Elemental sulphur oxidation in Australian cropping soils

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Abstract

Sulphur (S) is one of the essential nutrients for plant growth. Over the last few decades, soil S deficiency has become more common in many countries primarily due to the application of high analysis S-free fertilisers and stricter regulations for industrial S dioxide emissions. To ameliorate soil S deficiency, elemental S (ES) as a fertiliser source is of great interest as ES is cost-effective and less susceptible to leaching than sulphate sources. However, ES has to be oxidised to sulphate to become available for plants. Sulphur oxidation is a biological process and depends on many factors affecting the size (genetic potential) and activity of the microbial population, but the predictability of ES oxidation by S-oxidising organisms has not been studied in soil. This work aimed to 1) examine the relationship between the genetic potential of a soil to oxidise ES and the oxidation rate of ES; and 2) investigate causes for the slower oxidation of granular ES compared to powdered ES.

The relationship between the oxidation rate of ES, and soil physico-chemical properties and microbial populations (indicated by gene abundances) was investigated in ten Australian cropping soils covering a wide range of soil physico-chemical properties in a laboratory incubation experiment. The oxidation rate of ES, estimated from decreases in ES concentrations, varied greatly from 5.1 to 51.7 $\mu g\ cm^{-2}\ d^{-1}$ across soils and was positively correlated with the initial soil pH ($R^2 = 0.54$, $P < 0.05$). A regression equation including pH and organic C content as independent variables explained 79% of the variation in the oxidation rate ($P < 0.01$).
The copies numbers of a functional gene *soxB* was quantified to indicate the abundance of S-oxidising bacteria, and 16S ribosomal ribonucleic acid (16S rRNA) and 18S ribosomal

ribonucleic acid (18S rRNA) to indicate the abundance of total bacteria and fungi, respectively. The abundances of *soxB* and 16S rRNA were positively correlated ($P < 0.05$) with ES oxidation rate ($R^2 = 0.67$ for *soxB* and 0.66 for16S rRNA), but no significant correlation was observed between the oxidation rate and 18S rRNA abundance. This suggests that ES oxidation is dependent primarily on bacterial populations in soils. A combination of bacterial gene abundance (*soxB* or 16S rRNA) and soil pH could explain more than 80% of the variation in ES oxidation rate ($P < 0.01$). A distribution of *soxB* gene across diverse taxonomic and physiological bacterial groups was observed in the soils, which explains the strong relationship between *soxB* and 16S rRNA abundances ($R^2 = 0.99$, $P < 0.01$).

Elemental S is often combined with macronutrient fertilisers and this is generally found to reduce the ES oxidation rate as compared to ES in powdered form. We hypothesised that this reduction may be due to 1) acidification in the soil around the granule (in addition to ES oxidation, acidification can also be induced by monoammonium phosphate with which ES is often co-granulated); or 2) increased ionic strength of the soil solution in the vicinity of the granule from water-soluble fertilisers. Therefore, the effect of increases in acidity or ionic strength on ES oxidation in a sandy soil was studied. Interestingly, neither increases in acidity nor in ionic strength significantly affected ES oxidation in this soil, even though significant shifts in bacterial abundance and community composition were observed due to these changes. An additional experiment carried out at two ES application rates with two different soils showed similar results. This indicates that changes in bacterial abundance and community composition brought about by temporary changes in pH and ionic strength do not necessarily affect ES oxidation. The lack of agreement between bacterial population and ES oxidation might be due to the measurement of the total populations of bacteria, including dormant ones. The consistent ES oxidation (%ES oxidised) across treatments in this experiment suggests that

there were sufficient active populations of S-oxidisers even at high acidity and ionic strength levels. Furthermore, while soil pH related to ES oxidation rate across soils as indicated by our previous study, no relationship was found in the soil acidified for < 15 weeks. This inconsistent effect of pH on the oxidation of ES (across soils *versus* within a soil) can be reconciled by the fact that pH differences across soils are associated with differences in many soil chemical and biological properties, which is not the case for short-term acidification.

As the slower oxidation for co-granulated ES, compared to powdered ES, is likely not related to the chemical changes (pH, ionic strength) around the granule, we speculated that the slower oxidation is due to a reduction in the surface area of ES exposed to S-oxidisers in soil. To test this hypothesis, an experiment was conducted in which ES oxidation, soil chemical properties and bacterial abundance and community composition were compared between powdered (mixed through soil) and granular fertiliser (diammonium phosphate +10% ES). Soil in the vicinity of the granule including the granule was sampled for analysis. Oxidation of the co-granulated ES was much slower than for the powdered ES, with 36% oxidised for the former and 95% for the latter by the end of 20 weeks incubation. This difference was not related to differences in soil pH, bacterial abundances and community composition between these two treatments. Instead, the difference in ES oxidation rate between the two treatments corresponded to the difference in surface area of the granule and that of the individual ES particles, strongly suggesting that the slower oxidation rate for co-granulated ES was due to a reduction in the effective surface area available for S-oxidisers to colonise. Hence, oxidation of ES is not limited by the population of ES-oxidising bacteria in soil, but by the amount of ES exposed to soil organisms.

This work shows that ES oxidation is influenced by both soil biological and physico-chemical properties across soils, and it could be well predicted by two variables i.e. soil pH and bacterial

gene abundance under a steady environment. However, alterations in the abundance and community composition of bacteria resulted from temporary ambient changes within a soil do not necessarily affect ES oxidation, which suggests that the slow oxidation of ES in granules is not related to chemical changes but due to the low degree of dispersion of granules in soil. Therefore, in an effort to improve the effectiveness of granular ES, it is key to improve the exposure of ES to soil microorganisms, e.g. by technically improving granule dispersion in soil or by inoculating S-oxidisers into the granule.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or tertiary institution without the prior approval of The University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Publications arising from this thesis

1. Zhao. C, F. Degryse, V. Gupta and M.J. McLaughlin. 2015. Elemental sulfur oxidation in Australian cropping soils. Soil Science Society of America Journal 79:89-96.

2. Zhao. C, V.V.S.R. Gupta, F. Degryse, and M.J. McLaughlin. Abundance and diversity of sulphur-oxidising bacteria, and their role in elemental sulphur oxidation in Australian cropping soil. Submitted to Soil Biology and Biochemistry.

3. Zhao. C, V.V.S.R. Gupta, F. Degryse, and M.J. McLaughlin. Effects of pH and ionic strength on elemental sulphur oxidation in soil. Submitted to Soil Biology and Biochemistry.

4. Zhao. C, F. Degryse, V.V.S.R. Gupta and M.J. McLaughlin. Oxidation of elemental sulphur in co-granulated fertiliser. Submitted to Soil Science Society of America Journal.

CHAPTER 1

Introduction

1. Background

Sulphur (S) is considered as the "fourth essential nutrient" after nitrogen (N), phosphorus (P) and potassium (K), and its importance in crop production is increasingly recognised. Soil S deficiency is becoming more widespread due to imbalanced soil S input and output fluxes. In an effort to ameliorate this issue, S fertilisers are applied to soil. The most commonly used S fertilisers contain sulphate-S or elemental S (ES) as S sources. Compared with sulphate-S, ES is more cost-effective and less susceptible to leaching. However, the effectiveness of ES fertiliser has been found to be variable and unpredictable (Solberg et al., 2005).

Elemental S is not readily available for plants until it is oxidised to sulphate. Elemental S oxidation in soil is predominantly mediated by microorganisms. Therefore, the oxidation of ES is affected by environmental conditions and soil physico-chemical properties that affect the microbial population size and activity. In particular, a significant correlation between ES oxidation and soil pH and/or organic carbon (C) has been observed across soils from several countries (Barrow, 1971; Fox et al., 1964; Lawrence and Germida, 1988; Skiba and Wainwright, 1984). Still, little is known about the effect of physico-chemical properties on ES oxidation in Australian cropping soils.

Furthermore, it has been found that ES oxidation is correlated with soil microbial biomass C and soil respiration rate (Lawrence and Germida, 1988). However, another study has suggested that the oxidation is not limited by the number of S-oxidising organisms, given that the oxidation rate for a soil is independent of the ES application rate (Janzen and Bettany, 1987). The role of soil heterotrophs and autotrophs in oxidising ES has been reported in many studies using culture-based methods, but no conclusions have been reached regarding their relative importance. Functional gene methods are culture-independent and hence allow studying the

whole bacterial community, not just the culturable community. Only a few studies on S-oxidising bacteria in soil have been carried out based on functional gene measurements and it has been shown that diverse bacterial groups are involved in ES oxidation (Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014).

Powdered ES mixed through soil generally oxidises quickly and hence supplies sulphate to plants in the short term. However, powdered ES is not practical to be used as a commercial fertiliser, due to the difficulties in handling powders and the explosion hazard of finely divided ES. Commercial ES-containing fertilisers are therefore usually in granular form. However, the oxidation of (co-)granulated ES has been found to be slower than that of powdered ES (Friesen, 1996; Janzen and Karamanos, 1991). The reasons for the effect of granulation on oxidation rate are unclear, and it has been speculated that this is related to the poor dispersion of granular ES through the soil medium (Friesen, 1996).

This thesis determined the relationship between ES oxidation rate on the one hand and soil physico-chemical properties and bacterial abundance and diversity on the other, across Australian cropping soils. Furthermore, how chemical changes affected bacterial abundance and community composition and ES oxidation, and which factors limited the oxidation of co-granulated ES were also examined.

2. Thesis structure

This thesis is presented in publication format and includes papers that have been published or submitted for publication. Including the present chapter, this thesis is organised in seven chapters. A schematic structure of this work is given in Fig. 1.

Chapter 2 is a literature review, which summarises S cycling in soil and discusses performance of S fertilisers. This chapter also describes the effects of environmental conditions and soil physico-chemical properties on ES oxidation, and the role of soil microorganisms in this process in Australian cropping soils. Following the review of the literature, the specific objectives of this thesis are provided.

Chapter 3 represents the results from an incubation experiment using ten Australian cropping soils covering a wide range of soil physico-chemical properties, with the objective of determining the relationship between ES oxidation and soil physico-chemical properties. This chapter also provides an assessment of two methods for the estimation of ES oxidation in soil, i.e. based on ES or on sulphate measurements.

Chapter 4 describes a new assay of polymerase chain reaction for the quantification of *soxB* gene, a functional gene involved in S oxidation. The abundance of S-oxidising bacteria was determined on the same soils as used in Chapter 3, and six of these soils were selected to investigate the diversity of S-oxidising bacteria. The relationship between S-oxidising bacterial abundance or diversity and ES oxidation was examined.

Chapter 5 describes shifts in bacterial abundance and community composition brought about by changes in pH or ionic strength, and the effect of these changes on ES oxidation.

Chapter 6 compares the oxidation between granular and powdered ES fertilisers, and determines the changes in chemical and microbial properties in the fertilised soils. This chapter tests whether the slower oxidation of co-granulated ES than that of powdered ES can be related

to microbial and chemical differences or whether this is due to a reduction in available surface area of the ES for soil microorganisms to colonise when ES is co-granulated.

Chapter 7 summarises the main outcomes arising from this work, and provides directions for future research.

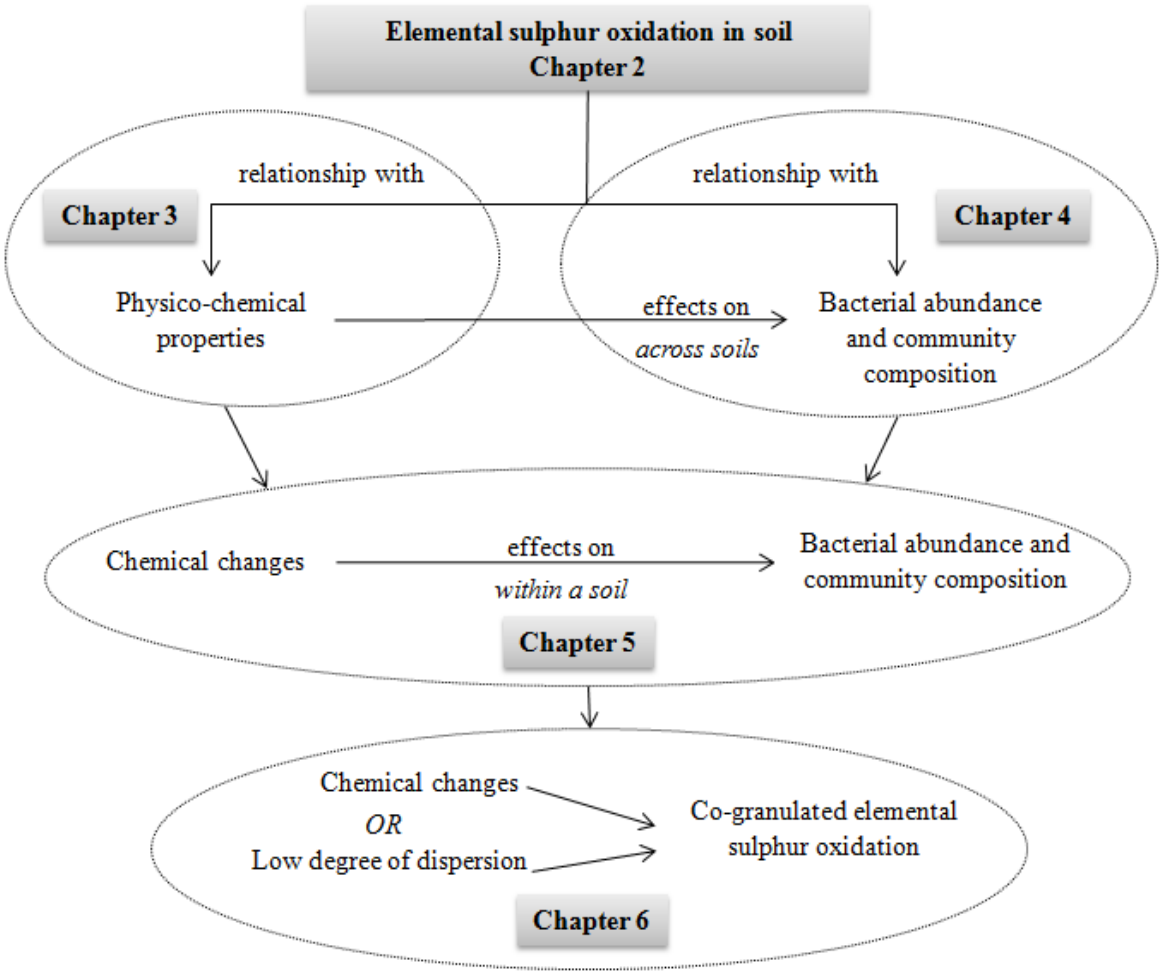

Fig. 1 Schematic illustration of thesis structure.

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CHAPTER 2

Review of the literature

1. Introduction

Sulphur (S) is used for the synthesis of many proteins and amino acids in plants and therefore S availability in soil affects the yield and quality of crops. The concentration of S in most plants is of a similar magnitude as that of P, ranging from 0.1 to 0.3% (Khurana et al., 2008).

Elemental S (ES) is used as a S fertiliser source to alleviate S deficiency in soil, but needs to be oxidised to become available to plants. This chapter reviews the scientific literature regarding S speciation and cycling in soil, discusses S fertilisers and the factors affecting oxidation of ES with a focus on the role of soil microorganisms in this process.

2. Sulphur in soil

2.1 Soil sulphur speciation and transformation in soil

The total S concentrations in most agricultural and mineral soils range between 50 and 1000 mg S kg^{-1} in the top 15 cm (Freney and Williams, 1983; Syers et al., 1987). Sulphur exists in various oxidation states ranging from -2 (e.g. sulphide, S^{2-}) to +6 (e.g. sulphate, SO_4^{2-}). Sulphur in soil consists of inorganic and organic S, with the latter representing the major proportion of the total S in most agricultural soils.

2.1.1 Inorganic sulphur

Inorganic S commonly represents less than 5% of the total S in soil, although it may account for a higher proportion (up to 25%) in agricultural soils (Saggar et al., 1998). Inorganic S concentrations in non-gypsiferous, non-calcareous soils typically range between 1 and 30 mg S kg^{-1} (e.g. Fox et al., 1964; Lee and Speir, 1979). Inorganic S may be present in the following forms in soil: sulphate, sulphite, thiosulphite, tetrathionate, ES and sulphide. Sulphate-S

dominates the inorganic S fraction in most agricultural soils due to its stability under aerobic conditions. The amount of S compounds at lower oxidation states is usually negligible (Bohn et al., 1986) as they are readily oxidised to sulphate under aerated conditions.

Sulphate in soil can be either dissolved in the soil solution, from where it is readily taken up by plants, adsorbed on mineral surfaces, or precipitated as gypsum in gypsiferous soils. The retention of sulphate in the soil profile depends on several chemical processes such as adsorption, desorption, precipitation, oxidation and reduction (Fig. 1). Adsorption can prevent sulphate from freely moving into the soil solution (Marsh et al., 1987; Parfitt, 1979). Adsorption of sulphate is very weak in most soils with pH > 6.0 (e.g. Curtin and Syers, 1990; Martini and Mutters, 1984), but can be considerable in soils with high oxide content and low pH (Singh et al., 1980). The sorption affinity of common ions in soil (in decreasing order) is as follows: hydroxyl > phosphate > sulphate > nitrate = chloride (Tisdale et al., 1984). The application of phosphatic fertilisers may therefore contribute to soil S depletion because sulphate can be desorbed by phosphate and then leached from the soil profile. Sulphate may be precipitated as gypsum in arid soils, which can replenish sulphate removed from the soil solution (Bettany et al., 1983). Sulphate may also be co-precipitated with $CaCO_3$ in calcareous soils (Williams and Steinbergs, 1962). It has been reported that in a Canadian calcareous soil, 42% of the sulphate was precipitated with $CaCO_3$ (Roberts and Bettany, 1985) and up to 93% in an Australian soil (Williams and Steinbergs, 1962).

Generally, sulphate has weak retention in soil and is therefore susceptible to leaching. Large leaching losses may hence occur in high rainfall areas. In arid areas, there is little net downward movement of water so that sulphate often accumulates at the soil surface in the form of gypsum

and other soluble sulphates, such as magnesium and sodium sulphate, and S deficiency is therefore unlikely in these soils.

2.1.2 Organic sulphur

Organic S accounts for more than 90% of the total S in well-drained non-calcareous soils. It is present as a mixture of soil organisms (biomass S), and decomposed plant, animal and microbial residues (Kertesz and Mirleau, 2004); hence the concentration of soil organic S is highly related with that of soil carbon (C) and nitrogen (N). A conventional approach to categorise organic S is based on its reaction with hydriodic acid (HI) (Freney, 1961). Organic S that can be reduced to sulphide by HI is named HI-reducible S and is assumed to consist of organic S bound to C, oxygen (O), N or S (e.g. organic sulphates, thioglucosides, sulphamates), while organic S that cannot be reduced to sulphide by HI is classified as C-bonded S (e.g. amino acids, sulphonic acids, heterocyclic compounds) (Biederbeck, 1978; Fitzgerald, 1978; Freney, 1986; Freney et al., 1969).

Hydriodic acid-reducible organic S comprises 30–70% of the total organic S (McLaren et al., 1985; Scherer, 2001). Ester sulphate-S is predominant in the HI-reducible organic S fraction (Lou and Warman, 1992b), and can be easily mineralised to inorganic S by extracellular enzymes or mild physico-chemical treatments (Bettany et al., 1979). Ester sulphate-S, along with soluble and adsorbed sulphate, is usually considered to be the plant-available organic S pool in soil. However, not all ester sulphate is labile in soil, and it has been suggested that the state of ester sulphate depends on its location in the structure of humic polymers (Lou and Warman, 1992a).

While ester sulphate-S can replenish the available S pool through rapid mineralisation, it is usually assumed that C-bonded S turns over very slowly and is therefore not available to plants in the short term. To gain a better understanding of C-bonded S, efforts have been made to reduce C-bonded S by Raney-Ni. However, only a small proportion of S that is bonded to C has been recovered (Freney et al., 1970; Stott and Hagedorn, 1980; Tabatabai and Bremner, 1972), indicating that most C-bonded S is chemically unreactive.

In an effort to understand S speciation in *situ* in the soil environment, since the 1990s X-ray adsorption near edge structure (XANES) spectroscopy has been employed to determine S speciation and found to be a reliable method to distinguish and quantify S species in different oxidation states (Morra et al., 1997; Prietzel et al., 2003; Solomon et al., 2005; Solomon et al., 2011). Several XANES studies have questioned the distinction of S species based on reduction using HI, since only weak relationships have been found between the results of wet chemical reduction and speciation as determined by XANES (Solomon et al., 2003; Solomon et al., 2005). Furthermore, XANES studies have also indicated that the conventional view that C-bonded S is recalcitrant while ester sulphate-S is easily mineralised may not hold. For instance, Zhao et al. (2006) found that gross mineralisation of S was more closely related to reduced S species (C-bonded S) than to oxidised S species (sulphate-ester S); Solomon et al. (2005) also found evidence that C-bonded S was more susceptible to microbial attack than ester sulphate-S.

