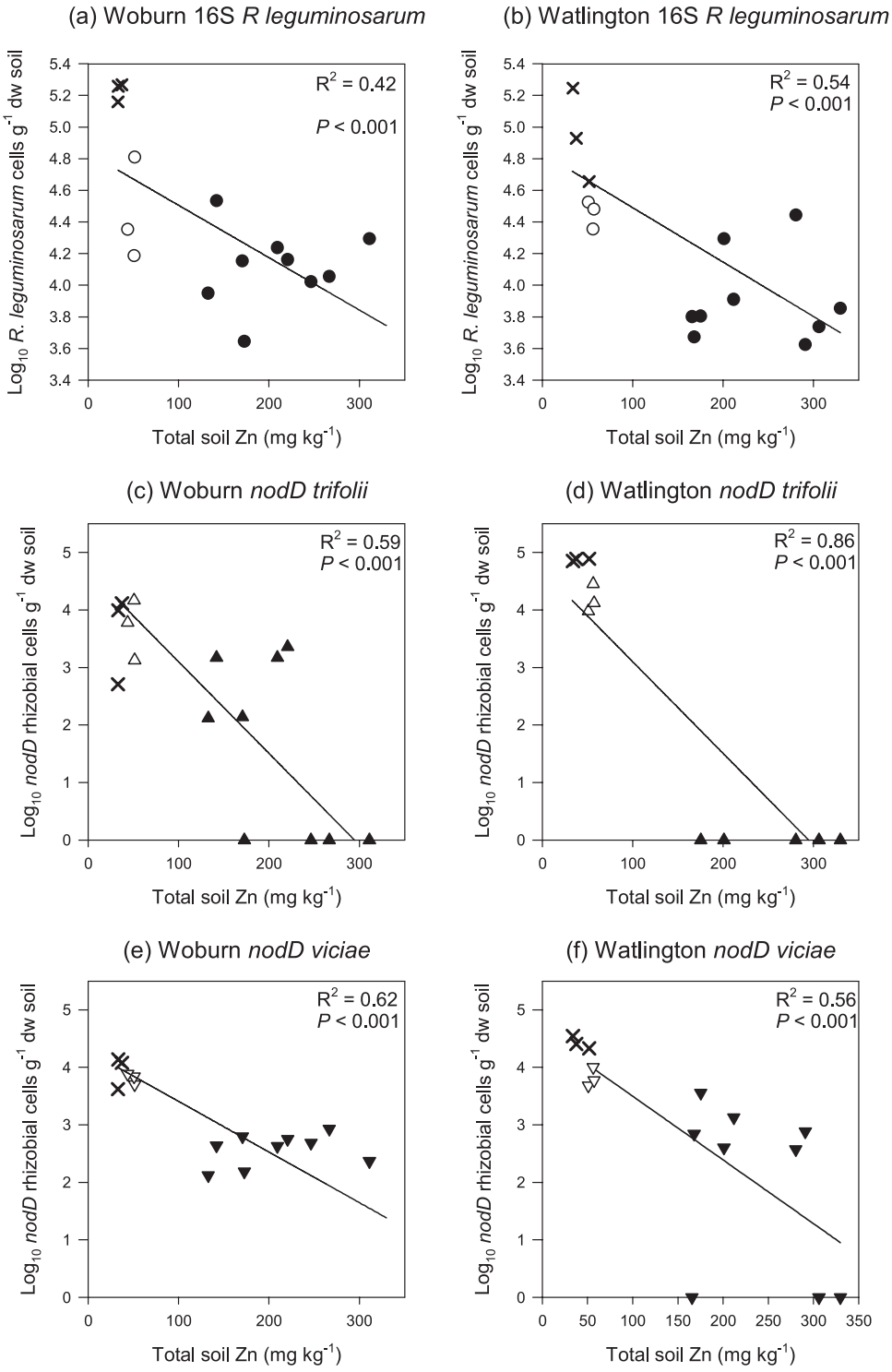

FIG. 2. The effect of Zn-enriched sludge addition on the number of indigenous *Rhizobium leguminosarum* cells based on 16S rRNA gene copy number at Woburn (a) or Watlington (b), on *R. leguminosarum* bv. trifolii *nodD* gene copy number at Woburn (c) or Watlington (d), or on *R. leguminosarum* bv. viciae *nodD* gene copy number at Woburn (e) or Watlington (f). Crosses represent no-sludge control soils, open symbols represent low-metal sludge-treated soils, and closed symbols represent Zn-rich sludge-treated soils.

for some time in soils following cell death (11), it is possible that the higher estimates observed using 16S rRNA gene targets may in part be attributed to the presence of dead cells. Methods to extract live bacterial cells from soils have been developed to try to overcome this potential bias (1, 14), but such approaches also suffer from inherent bias (28), and 100% extraction efficiency is unlikely. Nevertheless, the same trend of a reduction in rhizobia was observed in this data set, as has

TABLE 5. Pearson's correlation matrix showing the relationship between MPN and qPCR approaches for estimation of *R. leguminosarum* numbers in soils exposed to increasing levels of zinc contamination[a]