2.1.3 Sulphur immobilisation and mineralisation

Immobilisation and mineralisation are pivotal processes in S cycling in soil (Fig. 1). Sulphur immobilisation involves the assimilation of sulphate-S by soil biota through converting it to organic S, while mineralisation is the conversion of organically bound S to inorganic S (predominantly sulphate-S) (Schonenau and Malhi, 2008).

Immobilisation represents the incorporation of sulphate into ester sulphate-S and C-bonded S (Fitzgerald et al., 1982). Sulphate can also be immobilised by microorganisms to become a component of microbial biomass, and is, for instance, used in the synthesis of microbial cell walls (Myanard et al., 1983). Sulphate immobilisation can reduce the availability of S for plants, but the incorporation of sulphate into organic S can also effectively reduce sulphate leaching losses.

Mineralisation of organic S results in the release of plant-available sulphate-S. McGill and Cole (1981) proposed a model of organic S mineralisation in soil, which involves biochemical and biological processes. Biochemical mineralisation represents the release of inorganic S from organic matter (e.g. ester sulphate-S) driven by sulphate demand of soil organisms, whereas biological mineralisation is the conversion of C-bonded S to inorganic S to meet the energy requirement of organisms in soil. However, this model has been challenged, as the diverse S speciation and complicated reactions involved in S mineralisation in soil are not well represented by this dichotomous concept (Ghani et al., 1992). To date, the mechanisms of organic S turnover and the factors controlling it are still obscure.

Immobilisation and mineralisation occur simultaneously in soil, and the change in sulphate-S concentration over time hence depends on the net mineralisation, i.e. the difference between (gross) immobilisation and mineralisation. Net mineralisation of S from organic matter is the key to the supply of sulphate for plant growth in unfertilised soils. In agricultural soils, however, the crop demand for S is often greater than can be supplied by the mineralisation of organic S (Eriksen et al., 1995). It has been reported that net mineralisation from farmyard manure and crop residue was 3.5–5.6 mg S kg^{-1} soil after 95 days incubation (Boye et al., 2009), and that

from wheat straw and cabbage were –1.1 and 5.6 mg S kg^{-1} soil, respectively, after 43 days incubation (Nziguheba et al., 2006). Unless there is considerable atmospheric input, S fertilisation is therefore required in agricultural soils to supply sufficient S for plant growth.

2.2 Soil sulphur inputs and outputs

Sulphur compounds could be in gaseous state, in water-soluble form or in recalcitrant organic form. Sulphur is highly active in the environment, and can move freely between the hydrosphere, lithosphere and atmosphere. Fig. 1 illustrates the cycling of S between soil, plant, water and atmosphere.

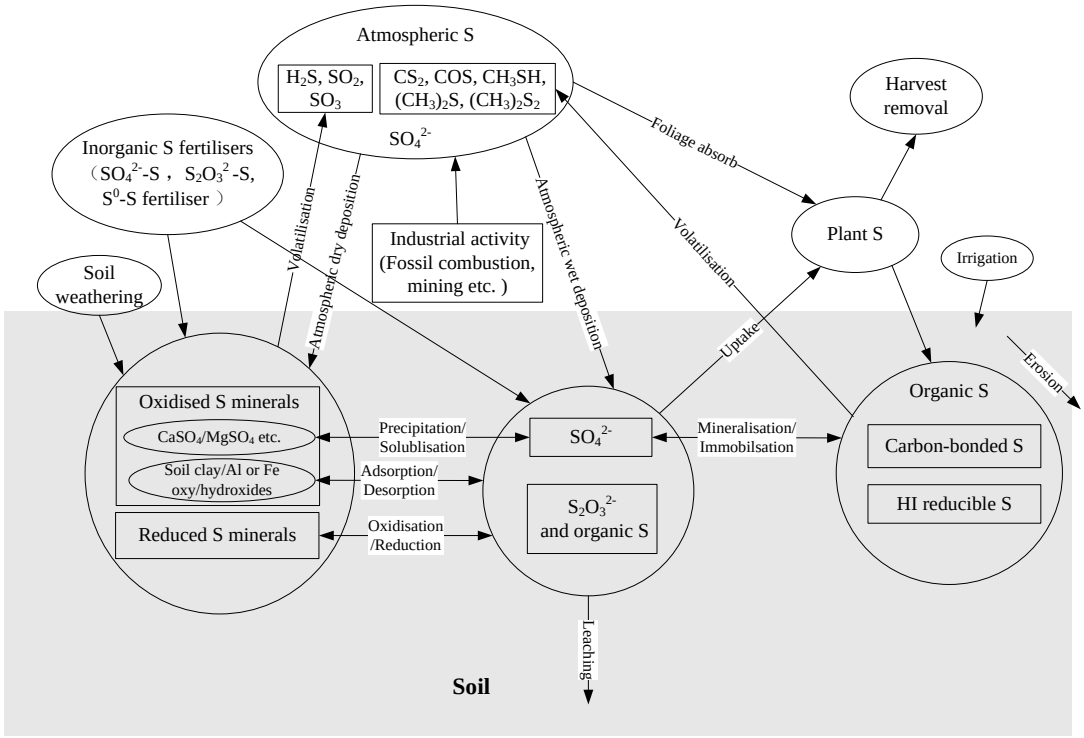


Fig. 1 Schematic illustration of sulphur cycling between soil, plant, surface water, and atmospheric components of the ecosystem (modified from Glendinning, 1999).

Sulphur inputs to soil may come from natural processes e.g. weathering of geological parental minerals, but a large proportion of soil S inputs originates from human activities such as atmospheric deposition from industrial activity, fertiliser application and irrigation. The

atmospheric deposition of S-containing rain or particulates resulting from combustion of fossil fuel was a major input of S to soils in heavily industrialised regions in the past (Robertson et al., 1995; Semb, 1978). Emission control has strongly reduced atmospheric deposition in many regions, but this is likely still a major input of S to soils in regions with less stringent pollution control, e.g. South-East Asia, Southern Africa and Eastern Europe. Additions of fertilisers to soil are also a considerable S input flux in agricultural soils. Sulphur may be incidentally added with N or P fertilisers, e.g. ammonium sulphate, single superphosphate. However, due to a shift to high analysis S-free fertilisers (e.g. ammonium nitrate, ammonium phosphate, triple superphosphate) and other reasons (discussed below), S fertilisers are now more often deliberately added to prevent or alleviate soil S deficiency. Irrigation may be another S input flux to soil (Bloem, 1998; Noggle et al., 1986).

Soil S outputs include removal of harvested crop parts, sulphate leaching, soil erosion and gaseous losses. Intensive crop growth and high-yielding crop cultivars in part have contributed to S depletion in the Indo-Gangetic plains (Khurana et al., 2008). For example, Aulakh (2003) reported an annual removal of more than 1.3 million Mg S through harvested crops in India compared with only 0.6 million Mg S applied (FAI., 2006). In addition, sulphate leaching causes large amounts of S to be lost from soil, particularly in arable soils. Leaching losses of sulphate in arable soils have been ascribed to the facts that 1) sulphate adsorption is very weak in soils with pH > 6.0 (see section 2.1.1); and 2) retention of sulphate through immobilisation to organic S is relatively small because organic matter does not accumulate in these soils (Knights et al., 2000; Randall et al., 1981). Soil S can also be reduced because of soil erosion (McLaughlin et al., 1992) and volatilisation of gaseous S compounds, e.g. H_2S, CS_2, COS (Anderson, 1978).

3. Crop sulphur nutrition

3.1 Sulphur in crops

Sulphur in crops is contained in many important biological compounds, such as the amino acids cysteine or methionine, and all proteins that contain these amino-acids as well as in co-enzymes, vitamins, lipids, volatile oils, peptides, etc. Sulphur concentrations in crops differ greatly depending on the S requirement of plants. Based on S demands, crops are classified into three categories. High S requiring crops include rapeseed, alfalfa, cruciferous plants, etc., moderate S requiring crops include sugarcane, clover, cotton, etc., and low S requiring crops include sugar beet, cereals, peanuts, etc. (Spencer, 1975). In most plants, S concentrations range from 0.1 to 0.3%. Crops for oil production e.g. cruciferous plants, and those rich in protein e.g. legumes, contain high concentrations of S, with seed S concentrations ranging from 0.24 to 1.2%. Sulphur concentrations in cereals are comparatively low, with concentrations in the grain between 0.16 and 0.25% (Aulakh et al., 1985; Khurana et al., 2008).

3.2 Crop responses to sulphur deficiency

Soil S deficiency can adversely affect crop morphology (Fig. 2). Smaller leaves with chlorosis and thinner stems of soybean, sunflower, black mustard and tomato have been observed in S-deficient soils (Eaton, 1935; Eaton, 1941; Eaton, 1942; Eaton, 1951). In addition, glucosinolates, which contribute to the defence of plants against pests and pathogens, will be broken down to synthesise other S-containing compounds when S is not sufficient, making the plant vulnerable to disease (Falk et al., 2007). For example, it has been observed that under S-deficient conditions, oilseed plants were much more susceptible to attack by fungi due to the reduction in concentrations of glucosinolates (Dubuis et al., 2005). Also, grain quality has been found to degrade at low S supply, e.g. in pea (Millerd et al., 1979), in lupin (Blagrove et al., 1976) and in

wheat (Moss et al., 1983). This is explained by changes in protein composition in the grains. Wrigley et al. (1984) demonstrated that the synthesis of proteins in wheat grain was redirected with insufficient S supply (i.e. more low S proteins were synthesised at the expense of high S protein), and that these alterations affected bread dough quality.

Fig. 2 Symptoms of S deficiency in crops (corn, canola, wheat and lettuce from left to right) (photo source: International Plant Nutrition Institute).

4. Sulphur fertilisers

Addition of S to soil can alleviate S deficiency in crops as demonstrated by increases in crop yield and in grain quality in response to S fertilisation (Bimbraw, 2008; Grant et al., 2012; Malhi et al., 2005). Based on S forms, S fertilisers can be categorised into organic and inorganic fertilisers (Table 1). The former mainly include biosolids, manure and composts, while the latter primarily are sulphate-S, ES and thiosulphate-S fertilisers. Sulphur concentration in organic fertilisers depends on fertiliser type (Sammi Reddy et al., 2002), with animal manure having relatively high S concentrations. In contrast, S concentrations in inorganic fertilisers can be artificially formulated based on nutrient requirements by plants. Elemental S can be added as a single nutrient source to soil, whereas thiosulphate- and sulphate-based fertilisers always comprise an accompanying cation, e.g. calcium in gypsum and single superphosphate fertilisers, ammonium in ammonium sulphate and ammonium thiosulphate fertilisers, potassium in

sulphate of potash fertiliser and occasionally magnesium in some mixed potassium/magnesium fertilisers.

Table 1 Inorganic and organic sulphur fertiliser sources (Dick et al., 2008).

Fertiliser source	Nutrient concentrations (%)			
	N	P	K	S
Organic sources	*	*	*	
Biosolids				0.3-1.2
Manures				
Most animals				0.25-0.3
Sheep				0.35
Poultry				0.5
Composts				
Biosolids				0.44
Dairy manure				0.22
Crop residues				0.10-0.22
Inorganic sources				
Elemental S	0	0	0	88-89
Gypsum	0	0	0	18
Ammonium sulphate	21	0	0	24
Ammonium thiosulphate	12	0	0	26
Ordinary superphosphate	0	9	0	11-12
Magnesium sulphate	0	0	0	14
Potassium magnesium sulphate	0	0	18.2	22
Potassium sulphate	0	0	41.5	18

* NPK levels in organic fertiliser sources are widely variable, hence not provided.

4.1 Organic sulphur sources

It is estimated that circa 8 Tg S per year is present in excreta worldwide, accounting for approximately 13% of the world S production (Eriksen, 2002). Return of animal manure to soil is a potential S fertiliser source (recognising that it depletes other areas where the animal grazed). However, S in organic manures is not a predictable nutrient source because of the complex composition of organic matter, the unpredictable mineralisation rate and the low S concentration in some sources (Eriksen, 2002). Sulphur concentrations in plant residues vary

greatly, and so does the mineralisation of that S. For instance, Eriksen (2005) studied a range of plant residues and found that S concentrations in the residues ranged from 0.08 to 0.81% (w/w), and net mineralisation of S from 0.13 to 3.8 % of the total S contents in these residues after a 32-day incubation.

4.2 Inorganic sulphur fertilisers

Addition of inorganic S compounds to soil is the most economically feasible way to improve crop S nutrition as the rate and time for application can be well controlled. Sulphate fertilisers can correct S deficiency in soil rapidly as sulphate is readily available for plants. Gypsum is one of the commonly used sulphate fertilisers, which is usually obtained by mining or as a by-product from industrial processing. Another common sulphate fertiliser is ammonium sulphate that simultaneously provides N and S for plants. However, due to the high solubility of sulphate compounds, sulphate in fertilisers can be easily leached out of the root zone. As a result, S deficiencies may reappear later in the crop growth cycle in high-rainfall environments. Hence, it has been recommended to combine fertilisers that release S rapidly (sulphate) and slowly (ES) for soils where sulphate leaching is likely to happen (Barrow, 1971; Solberg et al., 2007). Another disadvantage of sulphate fertiliser is the lower S concentrations (w/w) compared to ES fertiliser, leading to higher costs in transportation and application.

Elemental S has the advantage of being cheaper to transport and apply, and is hydrophobic and hence not susceptible to leaching. As a by-product of oil manufacture, ES is also a ready and cheap source of S for fertiliser manufacture. However, pure ES, particularly powdered ES, has potential fire and explosion hazards for handling (Rothbaum and Groom, 1961), and is therefore mostly used in research to assess factors affecting ES oxidation in soil (see section 5.1), or to study the relationship between the effectiveness of ES fertilisers and ES particles

(Boswell and Friesen, 1993). For agronomic use, ES powder is either granulated with bentonite as an inert carrier to produce nearly pure ES granular fertilisers (often 90% ES, e.g. Tiger 90™, Sulfurgran™), or is co-granulated with macronutrients to produce compound fertilisers (e.g. MicroEssentials™, Thiogro™, Granulock™). Oxidation of granular ES has been found to be slower than that of powdered ES (Boswell et al., 1988; Friesen, 1996; Hu et al., 2002; Janzen and Bettany, 1986; Janzen and Karamanos, 1991), but the reasons are still unclear. It has been speculated that this is due to the lower degree of dispersion of (co-)granulated ES (Friesen, 1996). Additionally, studies have shown that the effectiveness of ES fertilisers is unpredictable, varying depending on application methods, fertiliser formulation, soil type, crop variety, etc. For example, Solberg et al. (2005) found that the performance of ES fertiliser differed greatly depending on whether the fertiliser was incorporated, broadcasted, banded or nested. In addition, oxidation of ES-bentonite fertilisers is often slower than ES co-granulated with macronutrient fertilisers (Janzen and Karamanos, 1991). While the former has greater residual effect than the latter and sulphate fertilisers in supplying S for plants, the latter can supply S and other macronutrients (NPK) simultaneously for plants. In summary, ES fertiliser has great potential as a S fertiliser source, but further study is needed to gain insight into the controlling factors for the performance of ES-containing fertilisers.

5. Sulphur oxidation in soil

5.1 Factors affecting elemental sulphur oxidation in soil

5.1.1 Soil and environmental factors

Factors that affect ES oxidation have been mostly studied with powdered ES mixed through soil. Elemental S oxidation is primarily mediated by soil microorganisms, hence ES oxidation is strongly affected by factors that affect soil microbial activity and population. The effects of

environmental factors (e.g. temperature, water content, aeration), soil properties (e.g. soil texture, pH, C content), and ES particle size on ES oxidation have been well studied, and been extensively reviewed by Germida and Janzen (1993). Here, it is only briefly discussed how ambient edaphic factors, fertiliser formulation, and microbial population affect ES oxidation.

Soil temperature can affect enzymatic activity and consequently affect ES oxidation to sulphate. It has been reported that ES oxidation rate increases by 14% for every 1 °C increase in temperature, corresponding to a Q_{10} of 3.6, in the temperature range of 3–30 °C (Janzen and Bettany, 1987). Soil properties also have been found to affect ES oxidation, which can be attributed to their effects on the population and activity of soil microorganisms (Gonzalez-Quiñones et al., 2011; Kemmitt et al., 2006). Finally, the size of ES particles has a strong effect on the oxidation rate, since ES oxidation is a surface-based process. For spherical particles, for instance, the specific surface area and hence oxidation rate is inversely proportional to the particle diameter (Blair, 1987). While soil and environmental factors that affect ES oxidation in powdered form have been extensively explored, those affecting the performance of granular ES fertilisers are yet to be fully identified.

5.1.2 Biological factors

In the review of Germida and Janzen (1993), the role of autotrophs (mainly *Thiobacillus* spp.) and heterotrophs in ES oxidation was highlighted, but without drawing conclusions on their relative importance for ES oxidation. The presence of S-oxidising autotrophs, identified as *Thiobacillus* spp. in most studies, depends on soil properties (e.g. pH, Rao and Berger, 1971) and on the addition of ES, and it has been found that the number of *Thiobacillus* or *Thiobacillus*-like species increased concomitantly with an increase in ES oxidation (Lee et al., 1987; Tourna et al., 2014). On the other hand, heterotrophic S-oxidising bacteria have been

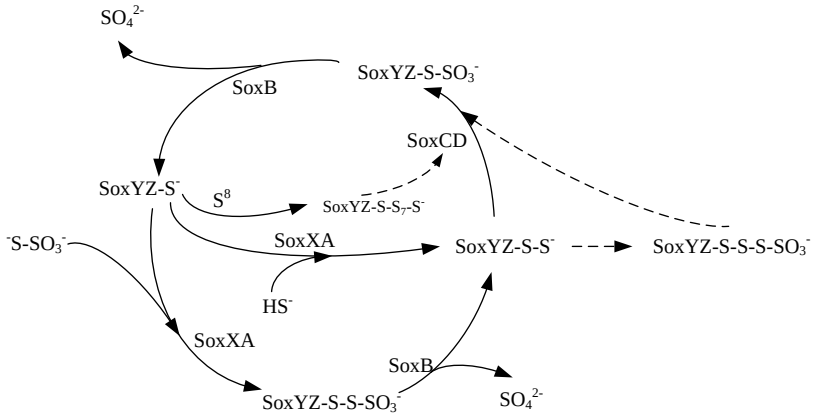
found to be ubiquitous in aerobic agricultural soils, and not as variable as that of autotrophs during ES oxidation (Lawrence and Germida, 1988; Lawrence et al., 1988), suggesting the importance of heterotrophs in ES oxidation. A synergic relationship between autotrophic and heterotrophic microorganisms for ES oxidation has been proposed by Germida (1985). Overall, the relative importance of autotrophic and heterotrophic oxidisers in the oxidation process is unclear, which may be attributed to the lack of studies on the whole community of S oxidisers. The culture-based methods that were applied in the study of S oxidisers in soil from the 1980s to the 2000s only cover less than 10% of the total soil microbial population. With the establishment of molecular techniques, studies on S oxidisers in soil have shown that ES oxidising microorganisms are widely diverse in soil (see section 5.3 for more detailed discussion) (Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014). However, there is still a need to further understand the relationship between the community composition and abundance of microbial population on the one hand, and ES oxidation on the other.

5.2 Molecular ecology of sulphur oxidation

Research on S oxidation pathways in bacteria has mainly focused on thiosulphate oxidation because thiosulphate is relatively stable and easily detected (Kelly et al., 1997; Welte et al., 2009). Three pathways of thiosulphate oxidation in chemolithotrophic and phototrophic S-oxidising bacteria are mainly proposed, namely the Sox pathway also known as the PSO pathway (Fig. 3A), the S_4 intermediate pathway (S_4I pathway) (a schematic illustration is not available) and the branched pathway (Fig. 3B). The Sox pathway has been described to involve Sox enzyme complexes, primarily SoxXAYZBCD, which are encoded by corresponding genes and associated with oxidising thiosulphate to sulphate without the formation of any intermediate (Fig. 3A) (Friedrich et al., 2001; Friedrich et al., 2005; Kelly, 1989; Kelly et al., 1997). Intermediates, e.g. tetrathionate, are formed in the S_4I pathway (Dam et al., 2007;

Hallberg et al., 1996; Kelly, 1989; Kelly et al., 1997); however, both enzymatic functions and

A netic bases are yet to be fully resolved for this pathway (Ghosh and Roy, 2007). The branched pathway (Fig. 3B) differs from the Sox pathway in the absence of SoxCD, and hence the transformations of S-compounds were postulated to be carried out by the interactions between Sox enzymes (except for SoxCD) and a reverse-acting dissimilatory sulphite reductase enzyme (DsrAB) (Dahl et al., 2005) that is known to reduce sulphite to sulphide.



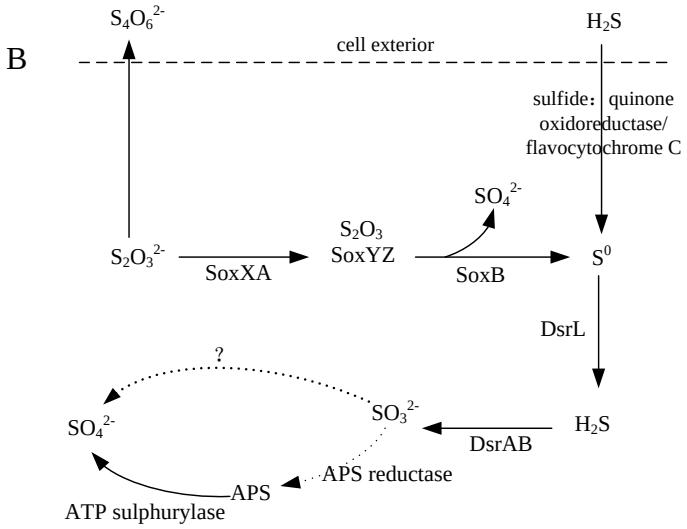


Fig. 3 Schematic overview of (A) Sox and (B) Sox branched pathways of thiosulphate oxidation in bacteria (modified according to Ghosh and Dam, 2009). APS is shorted for adenosine phosphosulphate and ATP for adenosine triphosphate.

5.3 Diversity and abundance of sulphur-oxidising bacteria in soil

The diversity and/or abundance of S-oxidising bacteria has been investigated in terrestrial environments (Loy et al., 2009; Tourna et al., 2014; Varon-Lopez et al., 2014; Xia et al., 2014), sulphide-removing bioreactors (Luo et al., 2011) and coastal aquaculture environments (Krishnani et al., 2010a; Krishnani et al., 2010b) by analysing functional genes such as *sqr*, *apr*, *dsr* and *sox*. The *sqr* gene encodes for sulphide:quinone oxidoreductase, which oxidises sulphide in photolithotrophic bacteria and chemolithotrophic archaea and bacteria (Chan et al., 2009; Friedrich et al., 2005), hence *sqr* is considered not suitable for the study of S-oxidising microorganisms in aerobic environment such as agricultural soils. The *apr* gene encodes for homologues of dissimilatory adenosine-phosphosulphate (APS) reductase (Apr). The reductase is observed in S-oxidising prokaryotes, which operates reversely by oxidising sulphite to APS. However, the *apr* gene is confined within organisms with anaerobic lifestyles only (Meyer and Kuever, 2007). The *dsr*AB gene encodes for dissimilatory sulphite reductase (dsr) in S-oxidising prokaryotes and has been used as a potential marker in alkaline lake sediments (Loy et al., 2009). The *soxB* gene encodes SoxB enzyme functioning in hydrolysing sulphate from thiocystine sulphate derivative (Quentmeier et al., 2003), and has been used in a few studies to determine the abundance and/or diversity of S-oxidising bacteria in soil environments (Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014). Using *soxB* gene as the molecular marker, researchers have found S-oxidising bacteria in the phyla of *Alpha-proteobacteria* (e.g. *Paracoccus spp*., *Bradyrhizobium spp.*), *Beta- proteobacteria* (e.g. *Thiobacillus spp*., *Pandoraea spp.*), *Gamma-proteobacteria* (e.g. *Halothiobacillus spp.*), and *Firmicutes* and *Actinobacteria* (Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014). Most S-oxidising bacteria are classified as chemolithoautotrophs (Stubner et al., 1998); however, many can grow both organoheterotrophically and mixotrophically (Anandham et al.,

2008; Graff and Stubner, 2003; Stubner et al., 1998), and some can even grow chemolithoheterotrophically (Das et al., 1996).

The abundance of functional genes involved in some biochemical processes in soil e.g. nitrification, has been determined and is proposed as a reliable predictor of the potential rate of that biochemical process (Petersen et al., 2012). However, the abundance of functional genes involved in S oxidation in soil has only been reported in recent studies (Tourna et al., 2014; Xia et al., 2014). In the study of Tourna et al. (2014), they found that the copy numbers of *soxB* gene in pasture soils varied greatly, with an order of magnitude difference between soils (7.9×10^{7} to 9.4×10^{8} copies g^{-1} dry soil), and increases in *soxB* abundance and sulphate production were observed after ES addition in all the soils. In the study of Xia et al. (2014), a weak linear relationship was found between *soxB* abundance and sulphur oxidation potential in landfill soils. Little is known regarding the abundance and diversity of *soxB* gene-harbouring bacteria in Australian cropping soils, and their relationship with a soil's potential to oxidise ES.

6. Objectives of this study

Alleviation of S deficiency by application of ES fertilisers is still challenging. To improve the performance of ES fertiliser, a comprehensive understanding of the role of microorganisms in ES oxidation in soil is needed. Molecular techniques based on functional genes provide a useful tool to better understand the genetic potential (functional gene abundance) of a soil to oxidise ES, and the role of the diversity of S-oxidising microorganisms in the oxidation process. Information on the population of S-oxidising microorganisms may give insight into the oxidation of (co-)granulated ES in soil, and assist in improving the effectiveness of ES fertilisers.

Specifically, the objectives of this work were:

1) to determine the oxidation rate of ES in Australian cropping soils covering diverse physico-chemical properties;

2) to examine the relationship between the genetic potential (functional gene abundance) of a soil to oxidise ES, S-oxidising bacterial diversity and soil properties on the one hand, and the oxidation rate of ES on the other; and

3) to investigate the potential factors that cause the slower oxidation of ES in co-granulated fertiliser compared to that of powdered ES.

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CHAPTER 3

Elemental sulphur oxidation in Australian cropping soils

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CHAPTER 4

Abundance and diversity of sulphur-oxidising bacteria, and their role in elemental sulphur oxidation in Australian cropping soils

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Abstract

There is increasing interest in elemental sulphur (ES) as a sulphur (S) source for fertilisers, but to be available to plants, ES has to be oxidised to sulphate. As a biologically mediated process, ES oxidation is associated with the diversity and abundance of S-oxidisers in soil. However, what remains unclear is the linkage between ES oxidation and the population of S-oxidising bacteria in cropping soils. In this study, we investigated the abundance and diversity of S-oxidising bacteria by targeting a functional gene (*soxB*), and assessed the relationship with ES oxidation in ten Australian cropping soils. Positive correlations between soil carbon, nitrogen and sulphur contents on the one hand and the gene copy numbers of *soxB* and 16S ribosomal ribonucleic acid (16S rRNA) on the other suggested that the abundance of bacteria in general, and of S-oxidisers in particular, depends on the soil carbon and nutrient supply. Both the copy numbers of *soxB* and 16S rRNA genes were significantly correlated with the oxidation rate of ES ($P < 0.05$). In contrast, we did not observe any significant correlation between 18S ribosomal ribonucleicacid (18S rRNA) gene copies and oxidation rate. These results indicate the importance of bacterial abundance for ES oxidation in Australian cropping soils. In addition, more than 80% of the variation in the oxidation rate of ES could be explained by the combination of *soxB* or 16S rRNA gene copy numbers and soil pH. Six clone libraries constructed with the *soxB* primers showed genera belonging to *Alpha-* and *Beta-proteobacteria*. The phylogenetic diversity and relative distribution of clones revealed great differences across soils. However, no linkage was found between the diversity of S-oxidising bacteria and the oxidation rate of ES. We concluded that heterotrophic S-oxidising bacteria significantly affected ES oxidation in studied soils. As far as we know, this is the first study showing a quantitative relationship between the abundance of S-oxidising bacteria and the oxidation rate of ES in cropping soils. These findings could help better understand the importance of S-oxidising bacterial population in ES oxidation in agricultural soil.

1 Introduction

Elemental sulphur (ES) is of interest as a sulphur (S) fertiliser source due to its high S content, but only becomes available to plants as sulphate after oxidation. Unravelling the major factors contributing to ES oxidation is pivotal for the effective use of fertiliser ES. Many studies have reported the effects of environmental conditions (e.g. Janzen and Bettany, 1987b; Chapman, 1996; Jaggi et al., 1999) and soil physicochemical properties (e.g. Janzen and Bettany, 1987a; McCaskill and Blair, 1987; Chapman, 1989) on ES oxidation. As a biologically mediated process, ES oxidation has also been found to be related with soil microbial biomass carbon, respiration, and enzyme activity (Lawrence and Germida, 1988; Dick and Deng, 1991). However, few studies have shown a direct relationship between the biological potential to oxidise ES across soils with regard to microbial populations involved in specific S transformation processes.

Sulphur oxidation is primarily mediated by soil microorganisms including fungi and bacteria. Although fungi were found to be able to oxidise S almost a century ago (Abbott, 1923), studies on S-oxidising fungi are still confined to limited culturable species, likely due to the requirement of carbon substrates (Germida et al., 1992). Additionally, fungi have been reported to be less efficient than bacteria with regard to ES oxidation in a silty soil (Czaban and Kobus, 2000).

In contrast, sulphur-oxidising bacteria have been found to be ubiquitous and taxonomically and phylogenetically diverse in soil, covering a broad range of taxa consisting of *Alpha-*, *Beta-* and *Gamma- proteobacteria*, *Beta-* and *Gamma-actinobacteria* and *Firmicutes* (Stubner et al., 1998; Graff and Stubner, 2003; Deb et al., 2004; Ghosh et al., 2005; El-Tarabily et al., 2006; Ghosh

and Roy, 2006; Ghosh and Roy, 2007; Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014). Sulphur-oxidising bacteria in soil are primarily chemolithotrophs (Tourna et al., 2014), which obtain electrons from reduced S compounds for respiration and carbon dioxide assimilation (Friedrich et al., 2005). Sulphur-oxidising chemolithotrophs are biochemically and physiologically diverse, which is revealed by their different abilities to utilise different substrates, and by the variations in conserving energy from same substrates by different species (Ghosh and Dam, 2009). In addition, the biochemical and physiological diversity of S-oxidising chemolithotrophs is also relevant with their different electron transport mechanisms, varieties of oxidation pathways and enzymes, and the species-dependent efficiency as they are oxidising the same substrate (Ghosh and Dam, 2009). Even though they are usually assigned as chemolithotrophs, some S-oxidising bacteria can also grow organoheterotrophically (Stubner et al., 1998; Kelly and Wood, 2006; Ghosh and Roy, 2007; Anandham et al., 2008), which facilitate their living on different substrates. The tremendous diversity of S-oxidising bacteria makes the functional gene-based method preferable, as it enables us to investigate bacteria with similar functions, yet spread across a wide range of taxa.

The *soxB* gene is one of many functional genes involved in S oxidation. It codes for the SoxB enzyme which functions in hydrolysing sulphate from the thiocystine sulphate derivative (Quentmeier et al., 2003). The presence of *soxB* genes is considered to indicate the ability of bacterial S-oxidisers to oxidise thiosulphate (Krishnani et al., 2010), a common product by most S-oxidising bacteria in the biogeochemical S cycle (Jørgensen, 1990; Jørgensen and Bak, 1991; Meyer et al., 2007; Anandham et al., 2008; Xia et al., 2014). The *soxB* gene assay has been used to study the community composition and abundance of S-oxidising bacteria in various environments, e.g., salt marsh sediments (Thomas et al., 2014), mangrove sediments (Varon-Lopez et al., 2014), land-fill soils (Xia et al., 2014) and pasture soils (Tourna et al.,

2014). In the study of Tourna et al. (2014), who used the *soxB* gene as a molecular marker, a close link was reported between the diversity and abundance of chemolithotrophic S-oxidising bacteria and the oxidation of ES. However, no attempt has been made yet to relate the abundance and diversity of bacteria containing the *soxB* gene to ES oxidation rate across soils with a wide range of properties.

The aims of this study were 1) to develop an assay to quantify *soxB* gene copies in soil, as an indicator of the abundance of S-oxidising bacteria; 2) to assess if there is a relationship between *soxB* gene abundance and the oxidation rate of ES; and 3) to characterise the diversity and distribution of S-oxidising bacteria, and to assess their role in ES oxidation in Australian cropping soils. Furthermore, the abundances of the total bacteria (indicated by 16S rRNA gene copy numbers) and fungi (indicated by 18S rRNA gene copies) were also determined in this study to assess the relative importance of bacteria and fungi in ES oxidation in soil.

2 Material and methods

2.1 Soil incubation and sampling

Ten Australian cropping soils were incubated with 1000 mg ES kg^{-1} air-dry soil at 25 ℃ and 70% field capacity. Soils without ES were also included as controls. Soil sampling of both ES-amended and control soils was carried out at days 8, 16, 24, 48 and 96 for chemical analysis (see Zhao et al., 2015 for more details), and at days 8, 48 and 96 for biological analysis (see sections 2.2–2.4). Soil physico-chemical properties are shown in Table 1 (see Zhao et al., 2015 for methods). For biological properties of the soil, we selected the gene copy numbers of *soxB*, 16S rRNA and 18S rRNA (Table 1) to indicate the populations of S-oxidising bacteria, total bacteria and total fungi, respectively, as these may be related to ES oxidation in soil. In addition, due to the dryness in November in southern Australia, most of our soils were dry when

collected, and the biological properties could not be analysed in *situ*. Therefore, we conducted gene-based analysis from control soils after 8 days incubation when soil microorganisms were expected to be activated.

<Table 1>

2.2 DNA extraction

Soil DNA was extracted from 0.25 g of soil using a PowerSoil Kit (MoBio, USA). For soil 9 with a clay content of 53%, 200 µl of Na_2HPO_4 (0.2 M, pH 8.0) and 200 µl of Tris buffer (0.5 M, pH 6.8) were added to a PowerBead tube in the first step to disperse soil micro-aggregates to improve DNA extraction efficiency. After extraction, total DNA concentration was determined on a NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Thermo Scientific).

2.3 Quantitative polymerase chain reaction analysis

2.3.1 Measurements of quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed to determine the copy numbers of *soxB*, 16S rRNA and 18S rRNA genes. All qPCR assays were run in duplicate on a Stratagene Mx3000P qPCR system (Agilent Technologies, Inc.). Table 2 shows primer sequences and thermal profiles of qPCR for these three genes. Single qPCR reactions were carried out with a 20 µl reaction volume 1× iQTM SYBR® Green supermix (Bio-Rad, USA), 2 µl of DNA templates, and primer with concentrations of 600 nM, 200 nM and 1.25 µM of each primer for *soxB*, 16S rRNA and 18S rRNA genes, respectively. Two negative controls were included in each assay for contamination detection. A melting curve was generated after each assay to check PCR specificity.

<Table 2>

For qPCR analysis, three environmental clones were used to make standards for each gene. Sequences of these standards were analysed to ensure their specific to the primer set (see section 2.4 for cloning and sequencing procedures). Three clones were shaken in Luria-Bertani medium overnight prior to plasmid DNA extraction. Concentration of extracted plasmid DNA was determined on a Nanodrop ND-1000 UV-Vis Spectrophotometer and gene copy numbers of plasmid DNA were directly calculated. A 10-fold dilution series was made from a known copy number of plasmid DNA to generate an external standard curve. A series from 10^7 to 10^1 copies μl^{-1} was made for *soxB* genes and, 10^7 to 10^2 copies μl^{-1} for 16S rRNA and 18S rRNA genes to ensure that unknown copy numbers fell within the corresponding series.

2.3.2 Evaluation of the qPCR assay for soxB gene abundance

The qPCR assay for *soxB* genes was validated by examining the correlation coefficient between log-transformed copy numbers and threshold values (Ct), amplification efficiency and inhibition from co-extracted contaminants. Standards ranging from 10^7 to 10^1 copies were amplified with a coefficient > 0.99. The amplification efficiency was > 85%. Inhibition from co-extracted contaminants was tested by analysing known copy numbers of DNA in soil DNA samples. Specifically, DNA from a standard (10^3 copies μl^{-1}) was mixed with each sample at a ratio of 1:1 (v/v). These mixtures were subjected to qPCR analysis together with standards and samples. Inhibition was assessed by comparing the measured and expected values. For samples with above 5 times dilution, the measured were in the same order of magnitude as expected values (Fig. 1). However, we conducted less than 5 times dilution for samples with low DNA concentrations (< 5 ng μl^{-1}) when gene copy numbers of these samples fell out of the standard

series. These samples accounted for less than 2% of all samples. Though less diluted, qPCR did not show much variation between duplicate analyses of each sample, with the coefficient of variance ranging from 2.5–17.2%. Specificity was assessed by generating a dissociation curve after each run. Compared to the control soils, we observed evident shifts of peaks on the melting curves of *soxB* genes in the ES-amended soils. According to Tourna et al. (2014), this is possibly due to community shifts of S-oxidising bacteria after ES application.

<Fig. 1>

Substantial variation in *soxB* gene copy numbers was observed in replicates of soil 9 after incubation for 48 and 96 days, and gene copy numbers were therefore considered unreliable, and hence are not reported here.

2.4 Cloning and sequencing of soxB genes

Cloning was performed to investigate the diversity of S-oxidising bacteria in unamended soils after 8 days incubation. Six soils were selected based on variations in the oxidation rate of ES and soil properties. Six clone libraries were constructed (one for each soil) by generating end-point PCR products with *soxB* primers as used in qPCR analysis (Table 2). The PCR reaction contained 0.2 U of HotTaq polymerase (Qiagen), 1.5 mM of $MgCl_2$, 1 × PCR buffer (Qiagen), 600 nM of each primer, 0.8 mM of dNTPs and 4 µl of DNA template. The PCR mixtures were subject to amplification under the same conditions as for qPCR (Table 2), except that 30 cycles were applied for amplification on a thermocycler (Bio-Rad). These PCR products of *soxB* genes were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, USA), ligated and cloned with a TA Cloning® Kit (Invitrogen, USA) following the manufacturer's instructions. Fifty colonies for each soil were randomly picked from a Luria-Bertani agar plate. The

nucleotides of inserted fragments in all clones were subjected to sequencing by ABI 3730xl (BGI Tech Solutions, (Hong Kong) Co., Ltd.). A total of 217 sequences were obtained from PCR and cloning analyses. All sequences were subjected to BLAST analysis at the National Centre of Biotechnology Information (NCBI) and 200 of them were found to be homologous to known *soxB* genes. *soxB* gene-like sequences from this study together with sequences retrieved from GenBank were aligned using the online tool ClustalW2 (Larkin et al., 2007). A phylogenetic inference was performed using MEGA 6.0 software (Tamura et al., 2013) based on P-distance and neighbour joining methods. Bootstrap consensus from 100 replicates was obtained for each gene analysis with bootstrap values > 50 being shown. The sequences of *soxB* genes in this study were deposited in the GenBank database under accession numbers from KR871015 to KR871214.

2.5 Data analysis

The copy numbers of the three genes were log-transformed to homogenise variance prior to statistical analyses performed with SPSS (IBM, version 20). To compare the differences in gene copy numbers between ES-amended soils and controls, log-transformed gene copies were subjected to independent-samples T test at the 95% confidence interval. ANOVA analysis was performed on the log-transformed gene copies in ES-amended soils to assess difference between soils or between incubation times. Linear regression was performed to assess the correlation between oxidation rate of ES and soil variables. The relative *soxB* gene abundance, expressed as percentages of absolute *soxB* to 16S rRNA gene copy numbers, were also subject to T test and ANOVA analyses as performed for log-transformed absolute *soxB* gene copies.