Predictor used	Correlation between estimates						
	Woburn				Watlington		
	MPN, bv. trifolii	MPN, bv. viciae	16S rRNA gene	*nodD* trifolii	MPN, bv. trifolii	16S rRNA gene	*nodD* trifolii
MPN, bv. viciae	0.734***						
16S rRNA gene	0.692**	0.817***			0.637*		
nodD trifolii	0.743**	0.698**	0.594*		0.926***	0.785**	
nodD viciae	0.871***	0.924***	0.765***	0.687**	0.776**	0.759**	0.770**

[a] Significant correlations are shown as follows: ***, $P < 0.001$; **, $P < 0.001$; *, $P < 0.05$.

been consistently observed using the MPN approach. Further, there was a strong correlation between numbers of rhizobia estimated by the MPN approach and by both the 16S rRNA gene and the *nodD* gene approaches (Table 5), demonstrating that the qPCR approach developed provides estimates that are broadly in accordance with the conventional MPN estimates for *R. leguminosarum*.

A long-standing uncertainty in use of plant bioassay MPN estimates of rhizobial numbers in soils has been whether an observed lack of nodulation stems from the death of rhizobial cells or a loss of the ability of these cells to nodulate (Nod^-). Here we have demonstrated declines in both 16S rRNA gene copies and *nodD* gene copies in response to increasing Zn contamination. Although 16S rRNA gene copy numbers did not fall below detection limits with increasing Zn concentrations, as was generally observed for *nodD*, the appreciable declines in 16S rRNA gene copies in Zn-contaminated soils compared to those in uncontaminated soils provides compelling evidence that declines in the number of rhizobia observed under Zn-contaminated soils is a result of a toxic effect that kills the *R. leguminosarum* cells and closely related species and not merely a loss in the ability of these cells to nodulate. The qPCR assay using *nodD* primers gave reliable results down to 1.3×10^2 copies g^{-1} of soil, which is lower than previously reported for other rhizobial species using other gene targets (19, 33). The higher qPCR estimates for the *nodD* bv. viciae approach than for the MPN approach at Woburn indicate that this may be a more sensitive and rapid assessment of symbiotically competent rhizobial populations that have the potential to nodulate. For *nodD* of bv. trifolii, the MPN approach showed a slightly higher level of sensitivity than the qPCR approach. This may in part be a reflection of the differences in the volume of soil used for each approach, and the sensitivity of the qPCR approach may be improved by increasing the volume of soil from which the DNA is extracted. Further work is needed to determine whether the greater sensitivity equates to better reliability.

In conclusion, the qPCR approach developed here that targets 16S rRNA and *nodD* genes, primarily of *R. leguminosarum*, confirms the toxic effect of Zn on rhizobia populations. This confirms and extends the information available from the MPN approach, since we can conclude that over the long term, following exposure to Zn-contaminated sludge addition, numbers of both free-living *R. leguminosarum* and those cells con-

TABLE 6. Linear regression between soil analyses and numbers of *Rhizobium leguminosarum* bacteria estimated by different MPN and qPCR approaches[a]

Predictor used	Variate	% variance accounted for by regression		*P* value		Slope	
		Woburn	Watlington	Woburn	Watlington	Woburn	Watlington
Rhizobium leguminosarum bv. trifolii MPN	Total Zn	59	77	<0.001	<0.001	−0.01	−0.01
	Soil solution Zn	20	67	NS	<0.001	−1.46	−8.49
	NH_4NO_3-extractable Zn	34	74	<0.05	<0.001	−5.11	−15.47
Rhizobium leguminosarum bv. viciae MPN	Total Zn	79	*	<0.001	*	−0.02	*
	Soil solution Zn	40	*	<0.01	*	−3.06	*
	NH_4NO_3-extractable Zn	52	*	<0.01	*	−9.29	*
Rhizobium leguminosarum 16S rRNA	Total Zn	38	50	<0.01	<0.01	−0.0034	−0.003
	Soil solution Zn	13	47	NS	<0.01	−0.47	−2.50
	NH_4NO_3-extractable Zn	18	26	NS	<0.05	−1.531	−3.39
Rhizobium leguminosarum bv. trifolii *nodD*	Total Zn	56	85	<0.001	<0.001	−0.01276	−0.02
	Soil solution Zn	8	86	NS	<0.001	−1.327	−18.46
	NH_4NO_3-extractable Zn	7	77	NS	<0.001	−3.71	−25.06
Rhizobium leguminosarum bv. viciae *nodD*	Total Zn	59	53	<0.001	<0.001	−0.00582	−0.01
	Soil solution Zn	26	33	<0.05	<0.05	−0.770	−6.90
	NH_4NO_3-extractable Zn	22	27	<0.05	<0.05	−2.31	−11.00

[a] *, not measured; NS, not significant.

taining the nodulation factor *nodD* show dramatic rates of decline which are in line with those observed using the conventional MPN approach. Targeting of specific functional genes involved in nodulation may provide a more efficient assessment of rhizobial numbers than the conventional MPN approach.

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