3 Results

3.1 Abundances of soxB, 16S rRNA and 18S rRNA genes

The copy numbers of *soxB*, 16S rRNA and 18S rRNA genes were determined in ten Australian cropping soils with a wide variation in physico-chemical properties. To quantify *soxB* genes, we developed a qPCR assay using *soxB* primers (Table 2) from the literature (Petri et al., 2001). This assay provided reproducible results of *soxB* gene abundance in soils with a wide range of properties.

soxB gene copy numbers ranged from 6.3×10^5 to 1.8×10^7 copies g^{-1} air-dry soil in the soils (Table 1). Compared with controls, *soxB* gene copies only exhibited positive responses to ES amendment at day 48 with significant increases in soils 1 and 10 ($P < 0.05$) (Fig. 2B). A significant decrease in *soxB* gene copy numbers in response to ES amendment was observed for soil 8 at day 48 ($P = 0.006$) (Fig. 2B). In soils amended with ES, *soxB* gene copies did not show any significant changes over time except for soils 4 and 5, but even then changes were of a small magnitude (Table S1). The copy numbers of 16S rRNA genes in soils varied from 5.3×10^7 to 1.8×10^9 copies g^{-1} air-dry soil (Table 1). Similar to *soxB* genes, 16S rRNA gene copies did not show any positive response to ES application, and even decreased in response to ES application in some soils (Fig. 2D–F). This reduction was greatest at day 96 with 5 soils showing significantly lower copy numbers of 16S rRNA genes in the amended soils ($P < 0.05$) (Fig. 2F). This negative effect of ES application was also evident from the changes in 16S rRNA gene copies over time in the amended soils, with 5 soils showing significantly lower abundances at day 48 or day 96 than at day 8 ($P < 0.05$) (Table S1). We also analysed the relative *soxB* gene abundance (percentage of *soxB* gene copy numbers relative to 16S rRNA gene copy numbers) (Fig. 3) to gain a better understanding of variations in *soxB* gene copies as 16S rRNA gene copy numbers were decreasing. Compared to the controls, the relative *soxB*

gene abundance was significantly higher in the amended soils for soils 5 and 9 at day 8 and soils 5 and 10 at day 48 ($P < 0.05$), while no significant differences were observed at day 96 (Fig. 3). Unlike 16S rRNA genes, ES amendment did not cause much change in 18S rRNA gene copy numbers (Fig. 2G-I), with only 2 soils having significantly lower gene copy numbers in amended than in the control soils at day 48 ($P < 0.05$) (Fig. 2H). In addition, there was no significant change in the 18S rRNA gene copies in amended soils over time (Table S1).

<Fig. 2 and Fig. 3 >

Gene copy numbers of these three genes from control soils incubated for 8 days were used to represent the original gene copy numbers in soils (Table 1). There were significant correlations between the copy numbers of the three types of genes ($P < 0.05$), with a very strong correlation between log *soxB* and log 16S rRNA ($R^2 = 0.99$). Soil properties relevant to microbial nutrient supply, i.e. total and organic carbon, total and organic sulphur, and total nitrogen, showed significant correlation with gene copies ($P < 0.05$) (Table 3).

<Table 3>

3.2 Relationships between the oxidation rate of elemental sulphur and gene abundances

Both *soxB* and 16S rRNA gene copy numbers showed a significant positive correlation with the oxidation rate ($P < 0.01$) (Fig. 4). However, we did not observe significant correlation between 18S rRNA copy numbers and the oxidation rate. In a two-factor analysis, more than 80% ($P < 0.01$) of the variation in oxidation rate could be explained by the combination of *soxB* or 16S rRNA gene copy numbers ($P < 0.05$) and soil pH ($P < 0.05$) (Fig. 5).

<Fig. 4 and Fig. 5>

3.3 Diversity of S-oxidising bacteria

The phylogeny of *soxB* clones were classified into 2 groups based on sequence similarities, i.e. *Beta-proteobacteria* and *Alpha-proteobacteria* (Fig. 6), with 60% of clones attached to *Beta-proteobacteria.* Even though all aligned sequences were *soxB* gene-like, only 17% of them showed similarities higher than 50% to identified S-oxidising bacterial species (Fig. 6).

<Fig. 6>

The relative distribution of clones (class order) showed great differences in the structure of *soxB* genotypes across soils (Fig. 7). Specifically, clones in soil 2 were almost evenly distributed to *Alpha-* and *Beta-proteobacteria* with 47% and 57%, respectively. Clones clustered to *Beta-proteobacteria* accounted for 63–78% in soils 4, 5, 6 and 10, but only for 36% in soil 7.

<Fig. 7>

4 Discussion

4.1 Abundances of S-oxidising bacteria are related to organic carbon and nutrients concentrations

Variations in soil physic-chemical properties mainly drive the differences in microbial habitats across soils, e.g. substrate availability, aeration and pH, and therefore result in the differences in microbial community size and composition. Soil type-based differences in the abundance of functional genes involved in nitrogen cycling have been reported in south-eastern Hayden et al.

(2010) and western Australia (O'Sullivan et al., 2012). Similarly, our study showed that *soxB* gene copy numbers varied greatly in the ten cropping soils used and the variation was related to soil physico-chemical properties including soil pH, organic carbon, organic S etc. The abundance of *soxB* genes in our study (6.3×10^5 to 1.5×10^7 copies g^{-1}) was lower than in New Zealand pasture soils (Tourna et al., 2014) (7.9×10^7 to 9.4×10^8 copies g^{-1}). This could be related to the higher organic matter content in pasture soils than in the cropping soils of our study. The availability of carbon has been reported to be a limiting factor affecting the size and activity of microbial biomass in soils (Gonzalez-Quiñones et al., 2011). Although, autotrophic S-oxidisers such as *Thiobacilli* were mostly attributed to ES oxidation, heterotrophic bacteria were found to play a significant role in S oxidation especially in cropping soils (Germida and Janzen, 1993). Our result on *soxB* gene diversity showed the distribution of *soxB* genes in taxonomically diverse group of soil heterotrophic bacteria suggesting the likely impact of C availability on the abundance of *soxB* functional genes. Our study also showed that soils with higher organic carbon content had higher copy numbers of *soxB* and 16S rRNA genes, i.e. bacterial abundances (Table 3). This indicates that, like total bacteria, S-oxidising bacteria also respond to the variation in soil carbon and nutrient availability. Being capable of utilising different carbon sources as well as mixed S forms (both organic and inorganic S) offers S-oxidising bacteria a strong ability to cope with environmental variation (Graff and Stubner, 2003). Furthermore, this may also suggest that ES oxidation in these soils would be improved by adding carbon sources to soil such as crop residues. This stimulating effect of adding carbon on ES oxidation was reported in soils amended with wheat straw and pressed sugar beet (Wainwright et al., 1986) or amended with glucose (Lawrence and Germida, 1988).

4.2 Elemental sulphur oxidation is associated with bacterial abundances and chemical properties

Previously, culture-based methods were used to correlate populations of S-oxidising microorganisms with ES oxidation, which showed variable relationships between the abundance of S-oxidising microbes and ES oxidation (Germida and Janzen, 1993). These methods, however, are known to only cover < 10% of the total bacterial community in soil, whereas culture-independent DNA-based methods allow the interrogation of all bacterial communities in soil (Tiedje et al., 1999). In this study, the copy numbers of *soxB* genes, the functional genes involved in ES oxidation, were quantified using qPCR to represent the total S-oxidising bacterial abundance. The total bacterial population was also represented by the 16S rRNA gene copy abundance. We found that 16S rRNA and *soxB* gene copies were highly correlated ($r = 0.99$, $P < 0.01$) and therefore the same proportion of the variation in oxidation rates could be explained by the copy numbers of these two genes (Fig. 5). However, we did not observe any significant correlation between 18S rRNA abundance and the oxidation of ES in the soils. These findings suggest the importance of bacterial abundance in ES oxidation.

A strong correlation between the 16S rRNA and *soxB* gene abundances could be attributed to the broad coverage of the primers used to a wide range of bacterial phyla (Petri et al., 2001), which is supported by the non-specificity of the primer set to *soxB* genes in soils (92% of the clones were putative *soxB* gene sequences) (see section 2.4). In addition, our DNA-based measurements represent the abundance of total populations, including dormant and highly active bacteria, under the experimental conditions. By considering the broad distribution of *soxB* genes, it is logical to assume only a subset of organisms, i.e. bacteria adapted to the experimental conditions, would be active and involved in S oxidation in the different soils in

our study. Thus, a measurement of active S-oxidising bacterial populations may have provided a better relationship between the *soxB* gene abundance and ES oxidation rates.

It has been suggested that the size and activity of the microbial biomass determines ES oxidation, and that soil properties control the size and activity of the microbial biomass (Lawrence and Germida, 1988). In many soil properties, pH has been demonstrated to be a major factor shaping bacterial community composition and determining their abundance in soil (Fierer and Jackson, 2006; Lauber et al., 2008; Rousk et al., 2010). In our previous study (Zhao et al., 2015), we found that soil chemical properties (pH and organic carbon) explained 79% of the variation in the oxidation rate of ES across soils. In the present study, higher than 80% of the variation in ES oxidation rate was explained by the size of the populations of total or S-oxidising bacteria (determined using molecular methods) along with soil initial pH (Fig. 5).

4.3 Effect of application of elemental sulphur on soil bacterial population, and the subsequent ES oxidation

The addition of ES to soil is expected to stimulate the growth of S-oxidising bacteria, especially for *Thiobacillus* species that are responsive to the presence of ES (Lee et al., 1987; Lee et al., 1988; Chapman, 1990; Tourna et al., 2014). By using *soxB* primers exclusively for chemolithotrophic S-oxidising bacteria, Tourna et al. (2014) demonstrated a stimulatory effect of ES and the proliferation of Thiobacillus-like S-oxidisers in soil. However, we did not observe positive responses of the abundance of S-oxidising bacteria to ES application in most soils. It is possible that most *Thiobacillus* species were not present in our soils (see section 4.4).

As for total bacterial abundance, we found a reduction in half of the soils incubated with ES (1000 mg kg^{-1}) for 96 days (Fig. 2F). Application of ES has been found to exhibit suppressive

effects on soil heterotrophic microbial populations, enzymes and biomass (Lee et al., 1987; Gupta et al., 1988; Lawrence et al., 1988; Deng and Dick, 1990; Lawrence and Germida, 1991), which are probably due to the decrease in pH and increase in sulphate concentration as ES is oxidised. Decreases in soil pH has been reported to strongly influence bacterial community composition and diversity with acidic soils generally showing lower abundances of many bacterial phyla (e.g. Rousk et al., 2010). In this experiment, the pH was 0.1–2.7 units lower in ES-amended soils than in control soils by the end of incubation (Zhao et al., 2015), which may explain the reduction of total bacterial abundance in half of the soils incubated for 96 days (Fig. 2F). It has been shown that application of $CaCO_3$ effectively prevented decreases in bacterial abundance in soils treated with 0.1% and 0.5% ES, and soils with higher $CaCO_3$ addition (0–2%) had more ES oxidised (Adamczyk-Winiarska et al., 1975). It has been reported that high sulphate concentration had repressive/inhibitory effects of on enzymatic activity involved in ES oxidation (Ray et al., 1985; Gupta et al., 1988; Deng and Dick, 1990), which could consequently influence ES oxidation. These effects, however, were observed during or after ES oxidation when pH had decreased, hence it is possible that the effect of high sulphate concentration was confounded by that of pH decrease. By adding 1.0% of $CaCO_3$, no effect of sulphate on ES oxidation was observed even when sulphate-S accumulated from 3000 to 6000 mg kg^{-1} from ES oxidation (Adamczyk-Winiarska et al., 1975). Therefore, pH decrease is the major factor influencing ES oxidation, and the effect of high sulphate concentration, if there were any, might be dependent on the co-varied pH.

4.4 The diversity of sulphur-oxidising bacteria is soil-type dependent

The phylogenetic tree (Fig. 6) revealed a great diversity in S-oxidising bacteria. The list of S-oxidising bacteria in Australian soils included genera commonly associated with S oxidation such as *Pandoraea*, *Thiobacillus*, *Paracoccus* etc., but also genera that are not commonly

linked to S oxidation like *Ralstonia* and *Bradyrhizobium*, as well as a number of unidentified species. *Thiobacillus* is a well-studied group among S-oxidisers and can respond to ES application rapidly with substantial increases in population (Lee et al., 1987; Chapman, 1990; Tourna et al., 2014), whereas the importance of genera such as *Bradyrhizobium* is well known for N-fixation in soil (Kaneko et al., 2002). Tourna et al. (2014) found new *soxB* sequences in New Zealand pasture soils suggesting that the diversity of S-oxidising bacteria in soils remains to be further explored. Soil type has been shown to determine the general bacterial community composition (Girvan et al., 2003), hence might also influence the diversity of S-oxidising bacteria that are evidently phylogenetically and taxonomically diverse. Different *soxB* gene-harbouring bacterial species were found in soil environments varying in physico-chemical properties. For example, in land-fill cover soil, *Halothiobacillus* species were found due to limited oxygen in that environment (Xia et al., 2014), whereas *Firmicutes* were isolated from rhizosphere soils of crop plants (Anandham et al., 2008). However, *Halothiobacillus* were not observed in our soils because that they are not typical for most agricultural soils (Tourna et al., 2014), and *Firmicutes* were not observed in bulk soils such as our soils or New Zealand pasture soils (Tourna et al., 2014). Our study also revealed the phylogeny and relatively distribution of S-oxidising bacteria varied greatly in soils across Australia (Fig. 6 and Fig. 7). The diversity and composition of S-oxidising bacteria have also been shown to be influenced by the presence of ES in soil. For example, Tourna et al. (2014) found that New Zealand pasture soils amended with ES harboured different *Thiobacilli* species of variable abundances, related to different ES oxidation trends across soils. However, we did not observe any link between the diversity of S-oxidising bacteria and ES oxidation across soils. To investigate the potential of a soil to subsequently oxidise ES, we only examined the diversity of S-oxidising bacteria in soils without ES amendment under controlled environmental condition. Under this condition,

Thiobacillus were either not present in most of the soils as indicated by the phylogenetic tree of *soxB* genes (Fig. 6).

4.5 Heterotrophic sulphur-oxidising bacteria have significant effect on elemental sulphur oxidation

The lack of presence of significant populations of chemolithotrophic *Thiobacilli* in agricultural soils has been reported before in Canadian (Lawrence and Germida, 1988) and Scottish soils (Chapman, 1990). It was suggested that in these soils aerobic heterotrophic bacteria are the major contributors to ES oxidation. Chemolithotrophic *Thiobacilli* have specific pH and other chemical environmental requirements for growth, for example, *Thiobacillus thiooxidans* generally requires low pH (< 5.5) for growth (Rao and Berger, 1971). Soils used in this incubation study generally had soil pH above 5 (Table 1). Yang et al. (2010) reported an increase in populations of both the *Thiobacillus* spp. and aerobic heterotrophic bacteria during the first four weeks of incubation with ES but did not find any *Thiobacilli* after 84 days, suggesting that ES oxidation relied on aerobic heterotrophic bacteria. This may also suggest that *soxB* gene copy numbers in the control soils in our study mainly indicated the population size of heterotrophic S-oxidising bacteria. If so, the population of heterotrophic S-oxidising bacteria is a good predictor of ES oxidation rate in cropping soils in this study. This agrees with Lee et al. (1987) who found that ES oxidation could be best predicted by the number of S-oxidising heterotrophic bacteria among other properties (i.e. soil pH, the numbers acidophilic and neutrophilic S-oxidisers) for 48 New Zealand pasture soils. Similarly, Lawrence and Germida (1988) didn't find measurable populations of S-oxidising autotrophs in agricultural soils from across Saskatchewan in Canada and used the size of microbial biomass as an indicator of total microbial populations in soils. In an investigation of 288 Australian soils, Vitolins and Swaby (1969) found that heterotrophic S-oxidisers were more common than

thiobacilli in general. It is possible that in some of the soils of this study, chemolithotrophic bacterial population may have increased following incubation with ES but was missed due to the long interval between sampling occasions (40–48 days). However, similar to that observed in a number of previous studies with agricultural soils, our results suggest that aerobic heterotrophic bacteria have a significant role in ES oxidation in Australian cropping soils.

4.6 Conclusions

The abundance and diversity of *soxB* genes were dependent on soil type. *soxB* gene copy numbers were strongly correlated with potential for ES oxidation. As *soxB* gene abundance was a relatively constant proportion of the 16S rRNA gene abundance, the 16S rRNA gene abundance was also strongly related with subsequent ES oxidation and could be used to predict the oxidation rate of ES. A combination of soil pH and gene abundances predicted ES oxidation rate much better, indicating that ES oxidation is influenced by both soil biological and physico-chemical properties. Additionally, soil bacterial abundances were reduced in some soils during and after ES oxidation, which is mostly likely related to an increase in acidity as a result of ES oxidation. Sulphur-oxidising bacteria exhibited great diversity across soils, but we did not observe any relationship between the diversity of S-oxidising bacteria and ES oxidation. Similar with previous studies, we concluded that heterotrophic S-oxidising bacteria are more important than autotrophic bacteria for ES oxidation in agricultural soils.

Acknowledgements

Special thanks go to Marcus Hicks for his significant assistance with extraction and characterisation of soil DNA. Nilangani Harris, Belinda Stummer, Rosemary Warren, Adrienne Gregg are also acknowledged for assisting with molecular techniques.

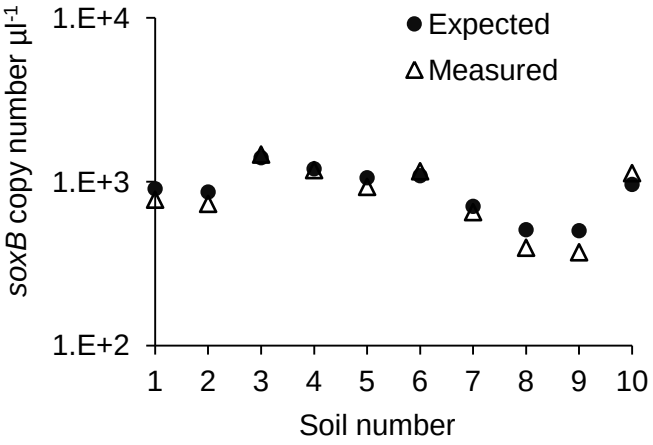

Fig. 1. Measured and expected *soxB* gene abundances in soil DNA samples inoculated with known DNA copies to test inhibition in the qPCR assay of *soxB* genes.

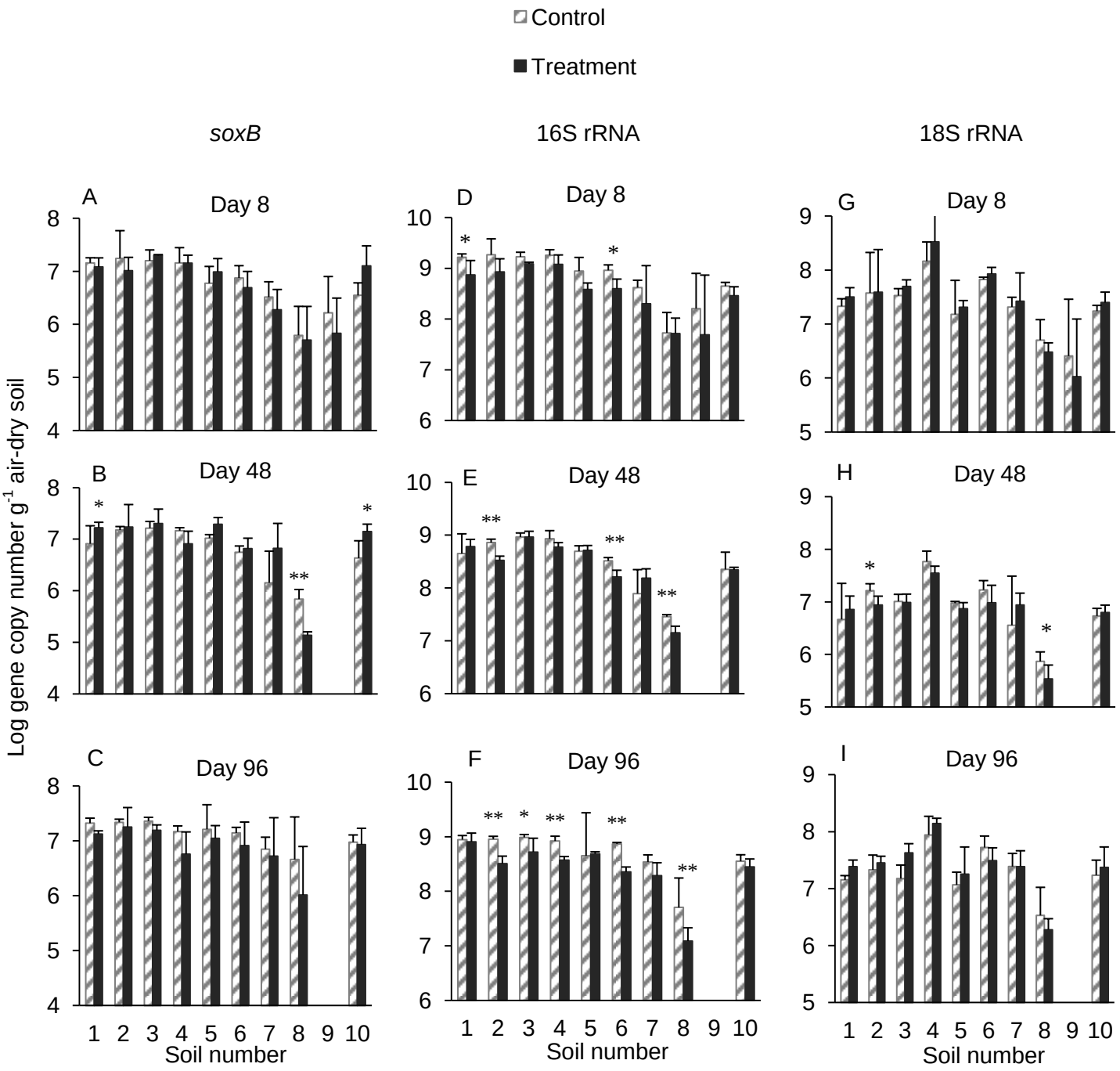

Fig. 2. The abundance of *soxB*, 16S rRNA and 18S rRNA genes in control and ES-amended soils at different times of incubation. Error bars indicate standard deviation of four replicates. * indicates a significant level of $P \leq 0.05$, and ** indicates $P \leq 0.01$.

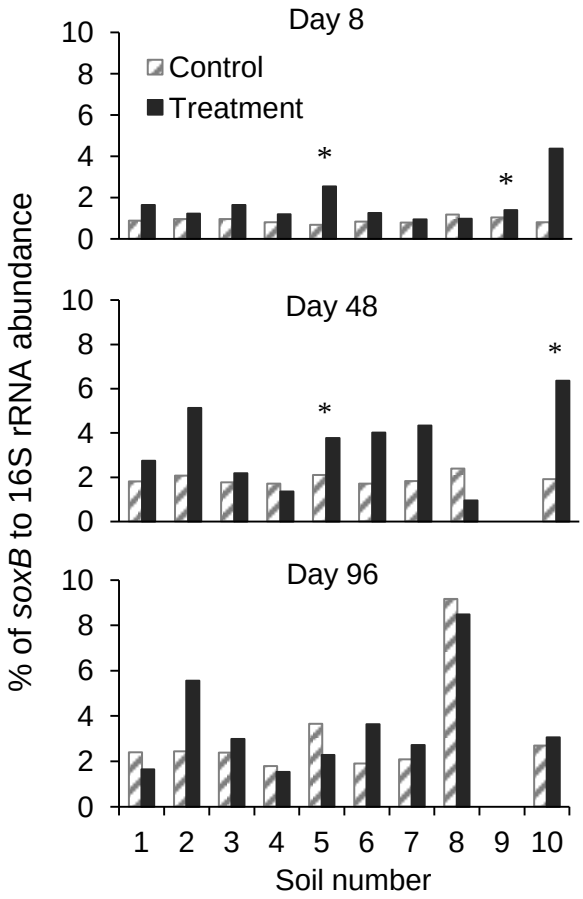

Fig. 3. Relative abundance of *soxB* genes expressed as percentage of *soxB* to 16S rRNA gene copy numbers at the three sampling occasions. * indicates a significant level of $P \leq 0.05$.

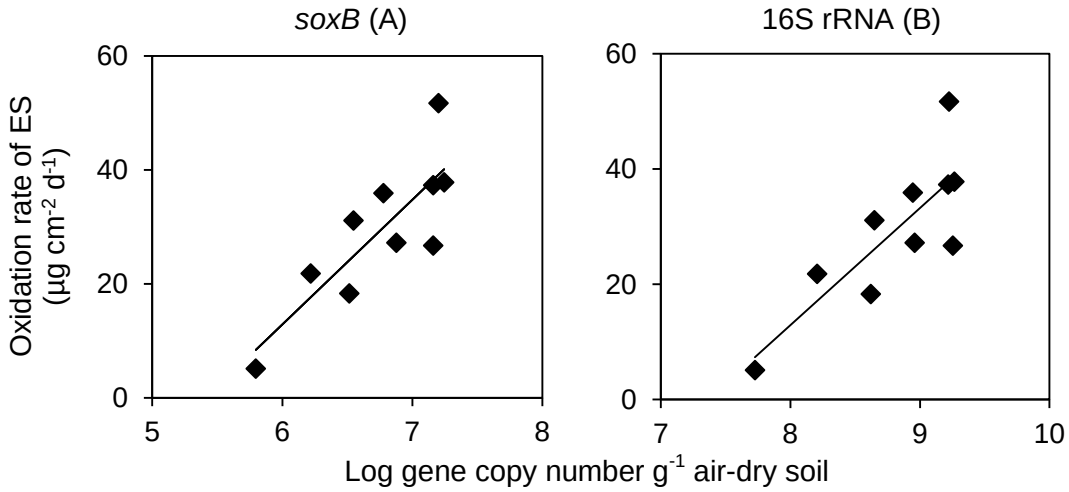

Fig. 4. Relationship between elemental sulphur oxidation rate and logarithm-transformed *soxB* /16S rRNA gene copy numbers. A: Oxidation rate = 21.14 × log(*soxB*) – 113.40 (R^2 =0.66, P = 0.004, n = 10); B: Oxidation rate = 20.30 × log(16S rRNA) – 149.46 (R^2 = 0.67, P = 0.004, n = 10).

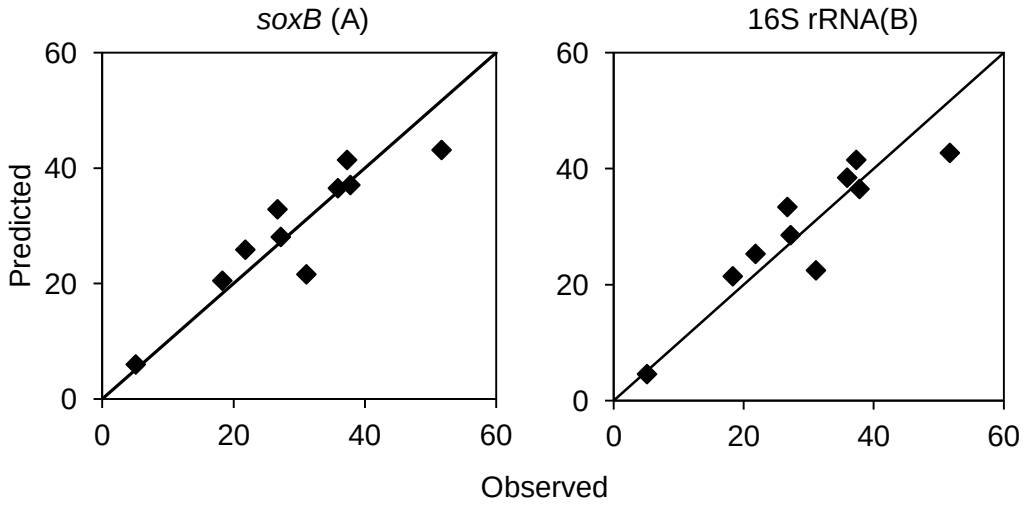

Fig. 5. Predicted *versus* observed ES oxidation rate (μg cm^{-2} d^{-1}). The line is the 1:1 line. A: Oxidation rate=15.49 × log(*soxB*) (*P* = 0.016) + 3.80 × pH (*P* = 0.049) – 100.31 (R^2 = 0.82, *P* = 0.003, n = 10); B: Oxidation rate=15.02 ×log(16S rRNA) (*P* = 0.012) + 3.80 ×pH (*P* = 0.041) - 128.48 (R^2 = 0.83, *P* = 0.002, n = 10).

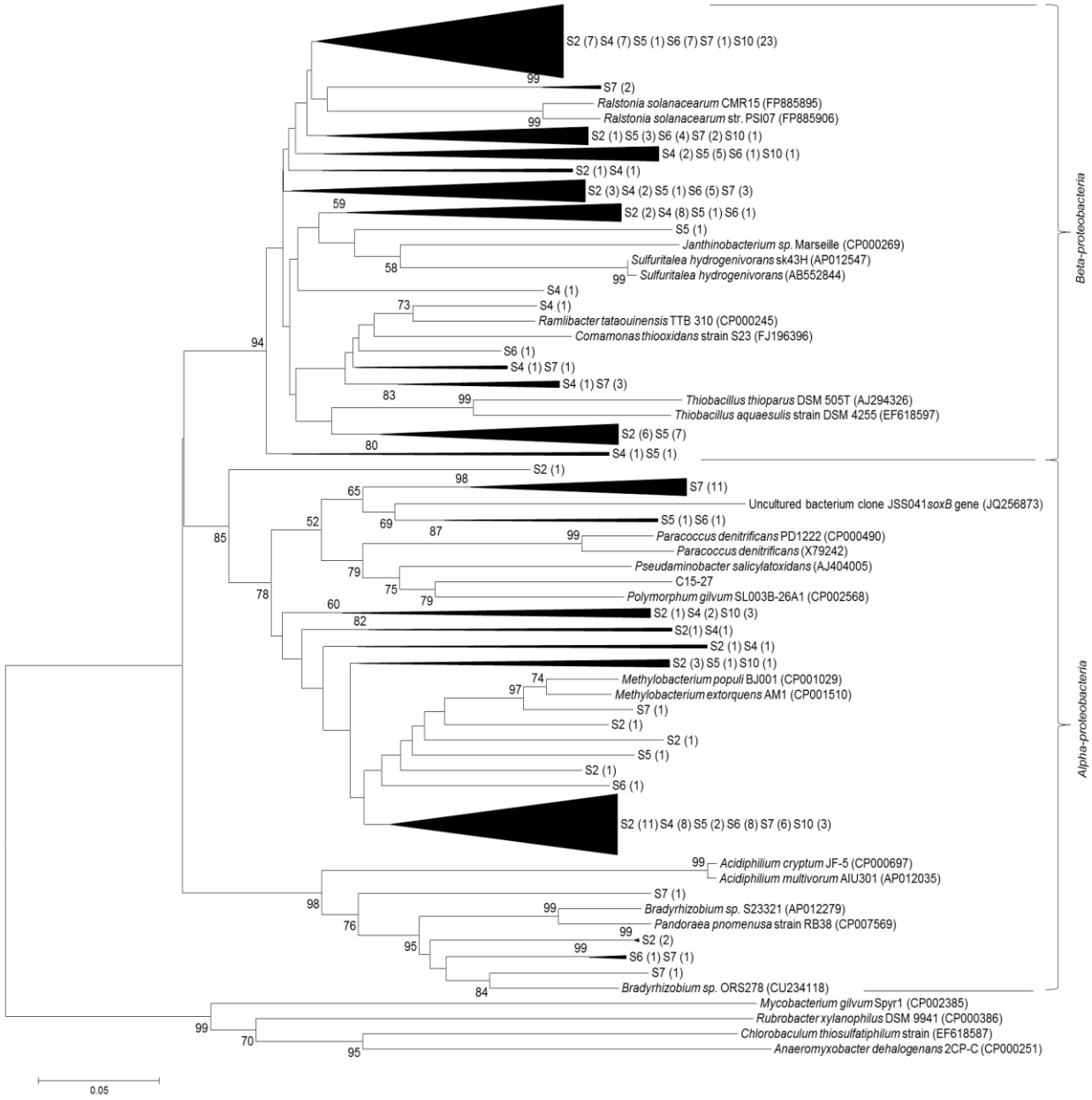

Fig. 6. Phylogenetic tree based on clone libraries of *soxB* genes from 6 clone libraries (one for each selected soil: S2, S4, S5, S6, S7 and S10), followed by the number of clones from each library in the parenthesis.

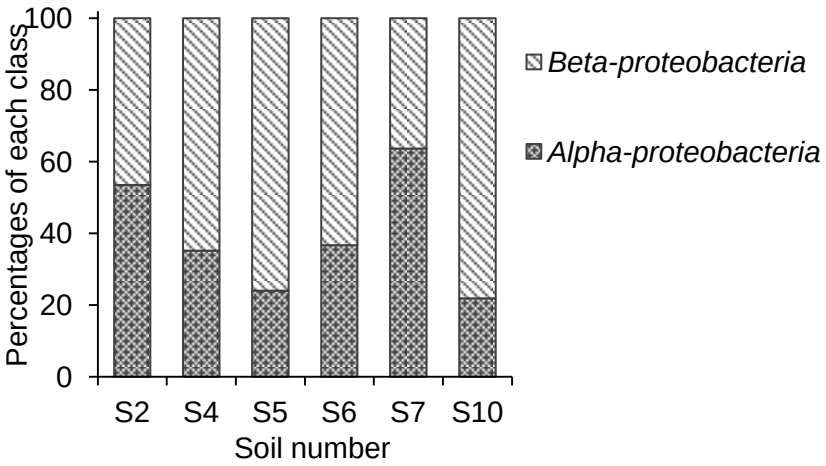

Fig. 7. Relative distributions of clones attached to *Alpha-* and *Beta-proteobacteria* within the clone libraries form six soils.

Table 1 Physico-chemical properties of the Australian cropping soils used.

Soil no.	State[a]	CEC[b]	Clay	FC[c]	pH	TS[e]	Sulphate-S	TC[f]	TN[g]	$CaCO_3$	OC[h]	Oxidation rate	*soxB*	16S rRNA	18S rRNA
		$cmol_c\ kg^{-1}$	%	%	(water)	----mg kg^{-1}---		----------%----------				$\mu g\ cm^{-2}\ d^{-1}$	log gene copies g^{-1} air-dry soil		
1	SA	17	19.9	23.1	8.2	182	9.6	1.6	0.12	3.0	1.3	37.3	7.2	9.2	7.3
2	SA	7	4.7	14.9	6.7	205	4.6	2.0	0.19	< 0.2	2.0	37.8	7.2	9.3	7.6
3	SA	15	16.4	23.4	8.5	279	7.1	3.0	0.15	11.0	1.7	51.7	7.2	9.2	7.5
4	Vic	9	10.3	16.9	6.0	224	5.8	2.2	0.20	< 0.2	2.2	26.7	7.2	9.3	8.2
5	Vic	7	7.5	7.6	8.5	83	5.3	0.7	0.06	1.0	0.5	35.9	6.8	8.9	7.2
6	NSW	8	10.4	26.9	5.9	128	13.2	1.4	0.12	< 0.2	1.4	27.2	6.9	9.0	7.8
7	NWS	10	20.3	22.1	5.3	133	21.2	1.3	0.11	< 0.2	1.3	18.3	6.5	8.6	7.3
8	WA	4	10.9	8.6	4.4	64	28.2	0.6	0.04	< 0.2	0.6	5.1	5.8	7.7	6.7
9	Qld	59	53.4	57.4	8.0	52	5.3	1.0	0.05	< 0.2	1.0	21.8	6.2	8.2	6.4
10	Qld	16	49.4	28.8	5.5	202	15.2	1.7	0.14	< 0.2	1.7	31.1	6.5	8.6	7.2

[a] SA: South Australia; Vic: Victoria; NSW: New South Wales; WA: Western Australia; Qld: Queensland.

[b] cation exchangeable capacity.

[c] gravimetric water content at field capacity.

[d] electricity conductivity measured in 1:5 (g ml^{-1}) water extract.

[e] total sulphur.

[f] total carbon.

[g] total nitrogen.

[h] organic carbon.

Table 2 Primers and thermal profiles used for qPCR for three genes.

Oligonucleotide Primer and target sites	Primer sequence (5'-3')	Reference	Thermal profile	Cycle	Reference
*soxB*432F *soxB*693B	GAYGGNGGNGAYACNTGG TANGGRAANGCYTGNCCGAT	(Petri et al., 2001)	95 ℃ - 15 min 95 ℃ - 40 s/54 ℃ - 40 s/72 ℃ - 40 s	1 40	This study
16S 968F 16S 1378R	AACGCGAAGAACCTTAC CGGTGTGTACAAGGCCCGGGAACG	(Heuer et al., 1997)	95 ℃ - 15 min 95 ℃ - 30 s/54 ℃ - 30 s/72 ℃ - 60 s	1 35	(Gupta et al., 2014)
18S FR1 18S FF390	AICCATTCAATCGGTAIT CGATAACGAACGAGACCT	(Vainio and Hantula, 2000)	95 ℃ - 15 min 95 ℃ - 15 s/54 ℃ - 30 s/72 ℃ - 60 s	1 40	(Prevost-Boure et al., 2011)

Table 3 Correlation matrix between soil properties and bacterial/fungal abundances.

	log 16S rRNA	log 18S rRNA	pH	EC	TC	OC	TS	OS[a]	TN
log *soxB*	0.99**	0.78*	0.49	0.38	0.72*	0.65*	0.76*	0.80**	0.78**
log 16S rRNA		0.79**	0.48	0.36	0.70*	0.65*	0.75*	0.79**	0.78**
log 18S rRNA			-0.08	0.09	0.62	0.72*	0.69*	0.69*	0.84**

[a] indicates organic sulphur, expressed as the difference between total sulphur and sulphate concentration;

*indicates a significant level of $P \leq 0.05$, and ** indicates $P \leq 0.01$.

Table S 1 *soxB*, 16S rRNA and 18S rRNA copy numbers in elemental sulphur amended soils at 8, 48, or 96 days of incubation.

Soil No.	*soxB* (log copy number g^{-1} air-dry soil)[a]				16S rRNA (log copy number g^{-1} air-dry soil)				18S rRNA (log copy number g^{-1} air-dry soil)				*soxB*/16S rRNA (%)		
	D8	D48	D96		D8	D48	D96		D8	D48	D96		D8	D48	D96
1	7.1ab	7.2ab	7.1ab		8.9a	8.8b	8.9a		7.5bc	6.9b	7.4bc	A/B/A	1.6	2.7abc	1.7
2	7.0ab	7.2abc	7.3ab		8.9a	8.5c	8.5bc	A/B/B	7.6bc	6.9b	7.5bc		1.2	5.1ab	5.6
3	7.3a	7.3ab	7.2a		9.1a	9.0a	8.7ab	A/A/B	7.7abc	7.0b	7.6b	A/B/A	1.6	2.2bc	3.0
4	7.2ab	6.9abc	6.8abc	A/AB/B	9.1a	8.8b	8.6bc	A/B/B	8.5a	7.5a	8.1a	A/B/A	1.2	1.4bc	1.5
5	7.0ab	7.3a	7.0ab	B/AB/A	8.6ab	8.7b	8.7ab		7.3bc	6.9b	7.3bc		2.5	3.8abc	2.3
6	6.7bc	6.8bc	6.9abc		8.6ab	8.2de	8.4cd	A/B/AB	7.9ab	7.0b	7.5bc	A/C/B	1.2	4.0ab	3.6
7	6.3cd	6.8c	6.7bc		8.3bc	8.2e	8.3d		7.4c	6.9b	7.4bc		0.9	4.3abc	2.7
8	5.7e	5.1d	6.0d		7.7c	7.2f	7.1e	A/B/B	6.5d	5.5c	6.3d	A/B/A	1.0	1.0c	8.5
9	5.8de	N/A[b]	N/A		7.7cd	N/A	N/A		6.0e	N/A	N/A		1.4	N/A	N/A
10	7.1ab	7.1d	6.9c		8.5ab	8.3d	8.4cd		7.4bc	6.8b	7.4bc	A/B/A	4.4	6.4a	3.1

[a] different small letters within the row indicate significant differences ($P \leq 0.05$) between soils; different capital letters indicate significant differences over time (D8/D48/D96).

[b] indicates not available.

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CHAPTER 5

Effects of pH and ionic strength on elemental sulphur oxidation in soil

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Abstract

Elemental sulphur (ES) has to be oxidised to sulphate for plant uptake. The oxidation of ES in soil is a microbially mediated process, and could be influenced by changes to soil chemical properties such as soil acidity and ionic strength due to ES oxidation or dissolution of macronutrients in co-granulated products (e.g. nitrogen, phosphorus or potassium). This study assessed the effect of acidification and an increase in ionic strength on bacterial abundance and community composition and on ES oxidation during a14-week incubation. Prior to incubation, a sandy soil was treated with HNO_3 to bring the pH to 6.7–4.4 or with KH_2PO_4 to increase the ionic strength by 0–0.7 M. Soils were incubated with 200 mg ES kg^{-1} air-dry soil at 25 ℃ and 70% field capacity. Acidification or increasing ionic strength did not affect the % of ES oxidised, but significantly decreased the abundances of *soxB* and 16S ribosomal ribonucleic acid (16S rRNA) genes, and changed the bacterial community structure. An additional experiment with two other soils also showed that acidification did not, or only slightly decreased, ES oxidation at two different ES rates, even though acidification strongly decreased *soxB* and 16S rRNA gene abundances in one of the soils. The independence of ES oxidation on bacterial gene abundance indicates that ES oxidation is not limited by bacterial population abundance, as also evidenced by the fact that %ES oxidation was unchanged as ES application increased. The lack of agreement between bacterial community composition and ES oxidation could be attributed to the fact that the *soxB* gene is present in taxonomically and physiologically diverse bacterial groups, hence bacterial species outcompeted due to alterations of soil physico-chemical conditions are expected to be replaced by other species with the same function, but adapted to the changed environment. This study indicates that shifts in bacterial population brought about by temporary changes in pH and ionic strength have no, or only slight, effects on ES oxidation.

Keywords

Elemental sulphur oxidation, pH, ionic strength, bacterial abundance, bacterial community composition

1 Introduction

Elemental sulphur (ES) is used as a sulphur (S) fertiliser source, but is not readily available for plant uptake as it has to be first oxidised to sulphate. The oxidation of ES is a biological process and could therefore be strongly influenced by factors that affect microbial population and activity including soil temperature, water potential, aeration and pH. Elemental S oxidation has been found to be related to microbial biomass carbon, rhodanase activity, abundance of chemolithotrophic S-oxidising bacteria and pH across soils (Lawrence and Germida, 1988; Dick and Deng, 1991; Tourna et al., 2014). Our previous study also indicated that approximately 70% of the variation in surface-based oxidation rate of ES could be explained by the bacterial population size in ten Australian cropping soils (Zhao et al., submitted). The link between the diversity of S-oxidising bacteria and ES oxidation has been demonstrated by Tourna et al. (2014), who observed proliferation of different *Thiobacillus*-like bacterial species in two different soils where ES oxidation trends differed during a 30-day incubation.

Soil pH has a strong effect on bacterial diversity and community composition, as has been seen both for soils where pH was altered due to long-term management and for soils with natural pH variations across terrestrial ecosystems (Rousk et al., 2010; Fierer et al., 2012). Soil pH has also been reported to be positively related to bacterial abundance in soil with other properties remaining the same or similar (Rousk et al., 2010). The effect of soil pH on microbial processes may be due to the chemical form and solution concentration of substrates, and hence affects availability (Kemmitt et al., 2006). It has been found that ES oxidation is positively related with

soil pH (Lawrence and Germida, 1988; Zhao et al., 2015), but it is not really clear what the mechanism is behind this relationship. We hypothesised that this is due to soils with higher pH usually harbouring a larger number of S-oxidising bacteria as shown in our previous study (Zhao et al., submitted). The effect of soil pH on ES oxidation does not only occur across soils, but has also been reported within a single soil when the pH was varied by amendments. Greater ES oxidation was observed in a soil amended with $CaCO_3$ than in unamended soil, which was attributed to a larger number of bacteria in the limed soil that was protected against a drop in pH (Adamczyk-Winiarska et al., 1975). Similarly, more ES was oxidised in a soil amended with phosphate rock, in which the pH decreased less than in treatments without phosphate rock due to the acid-consuming dissolution of the rock (Lee et al., 1987).

Changes in ionic strength in soil solution may also affect bacterial movement and population size, and hence potentially influence ES oxidation. It was found that high ionic concentration encouraged the adherence of bacteria to solid surfaces by decreasing the thickness of electrical membrane layers (Tan et al., 1991; Tan et al., 1992). Ionic strength may also influence microbial activity or growth due to osmotic stress (Galinski and Trüper, 1994). In a culture-based study assessing the effect of pH and salts on S oxidation by *Thiobacillus thiooxidans*, it was found that low salt concentrations increased ES oxidation likely due to charge neutralisation of the cell surface, but high concentrations (~0.2 M) inhibited ES oxidation, which was attributed to high osmotic pressures (Suzuki et al., 1999).

For agronomic purposes, ES is generally not applied as a powder because of safety and practical considerations (Rothbaum and Groom, 1961), but is often co-granulated with a macronutrient carrier such as ammonium phosphate or urea. The chemical environment around a fertiliser granule in soil can be very different to that in the bulk soil. For instance, the pH

around the granule can be lower due to acidic compounds or compounds that undergo acid-forming reactions, such as oxidation of ammonium (nitrification) or oxidation of ES itself. Furthermore, the soluble compounds within the granule result in high salt concentrations in and near the granule (Sample et al., 1980). However, little is known about the changes in acidity and ionic strength around the granular fertiliser, which may affect the microbial population and in turn affect ES oxidation in the granular fertiliser.

Hence, we modified soil pH and ionic strength to different levels to reflect those changes occurring around a macronutrient fertiliser granule. The objectives of this study were to 1) investigate the effects of acidification or an increase in ionic strength on ES oxidation; 2) examine the changes of bacterial abundance and bacterial community composition under these conditions; and 3) examine if changes in ES oxidation as a result of chemical modifications can be related to shifts in the bacterial population in soil.

2 Material and methods

2.1 Effect of acidification or increased ionic strength (experiment 1)

A sandy soil from South Australia was used for this experiment. The soil properties are listed in Table 1 (for details of soil analysis, see Zhao et al., 2015).

To assess the effect of acidification, the soil in four containers (200 g each) was acidified with 0, 4, 7 and 10 ml of HNO_3 (1 M), respectively. Acidified soils were equilibrated overnight, followed by leaching with artificial rain water at a soil:liquid ratio of 1:2 kg l^{-1} to remove excess NO_3^-. The artificial rain consisted of $CaCl_2$ (0.015 mM), KNO_3 (0.008 mM), $MgCl_2$ (0.010 mM), Na_2SO_4 (0.010 mM), NaCl (0.064 mM) and NH_4Cl (0.006 mM) (Donn and Menzies, 2005). The leached soil was air-dried for one week. Thereafter, the soil pH was

determined in a 1:5 (kg l^{-1}) water suspension and was 6.7 (p1 or control), 6.3 (p2), 5.2 (p3) and 4.4 (p4) for the different acidification treatments. The air-dried soil of each treatment was separated into four replicates, and each replicate was amended with ES at a rate of 200 mg kg^{-1} air-dry soil, which was mixed thoroughly through the soil. The ES particles had an average diameter of 47 μm (see the distribution of ES particle sizes in Zhao et al., 2015). A rate of 200 mg ES kg^{-1} was selected to avoid elevated sulphate-S concentration which may have a suppressive effect on enzymatic activity (Deng and Dick, 1990).

The ionic strength was changed using KH_2PO_4 to avoid changes in soil pH and also because ES is often co-granulated with P fertilisers. The effect of ionic strength was assessed for conditions relevant for granular fertilisers, at 0.1–0.7 M KH_2PO_4 in soil solution, which corresponded to the range of phosphorus concentrations in the soil solution around a diammonium phosphate (DAP) granule (circa 38 mg) at distances of 0–4 mm, 4–10 mm and 10–15 mm after one day incubation, as determined in a preliminary experiment (data not shown). To obtain treatments with 0 (E1 or control), 0.1 (E2), 0.4 (E3), or 0.7 (E4) M KH_2PO_4, 21 ml of KH_2PO_4 solution with concentrations of 0, 14, 54 or 95 g l^{-1} was added to four aliquots of 200 g soil, respectively, which were thoroughly mixed. The soil of each treatment was divided into 4 replicates and amended with 200 mg ES kg^{-1} air-dry soil.

Following the addition of ES, the soil from each replicate was evenly distributed into 4 plastic containers for destructively sampling at weeks 2, 4, 8 and 14. The soil was incubated at 25 ℃ and 70% field capacity, and soil water content was adjusted on a weekly basis.

2.2 Effect of acidification at two different elemental sulphur rates (experiment 2)

Two other soils from Victoria (VIC) and New South Wales (NSW) were used for this experiment (Table 1). This experiment was conducted to verify the effect of acidification on bacterial abundance and ES oxidation at different application rates (200 or 1000 mg kg^{-1}).

<Table 1>

Before incubation, 10 and 7.5 ml of HNO_3 (1 M) were applied to 250 g of each soil to bring the soil pH within the range of 4–5, as determined in a preliminary experiment. The soils were then leached with artificial rain water (see section 2.1). The soil without acid amendment was leached in the same way. The pH was determined in water using air-dry soil. The pH in the control soil remained the same as that prior to leaching (Table 1), and the pH in the acidified treatments was 4.4 and 4.7 for VIC soil and NSW soil, respectively. Elemental S was mixed through the soils at two rates (200 and 1000 mg ES kg^{-1} air-dry soil). Water was added to the soils to reach 70% field capacity and the samples were incubated at 25 ℃ for 2, 4 and 6 weeks.

2.3 Sample analysis

2.3.1 Analyses of pH, ionic strength and remaining elemental sulphur

All samples were subject to analysis of pH, electrical conductivity (EC, as indicator of ionic strength) and concentrations of ES. Soil pH and EC were determined at a soil:water ratio of 1:5 kg l^{-1}. Elemental S analysis was carried out by extracting 2.5 g of fresh soil sample with 5 ml of chloroform. Two ml of water were also added to effectively disperse soil aggregates. The samples were shaken for 2 h before centrifugation at 4600 g for 20 min. Clear chloroform supernatant was transferred to a glass vial and diluted with methanol for analysis by high performance liquid chromatography (Agilent, USA) (see more details in Zhao et al., 2015).

2.3.2 Analyses of soxB and 16S rRNA gene abundances

Soil DNA was extracted from soil samples incubated for 2, 4 and 14 weeks in experiment 1 or for 2, 4, 6 weeks in experiment 2, for quantification of *soxB*, 16S rRNA and 18S ribosomal ribonucleic acid (18S rRNA) gene abundances. The abundances of *soxB* and 16S rRNA genes were used as indicators of the population size of S-oxidising bacteria and total bacteria, respectively. Quantitative polymerase chain reaction (qPCR) analysis was performed on a Strategene Mxpro 3000 system. *soxB* gene abundance was determined using *soxB* 432F/693B primers (Petri et al., 2001). The reactions consisted of 1 × iQTM SYBR® Green supermix (Bio-Rad, USA), 600 nM of each primer, and 2 µl of DNA templates. Quantitative PCR was run at 95 ℃ for 15 min, and 40 cycles at 95 ℃ for 40 s, 54 ℃ for 40 s, and 72 ℃ for 40 s. A melting curve was generated between 60 ℃ to 95 ℃ with readings every 0.5 ℃. The abundance of 16S rRNA genes was determined according to a published protocol (Gupta et al., 2014). The abundance of 18S rRNA genes was quantified according to Prevost-Boure et al. (2011).

2.3.3 Terminal Restriction Fragment Length Polymorphism analysis of total bacteria

Terminal restriction fragment length polymorphism (TRFLP) was performed on the samples from weeks 2 and 14 of the first experiment to assess the shift in bacterial community composition in acidified soils and soils with increased ionic strength. The analysis of TRFLP was conducted with 16S primers: 8F-FAM (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R-HEX (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989). The polymerase chain reaction (PCR) was run in duplicate. Single PCR reactions contained 1 × PCR buffer, 0.4 mM $MgCl_2$, 1 unit of HotStarTaq DNA polymerase (Qiagen, Australia), 400 nM of each primer, 0.2 mM dNTPs (Promega, Australia), and 5 µl of DNA templates. The PCR conditions consisted of enzyme activation at 95 ℃ for 15 min, 35 cycles of denaturation at 95 ℃ for 30 s,

primer annealing at 55 ℃ for 1 min and elongation at 72 ℃ for 2 min, and final extension at 72 ℃ for 10 min. The size of PCR products was examined by gel electrophoresis on a gel (0.8% w/v) stained with GelRed Nucleic Acid Stain (Biotium, Australia), and visualised by transilluminator (Bio-Rad, USA). Amplicons from duplicates were mixed and cleaned with a MiniElute 96 UF PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The concentrations of purified DNA were determined using a Quan-iT PicoGreen^{TM} dsDNA Assay Kit (Invitrogen, USA) on a Stategen Mxpro 3000. Approximately 150 ng of PCR products were digested with restriction enzymes *Alu1* and *Cfo1* (Promega) for 3 hr at 37 ℃, followed by 20 min denaturation at 65 ℃. The digested PCR products were analysed for sizes at the Australian Genome Research Facility (Adelaide, S. Aust.) using capillary separation on an ABI 3730 DNA analyser with a LIZ500 standard.

2.4 Data analysis

ANOVA analysis was performed with SPSS (IBM, version 20) to determine the differences in gene abundance, %ES oxidised and Shannon index (H') between treatments and over incubation times.

The TRFLP fragments were analysed using GeneMarker software (SoftGenetics Inc., State College, PA, USA) to obtain band intensity (peak height) data. Bands with intensity < 100 were excluded to distinguish restriction fragments from background noise. The band intensities (heights) were imported to Microsoft Excel 2010 for the calculation of relative intensity which was expressed as the intensity of each terminal restriction fragment (TRF) divided by the total intensity of TRFs in the profile. Bands with relative intensities ≥ 0.5% were included for diversity index calculation and multivariate statistical analysis using the Primer 6 software package (Primer-E Ltd, Plymouth, UK).The Shannon index (H') (Shannon and Weaver, 2002)

was calculated to estimate bacterial diversity. Similarity in bacterial community structure was analysed using the Bray-Curtis algorithm with a dummy variable (+d) based on square root-transformed abundance data. Permutation-based Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) was performed to test if acidification or increases in ionic strength or incubation time had any effect on bacterial community composition as estimated by the square root of components of variation ($\sqrt{CV}$). Dissimilarities in the abundances of individual TRFs between treated soil and control, and between two incubation durations were determined using SIMPER analysis based on the pair-wise comparisons (Warwick et al., 1990). Clusters of bacterial TRFs were generated to determine the similarity level at which TRF profiles in all the replicates were similar. Multivariate distances between samples were visualised by generating non-metric multidimensional scaling (nMDS) plots and points were grouped based on the similarity levels from cluster analysis. Linkage between bacterial community structure and environmental variables were examined by BIO-ENV test (Clarke and Ainsworth, 1993).

3 Results

3.1 Elemental sulphur oxidation and alterations in pH and EC

For the acidification series, the pH prior to incubation ranged from 6.7 to 4.4 (treatments p1, p2, p3, p4). These differences in pH did not have any significant effect on the oxidation of ES (Fig. 1A). Soil EC increased significantly over the incubation period, but was ≤ 0.28 mS cm^{-1} in all treatments by week 14 (Table 2).

In the soils with increased ionic strength (indicated by EC, treatments E1, E2, E3, E4), there was also no significant treatment effect on ES oxidation, with about 50% oxidised at week 2 and 95% at week 14 (Fig. 1B). The EC strongly increased in time for the control soil (E1) due

to the ES oxidation, but showed relatively less change in the amended soils (E2–E4) because of the higher starting values (Table 2). Compared to the initial pH, the soil pH dropped by 1.2 units in the control soil (E1) after 14 weeks incubation, but only by 0.3–0.6 units in the KH_2PO_4-amended soils (E2–E4) due to the additional buffering by phosphate (Table 2).

<Table 2 and Fig. 1>

3.2 Abundances of soxB, 16S rRNA and 18S rRNA genes

The abundance of *soxB* genes was significantly decreased in soils acidified with HNO_3 (p2 – p4) as compared to the control (p1) (Fig. 2A). In acidified soils (p2 – p4), *soxB* gene abundance showed a significant increase between weeks 2 and 4, followed by a slight decrease at week 14 in treatments p2 and p4 (Fig. S1A). For the 16S rRNA gene abundance, there was a significant increase in p2 treatment compared to the control (p1) at weeks 2 and 4, while the abundance was significantly decreased in the more acidified soils (p3, p4) compared to the control (p1) over the incubation (Fig. 2B).

An increase in ionic strength also significantly reduced *soxB* gene abundances (Fig. 2C) but the effects were smaller than those of acidification. Over time, the *soxB* gene copy numbers showed a significant increase at week 4, followed by a significant decrease at week 14 for all treatments (Fig. S1C). Increased ionic strength and incubation time had no or minor effect on 16S rRNA gene abundance (Fig. 2D and S1D).

<Fig. 2>

3.3 Bacterial community composition

The Shannon index (H') indicated that acidification significantly decreased bacterial TRF diversity in all treatments ($P \leq 0.05$) (Table 3). Over time, the bacterial TRF diversity significantly increased in the control (p1) and p3 treatment, but was unchanged in the other treatments (p2, p4) (Table 3). PERMANOVA analysis showed that acidification and the incubation time significantly altered the bacterial community structure (PERMANOVA $\sqrt{CV}$ = 36.7, P = 0.001 for acidification, and $\sqrt{CV}$ = 15.0, P = 0.001 for the incubation time). Furthermore, the interaction between acidification and time of incubation also showed significant effects on bacterial community composition (PERMANOVA $\sqrt{CV}$ = 27.5, P = 0.001). A similarity level of 50% was determined (Fig. S2 A) to partition TRFs profiles on nMDS plots (Fig. 3A), which showed that the shifts in bacterial community composition caused by acidification were evident between treatments (Fig. 3A); and time of incubation showed clearly separation in the most acidified treatment (p4) but not in other treatments (Fig. 3A). As observed from the SIMPER test, TRFs compositions were changed between treatments and over time of incubation. For example, TRFs no. 44, 136, 166 and 64 were the main contributors in the control (p1) at week 2, which however did not appear in the most acidified treatment (p4), and new TRFs no. 63, 259, 173 and 130 etc. were observed in this treatment. BIO-ENV analysis showed a close link between the shifts in bacterial community composition and soil variables, with pH and EC the main contributors (BIO-ENV test ρ = 0.571; P = 0.01).

In the soils with increased ionic strength, bacterial TRF diversity was significantly decreased compared to the control (E1), but the adverse effect was weaker at week 14 than week 2 (Table 3). Incubation significantly increased TRF diversity in the control (E1) and E3 treatment over time (Table 3). PERMANOVA analysis indicated that increases in ionic strength significantly altered the bacterial community structure (PERMANOVA $\sqrt{CV}$ = 28.7, P = 0.001). The

incubation also resulted in significant changes over time in the bacterial community composition (PERMANOVA $\sqrt{CV}$ = 22.8, P = 0.001). Furthermore, the interaction between increases in ionic strength and time of incubation also showed significant effect on bacterial community composition (PERMANOVA $\sqrt{CV}$ = 25.0, P = 0.001). A similarity level of 60% was determined (Fig. S2B) to group TRF profiles on nMDS plots which showed clear separation between different treatments or incubation times (Fig. 3B). The SIMPER test showed that TRFs compositions were changed between treatments and across incubation. For example, TRFs no. 171, 187, 186 and 67 contributed circa 20% to TRFs abundance in the control (E1) at week 2, the former three of which however did not appear in the treatment with the highest ionic strength (E4), and new TRFs no. 93, 292, 317 and 234 etc. were observed in the E4 treatment. A close link was observed between the shifts in bacterial community composition and soil variables, i.e. pH and EC (BIO-ENV test ρ = 0.562; P = 0.01).

<Table 3 and Fig. 3>

Overall, acidification and increased ionic strength resulted in significant decreases in (total and S-oxidising) bacterial abundances as well as in shifts in bacterial community composition, but did not affect ES oxidation. These results suggest that ES oxidation is independent of changes in bacterial population in soil. These results were unexpected given the results of our previous study, in which we found a positive relationship between ES oxidation and soil pH (Zhao et al., 2015) or bacterial abundance (Zhao et al., Submitted). This different finding might be related to the fact that this study assessed the effect of pH induced by short-term acidification of a single soil, whereas our previous study assessed the effect of pH across soils, but might also be related to the different ES rate used (1000 mg kg^{-1} in the previous study).

3.4 Effect of acidification on elemental sulphur oxidation at two different elemental sulphur rates (experiment 2)

3.4.1 Elemental sulphur oxidation

In the loamy sand (VIC), ES oxidation at week 6 was slightly decreased at the high ES rate (1000 mg kg^{-1}) and in the acidified soil compared to the non-acidified soil at the low ES rate (200 mg kg^{-1}) (Fig. 4A). In the sandy loam soil (NSW), only the acidified soil at the high ES rate had a small but significantly lower oxidation rate (Fig. 4A). Overall, the effects of ES rate or acidification were small, with 58–59 and 53–57 % of ES oxidised for the VIC soil and NSW soil, respectively, after 4 weeks incubation.

<Fig. 4>

3.4.2 Abundances of soxB and 16S rRNA genes

For the VIC soil, both *soxB* and 16S rRNA gene abundances were significantly reduced in the acidified soils at all sampling times (Fig. 4B, C). There were no significant effects of ES application rate on *soxB* or 16S rRNA gene abundance, except for a small decrease in *soxB* gene abundance in the non-acidified treatment at week 6 (Fig. 4B, C). The abundances of *soxB* and 16S rRNA genes showed different trends in NSW soil, but the acidified soil at the high ES rate tended to have the lowest gene abundances (Fig. 4B, C).

While the acidification treatment had significant effects on the ES oxidation at both rates, the effects were small and overall the findings of the first experiment were confirmed. There were minor effects of pH on ES oxidation and on the relationship between bacterial abundance (affected by acidification) and ES oxidation. For instance, in the VIC soil, there was no difference in ES oxidation between treatments at week 4 (with over 50% of ES oxidised) even

though the bacterial (*soxB* and 16S rRNA genes) abundance was significantly lower in the acidified soils. This was the case at both ES rates, indicating that the observation in the preceding experiment was not due to an effect of ES rate.

4 Discussion

Elemental S oxidation in soil has been reported to be related to soil pH and the abundance and diversity of bacterial oxidisers (Li and Caldwell, 1966; Nor and Tabatabai, 1977; Janzen and Bettany, 1987; Lawrence and Germida, 1988; Tourna et al., 2014; Zhao et al., 2015). In our previous study, we found that pH and bacterial abundance explained 80% of the variation in ES oxidation rate across soils (Zhao et al., submitted). In the present study, we modified soil pH to different levels reflecting the pH changes around a fertiliser granule containing ES; however, we did not find any relationship between pH and bacterial abundance on the one hand, and ES oxidation rate on the other. We would like to reconcile the different results between the present and previous studies by considering the different experimental designs. The literature studies including our previous study used different soils with different pH values, where pH is related to many other soil physico-chemical properties and vegetation differences. In the present study, however, we used one soil that was modified to different pH and ionic strength levels and these modifications took place one week before or at the commencement of incubation (see section 2.1). According to Zhang et al. (2015), soil acidification over 10 years only affects bacterial diversity through a mechanism of ecological filtering, i.e. changes in bacterial community structure are due to a reduction in bacterial richness, rather than the mechanisms of evolution or dispersal which induce new species or dispersion of species that are adapted to the acid environment over the tens of millions of years. Thus, acidification in the present study is expected to have a weaker effect on bacterial diversity and richness, in which the soil had only been acidified for 15 weeks, compared to soils with a longer-term acidification. However, our

study revealed that short-term acidification indeed significantly reduced bacterial abundance and shifted bacterial community composition, but these changes did not affect the soil's capacity to oxidise ES.

The lack of agreement between ES oxidation and bacterial population abundance could also be attributed to the fact that ES oxidation is not limited by the abundance of the microbial population in soil, except at very elevated application rates of ES. In a short-term (6 days) incubation experiment using Canadian soils, Janzen and Bettany (1987) reported that the weight-based ES oxidation was linearly correlated with the application rate (which ranged from 0–4000 mg ES kg^{-1}) – in other words, the relative oxidation rate (% oxidised or the surface-based oxidation rate) was independent of ES application rate. They concluded that the microbial population abundance was not a limiting factor for ES oxidation in soil. Similar results have also been observed over long time incubations (up to 60 days) in many other studies (Li and Caldwell, 1966; Nor and Tabatabai, 1977; Lettl et al., 1981; Janzen and Bettany, 1987). We also found that ES application rate had little or no effect on ES oxidation rate within a soil (Fig. 4). However, the abundance of S-oxidising bacteria varies greatly across different soils as indicated by our previous study (Zhao et al., submitted). Petersen et al. (2012) demonstrated that the abundance of a functional gene can predict the potential rate of a biochemical process because it has integrated long-term fluctuations in the physico-chemical environment. Thus, oxidation rate of ES may significantly correlate with *soxB* gene abundance across soils, even though the oxidation rate for a given soil may not be affected by changes in the ES rate or by changes in the bacterial numbers brought about by temporary changes in chemical properties of soil (e.g. acidification).

Alternatively, the lack of relationship between the oxidation rate and *soxB* gene abundance in this study might also be related to the DNA fingerprinting methods used in this study, which determines the total bacterial population, and not just the active population related to a real-time process (Rousk et al., 2009; Rousk et al., 2010; Petersen et al., 2012). In addition, *soxB* gene is only one of many genes regulating S oxidation in a sox pathway that is one of the three proposed pathways of S oxidation, with many genes involved in bacterial oxidisers (Ghosh and Dam, 2009). Even though the *soxB* gene regulates S oxidation by catalysing thiosulphate oxidation, it may not be the rate limiting step in the S oxidation pathway, explaining why ES oxidation showed no relationship with *soxB* gene abundance in this study.

The occurrence of additional functional activities of S-oxidising microorganisms in soil may be another reason for the lack of correspondence between *soxB* gene abundance and ES oxidation in our study. In soil, inorganic S compounds e.g. thiosulphate may not only originate from ES oxidation, but also from mineralisation of organic matter. The number of *Thiobacilli* has been reported to increase over incubation in soils even without ES amendment (Lee et al., 1987; Lee et al., 1988), indicating the supply of reduced inorganic S compounds as a result of mineralisation of organic matter (Starkey, 1966). Therefore, S-oxidising bacteria in soil are not only involved in ES oxidation but also other biochemical processes, e.g. mineralisation of organic S.

The present study not only showed a change in gene abundance, but also a significant shift in total bacterial community composition due to increases in acidity or ionic strength, and time of incubation. The BIO-ENV test showed that pH and EC were the main contributors to shifts in bacterial community composition. Regardless of changes in bacterial community composition, these shifts did not affect ES oxidation. Changes in microbial community composition do not

necessarily change the rate of biological processes. For instance, Marschner et al. (2003) investigated the effect of long-term application of organic and inorganic fertilisers and found changes in bacterial community composition, but not in enzyme activities including urease, xylanase, alkaline phosphatase and arylsulfatase. The fact that ES oxidation rate was not affected, despite the shift in community composition, may be related to the wide distribution of S-oxidising bacteria in terms of physiology and taxonomy in soil (Ghosh and Roy, 2006; Anandham et al., 2008; Tourna et al., 2014; Xia et al., 2014; Zhao et al., submitted). Therefore, it is likely that the role of some S-oxidising bacteria at one pH/ionic strength level had been fulfilled by other S-oxidising bacteria adapted to another pH/ionic strength level.

It is possible that fungi contributed more to ES oxidation in the most acidified soil (p4) than in other treatments, because 18S rRNA gene abundance was always the highest in the p4 treatment, with no difference between other treatments (p1, p2 and p3) (Fig. S3A). However, this could not be the case for ES oxidation in soil amended to different ionic strengths as 18S rRNA gene abundance did not differ between treatments at week 14 (Fig. S3B).

The point at which the acidity or ionic strength will start to disturb the functional capacity of indigenous bacteria and consequently ES oxidation rate, likely depends on soil type and incubation time. In our second experiment, we used two soils which showed different responses to acidification and ES application rate (Fig. 4). While the effect of acidification on ES oxidation was small compared to the effect on bacterial abundances, we observed some decreases in ES oxidation rate, which suggests that ES oxidation could be affected when S-oxidisers in soil are supressed, for example, to the point at which their abundance is not sufficient to fully colonise ES particles. This agrees with the findings that the decrease in ES oxidation was concomitant with a decrease in specific members of bacterial community such as

Thiobacilli or *Thiobacillus*-like oxidisers in soil (Lee et al., 1987; Yang et al., 2010; Tourna et al., 2014).

As a conclusion, in the present study, increased acidity and ionic strength significantly decreased S-oxidising and total bacterial abundances, and shifted total bacterial community composition, but had no or little effect on ES oxidation as measured in an incubation assay. Furthermore, the fraction of ES oxidised over a given time was independent of the ES application rate. These results suggest that ES oxidation within a soil is independent of bacterial abundance and community structure brought about by temporary increases in soil acidity and ionic strength. The broad range of physiological and taxonomic groups of S-oxidising bacteria in soil may contribute to the consistent ES oxidation at different pH/ionic strength levels. Therefore, chemical changes around a fertiliser granule resulting from ES oxidation or from dissolution of soluble macronutrients may not have any effect on ES oxidation. The results in this study also suggest that even though *soxB* genes have been used as a molecular marker for the presence of S-oxidising bacteria, our study indicates that the abundance of *soxB* genes in response to short-term chemical changes does not necessarily correlate with the rate of ES oxidation in soil.

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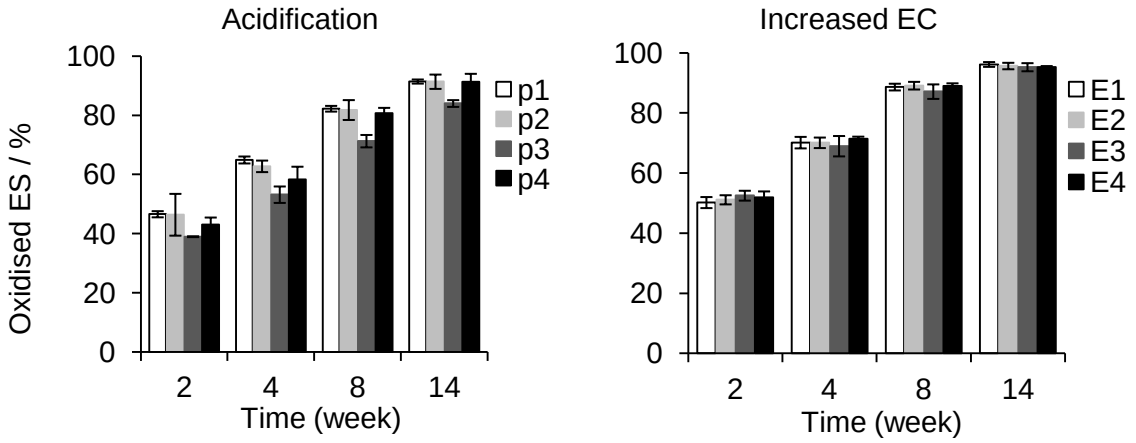

Fig. 1. Effects of treatment on the percentage ES oxidised in soils modified for (A) pH (treated with HNO_3 – p1, p2, p3, p4) or for (B) ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity/ionic strength. There was no significant difference at $P \leq 0.05$ in the percentage of ES oxidised between treatments within any sampling time.

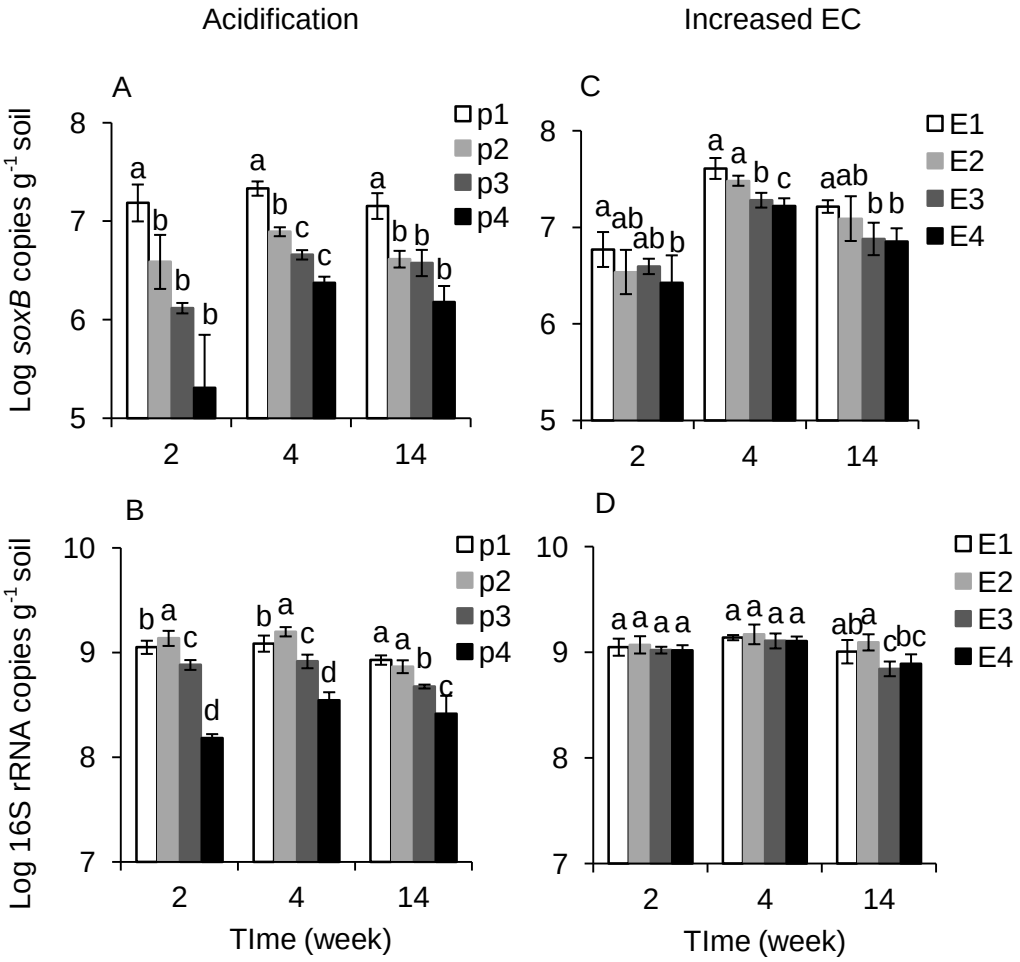

Fig. 2. Effects of treatment on the abundances of *soxB* and 16S rRNA genes in soils modified for (A, B) pH (treated with HNO_3 – p1, p2, p3, p4) or (C, D) ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or ionic strength. Gene abundances were calculated based on air-dry soil weight. Different letters indicate significant difference between treatments within each sampling time ($P \leq 0.05$).

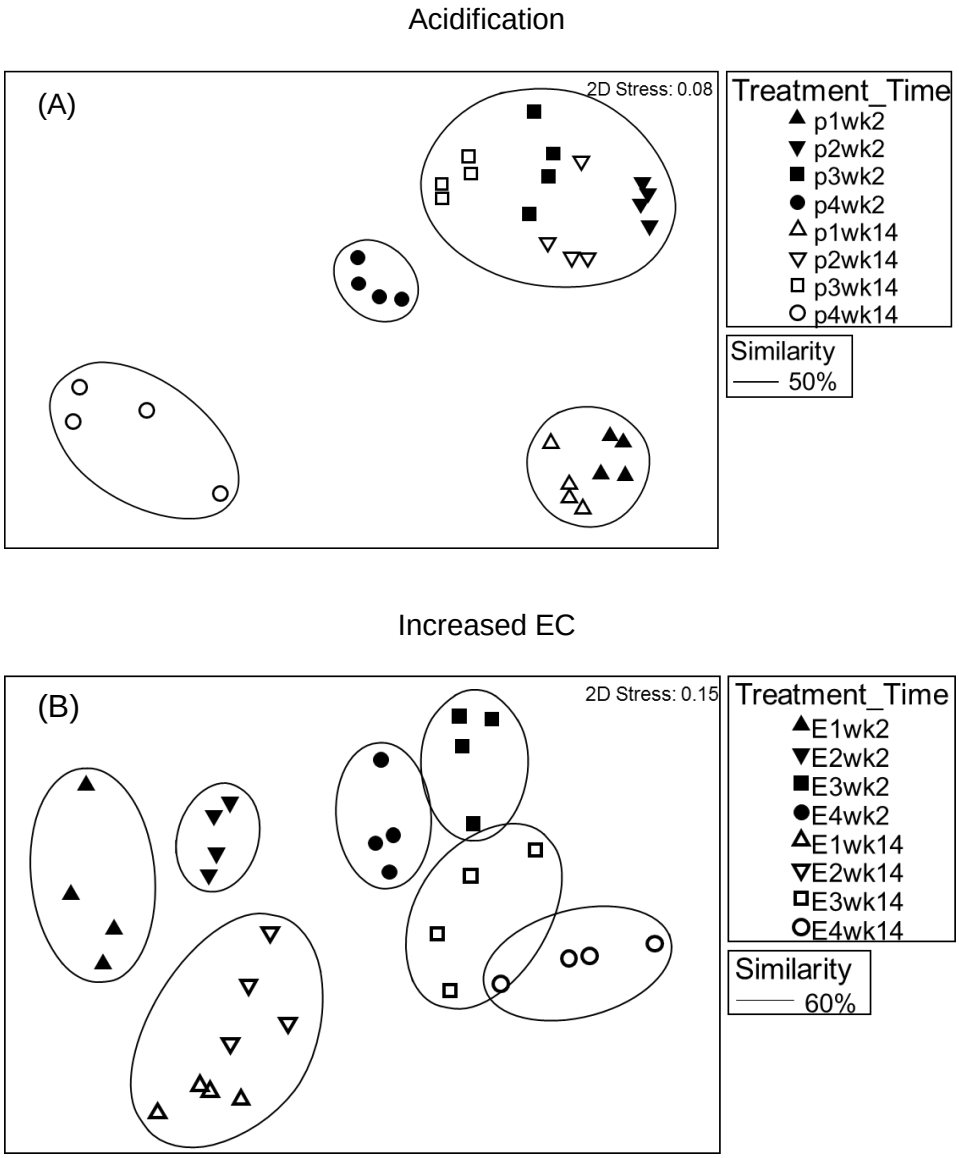

Fig. 3. nMDS ordination showing the similarities in bacterial community TRFLP profiles in soils modified for (A) pH (treated with HNO_3 – p1, p2, p3, p4) or (B) for ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or ionic strength. Points within the same circle share more than (A) 50% or (B) 60% similarities, and increasing distance between points reflects increasing dissimilarities in bacterial community structure.

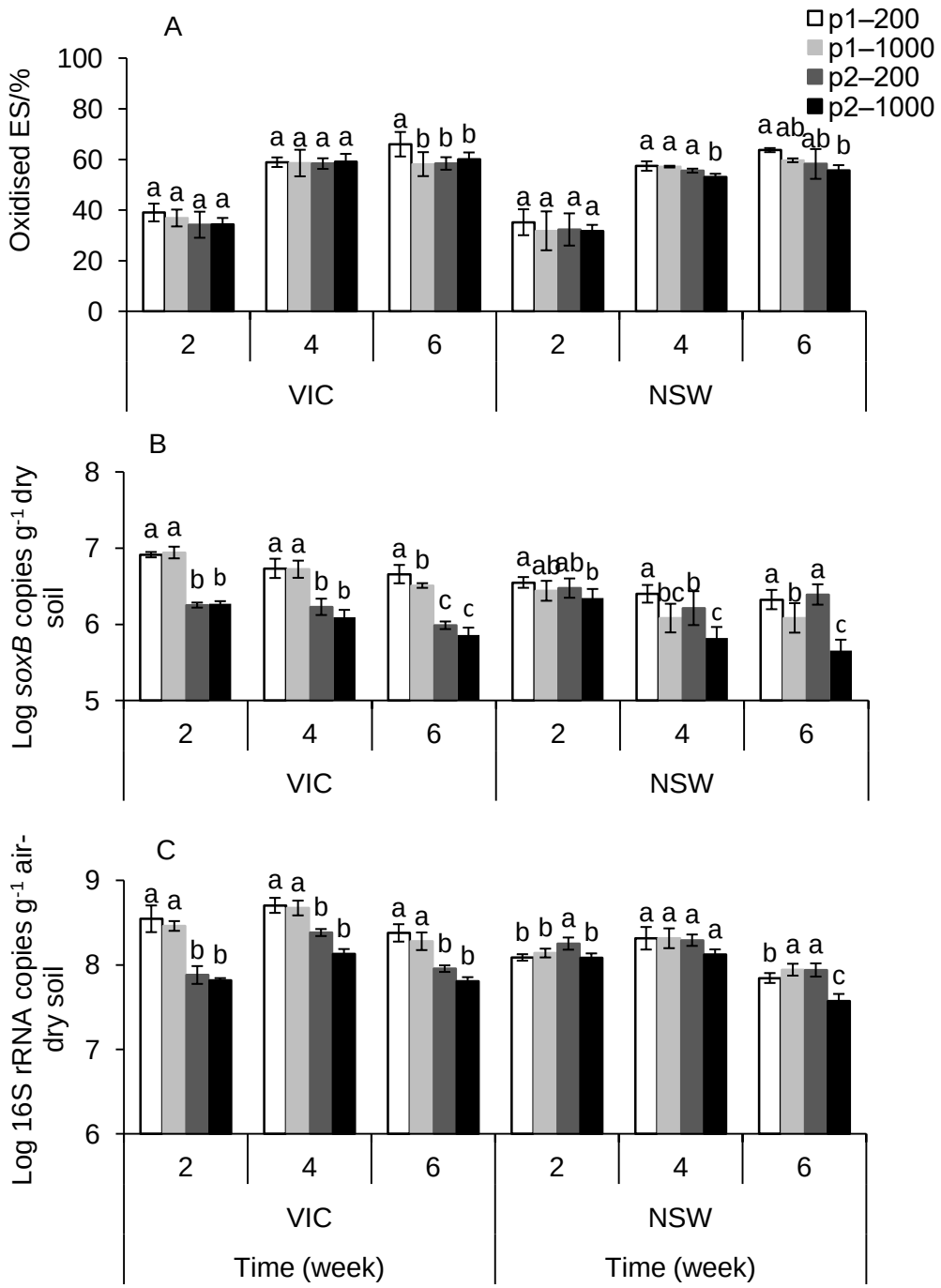

Fig. 4. Effects of ES rate in the incubation assay on (A) the percentage of ES oxidised, (B) *soxB* gene, and (C) 16S rRNA gene abundances in soils incubated with 200 or 1000 mg ES kg^{-1} air-dry soil. p1 is soil without acidification, and p2 is acidified treatment; 200 and 1000 indicate the ES rate (mg ES kg^{-1}). Gene abundances were calculated based on air-dry soil weight. Different letters indicate significant difference between treatments within each sampling time ($P \leq 0.05$).

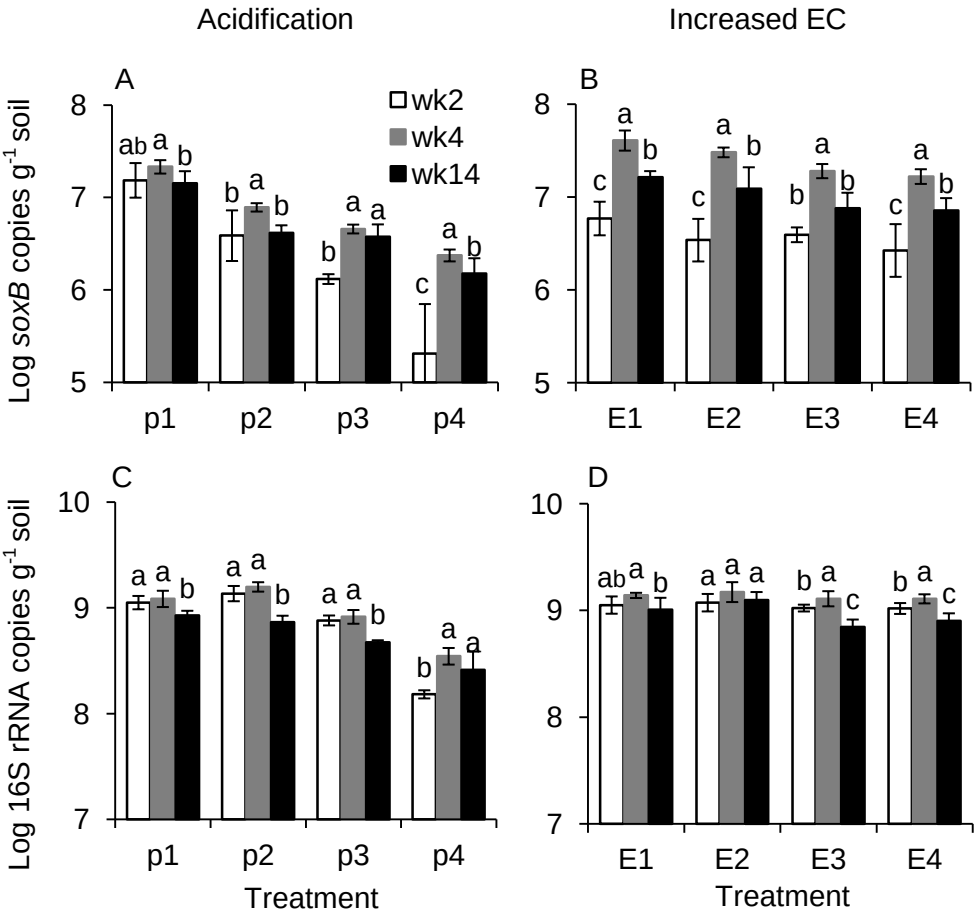

Fig. S1. Effect of incubation time on the abundances of *soxB* genes (A, C) and 16S rRNA (B, D) genes in soils modified for pH (treated with HNO_3 – p1, p2, p3, p4) or for ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or EC. Gene abundances were calculated based on air-dry soil weight. Different letters indicate significant difference between treatments within one sampling time ($P \leq 0.05$).

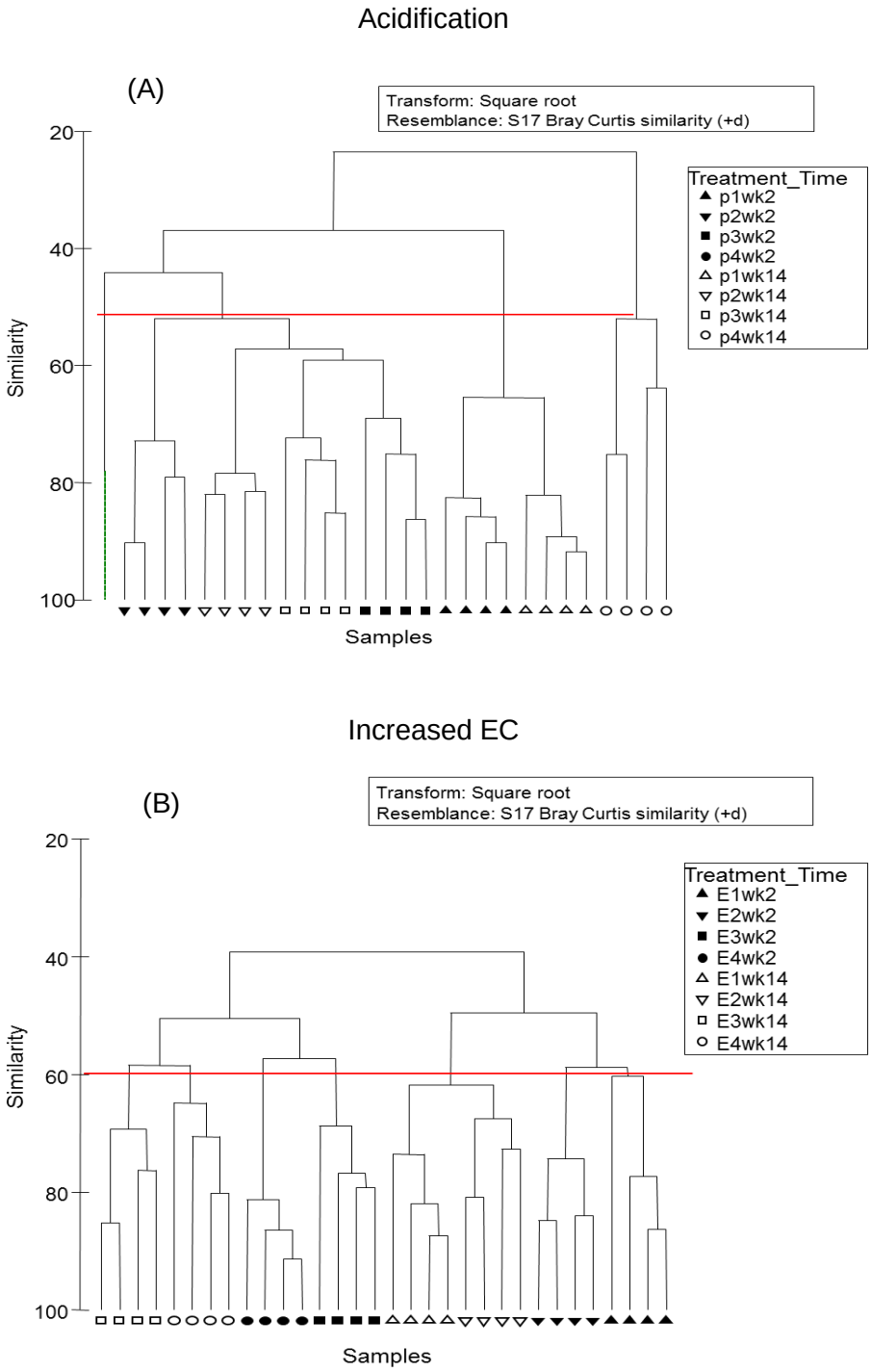

Fig. S2. Cluster analysis of bacterial community TRFs based on Bray-Curtis similarity in soils modified for (A) pH (treated with HNO_3) or (B) ionic strength (treated with KH_2PO_4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or EC. The horizontal line indicates that the TRFs profiles in all the replicates are similar under this level.

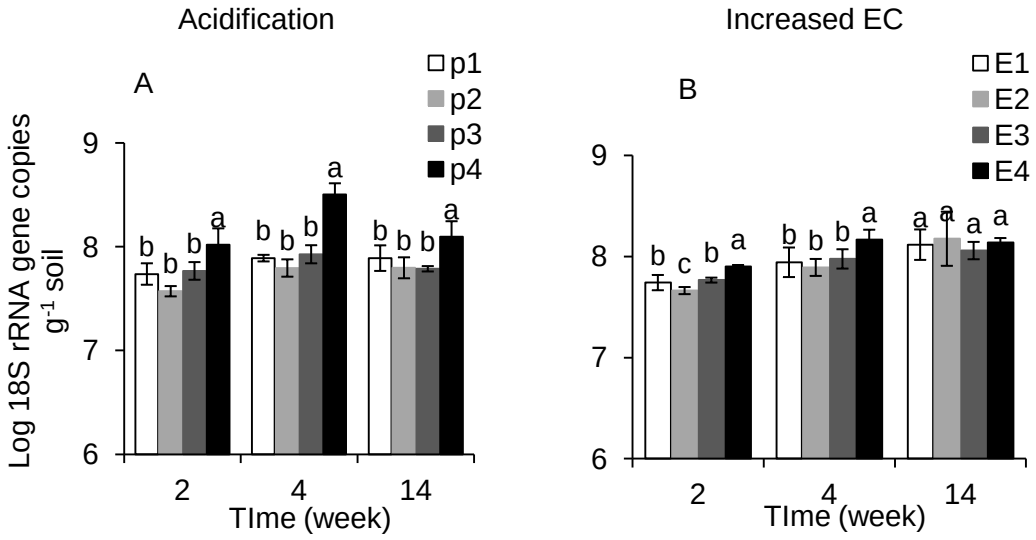

Fig. S3. Effect of treatment on the abundances of 18S rRNA genes in soils modified for (A) pH (treated with HNO_3 – p1, p2, p3, p4) or (B) ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control soil; p2/E2 – p4/E4 are soils with increasing acidity or EC. Gene abundances were calculated based on air-dry soil weight. Different letters indicate significant difference between treatments within one sample time ($P \leq 0.05$).

Table 1 Physico-chemical properties of soils.

Soil[a]	Texture	CEC[b]	pH	EC	TC[c]	TN[d]	$CaCO_3$	OC[e]	Sulphate-S	TS[f]
		cmol (+) kg^{-1}	water	mS cm^{-1}	----------%				-----mg kg^{-1}----	
SA	Sandy	7	6.7	0.06	2.0	0.19	< 0.2	2.0	4.6	205
VIC	Loamy sand	9	5.9	0.04	2.2	0.20	< 0.2	2.2	5.8	224
NSW	Sandy loam	8	6.0	0.08	1.4	0.12	< 0.2	1.4	13.2	128

[a] SA = South Australia, VIC = Victoria, NSW = New South Wales.

[b] cation exchange capacity;

[c] total carbon;

[d] total nitrogen;

[e] organic carbon;

[f] total sulphur.

Table 2 pH and EC in acidified soils (p1, p2, p3, p4), and in soils with increasing ionic strength indicated by EC (E1, E2, E3, E4) over the incubation period. p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or ionic strength.

Treatment		pH							EC (mS cm^{-1})					
		init.[a]	wk 2	wk 4	wk 8	wk 14	*LSD*[b]		init.	wk 2	wk 4	wk 8	wk 14	*LSD*
Acidification	p1	6.7	6.1	5.9	5.6	5.5	0.05	p1	0.06	0.11	0.17	0.24	0.28	0.02
	p2	6.1	5.8	5.7	5.5	5.5	0.06	p2	0.06	0.07	0.10	0.14	0.17	0.01
	p3	5.2	5.4	5.1	4.9	4.8	0.10	p3	0.06	0.05	0.08	0.13	0.16	0.01
	p4	4.4	4.5	4.4	4.3	4.3	0.07	p4	0.06	0.06	0.10	0.13	0.15	0.02
	LSD	n.a.[c]	0.09	0.06	0.06	0.08		*LSD*	n.a.	0.00	0.01	0.02	0.03	
Increased EC	E1	6.7	6.2	5.9	5.6	5.5	0.06	E1	0.06	0.13	0.19	0.27	0.29	0.04
	E2	6.7	6.5	6.4	6.2	6.1	0.09	E2	0.16	0.19	0.22	0.28	0.30	0.04
	E3	6.7	6.4	6.5	6.5	6.4	0.14	E3	0.46	0.58	0.60	0.66	0.66	0.04
	E4	6.7	6.2	6.3	6.3	6.3	0.13	E4	0.76	0.93	0.95	0.99	0.99	0.03
	LSD	n.a.	0.09	0.02	n.s.[d]	0.02		*LSD*	n.a.	0.03	0.02	0.02	0.02	

[a] initial values;

[b] Least square difference, with $P \leq 0.05$, which was estimated with pH/EC values at four sampling occasions;

[c] not available;

[d] not significant.

Table 3 Shannon index (H') of bacterial TRF diversity in soils modified for pH (treated with HNO_3 – p1, p2, p3, p4) or ionic strength (treated with KH_2PO_4 – E1, E2, E3, E4). p1/E1 is control treatment, and p2/E2 – p4/E4 are treatments with increasing acidity or ionic strength.

	Acidification		Increased EC	
week 2	p1	3.77a	E1	3.56a
	p2	3.15b	E2	3.49b
	p3	3.32b	E3	3.27d
	p4	3.27b	E4	3.37c
week 14	p1	3.93a*	E1	3.61a*
	p2	3.52b	E2	3.51ab
	p3	3.52b*	E3	3.57ab**
	p4	3.18c	E4	3.44b

Different letters within a row indicate significant difference between treatments within one sampling time ($P \leq 0.05$);

Asterisks indicate significant differences between week 2 and week 14 (* indicates $P \leq 0.05$ and ** $P \leq 0.01$).

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CHAPTER 6

Oxidation of elemental sulphur in co-granulated fertiliser

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Author Contributions

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CHAPTER 7

Summary, conclusions and suggestions for future research

1 Summary

Elemental sulphur (ES) is of great interest as a S fertiliser source to alleviate soil S deficiency. It is the most concentrated S form and therefore most cost-effective for transport, storage and application. Furthermore, ES is less susceptible to leaching losses than sulphate-S sources. However, ES only becomes available for plants after it is oxidised to sulphate. Elemental S oxidation is mediated by soil microorganisms, and therefore the population and diversity of S-oxidising microorganisms in soil may be of great importance for the effectiveness of ES fertiliser. Efforts have been made to study the role of soil microorganisms in ES oxidation in soil mostly through culture-dependent methods. However, these culture-based methods only provide information on less than 10% of the total microbial community in soil, whereas DNA finger printing methods allow the determination of all bacterial communities (Tiedje et al., 1999). In addition, in agronomy, ES is usually applied in granular form for safe handling, and the oxidation of (co-)granulated ES has been found to be much slower than that of powdered ES mixed through soil. Moreover, ES fertilisers perform variably depending on soil types, formulations, application methods, etc. To better understand the limiting factors for ES oxidation in soil, this work sought to 1) gain a better understanding of the role of the abundance and diversity of S-oxidising bacteria in ES oxidation in soil; and 2) determine the causes of the slower oxidation of granular ES compared to powdered ES.

To address the first objective, an initial study was carried out with ten Australian cropping soils covering a wide range of physico-chemical properties, to investigate the relationship between soil physico-chemical properties and the abundance of a bacterial functional gene involved in S oxidation on the one hand, and the oxidation rate of ES on the other. In a subsequent study, the effect of an increase in soil acidity or ionic strength on bacterial abundance and community

composition and ES oxidation was examined. A final study was conducted to determine the limiting factors in the oxidation of co-granulated ES.

1 Elemental sulphur oxidation in Australian cropping soil (Chapter 3)

The oxidation rate of ES ranged from 5.1 to 51.7 μg cm^{-2} d^{-1} in the ten Australian cropping soils. A significant correlation was observed between ES oxidation rate and soil pH (R^2 = 0.54, P < 0.05). A regression equation including both soil pH and organic C explained even more of the variation in oxidation rate (R^2 = 0.79, P < 0.05). These findings are in good agreement with previous studies that also found positive relationships of ES oxidation with pH and/or organic matter content (e.g. Dick and Deng, 1991; Janzen and Bettany, 1987; Lawrence and Germida, 1988). These positive correlations can be explained by the effect of physico-chemical properties on microbial size and activity and hence on ES oxidation. Soil pH is one of the most important properties affecting the abundance and diversity of soil bacteria (Nicol et al., 2008; Rousk et al., 2010), and the availability of soil C has been reported to limit the size and activity of microbial biomass in soil (Gonzalez-Quiñones et al., 2011).

Elemental S oxidation estimated based on sulphate production was significantly lower than that estimated based on ES disappearance, which was likely related to sulphate immobilisation and gypsum precipitation in the soils. We applied the regression equation with pH and organic C as independent variables to predict ES oxidation from literature studies. It was found that predicted and measured values agreed reasonably well for studies that determined oxidation rate based ES measurements, but that predicted ES oxidation rates were often larger than measured ones when sulphate was measured to assess ES oxidation, particularly when short incubation times were used. It was hypothesised that ES oxidation was underestimated in these studies due

to immobilisation of sulphate. This finding highlights the importance of ES-based measurements for the estimation of ES oxidation in soil.

2 Abundance and diversity of sulphur-oxidising bacteria and their role in elemental sulphur oxidation in Australian cropping soils (Chapter 4)

The abundance of *soxB* (an indicator of the abundance of S-oxidising bacteria) was determined in the same ten soils. The *soxB* abundance varied greatly across soils and was positively correlated with the oxidation rate of ES (R^2 = 0.67, P < 0.05). Similarly, the abundance of 16S (an indicator of the total bacterial abundance) could explain 66% of the variation in ES oxidation rate (P < 0.05). The combination of *soxB* or 16S and soil pH could explain higher than 80% of the variation in the oxidation rate (P < 0.01). In contrast, ITS abundance was not significantly correlated with the oxidation rate. These findings indicate the important role of bacterial abundance and soil pH in ES oxidation in the soils. It was also found that S-oxidising bacteria were distributed broadly in both taxonomy and physiology in the soils, which may explain the strong correlation between the abundance of *soxB* and 16S (R^2 = 0.99, P < 0.01). While the size of the bacterial population was strongly correlated with ES oxidation in soil, no association was found between the community composition of S-oxidising bacteria and ES oxidation.

Furthermore, this chapter also demonstrated that the abundance of *soxB* was related to soil physico-chemical properties, especially soil pH and organic C content. From the correlation between organic C and *soxB* abundance, we hypothesised that ES oxidation was significantly affected by heterotrophic S-oxidising bacteria in soil, which is in line with many previous studies (e.g. Lawrence and Germida, 1988; Lee et al., 1987).

It is notable that functional gene abundance alone explained less than 70% of the variation in ES oxidation rate. Two factors may account for the unexplained variation. First, DNA-based methods allow us to identify the total community of S-oxidising bacteria, not only the active population, and hence *soxB* gene abundance in this work only represents a soil's potential to oxidise ES rather than the rate of the real-time process. A better relationship would likely be obtained between the size of active population of S-oxidising bacteria (that are specific to soil and environmental conditions) and ES oxidation rate. Second, fungi may also have contributed to the oxidation of ES as some fungal species have been found to be able to oxidise ES in soils (Czaban and Kobus, 2000; Grayston and Wainwright, 1988).

3 Effects of pH and ionic strength on elemental sulphur oxidation in soil (Chapter 5)

Increases in soil acidity or ionic strength resulted in significant shifts in the abundance of S-oxidising bacteria and total bacteria, and in overall bacterial community structure. Bacteria have a narrow optimal pH range for their growth (Rosso et al., 1995) and a decrease in growth rate by 25% in certain species can result in those species being outcompeted by other bacterial species (Rousk et al., 2010), explaining the effect of pH. Changes in ionic strength can affect bacterial adherence to solid surfaces through changing the thickness of electrical membrane layers (Tan et al., 1992; Tan et al., 1991), and affect bacterial activity and growth through modifications in osmotic pressure (Suzuki et al., 1999). However, despite the effects on bacterial abundance and community composition, increases in acidity or ionic strength did not have any effect on ES oxidation. This contrasts with the positive correlation between the ES oxidation rate and pH and bacterial gene abundance found across the ten Australian cropping soils (Chapter 4). The inconsistent effect of pH on the oxidation of ES (across soils *vs* within a soil) can be reconciled by the fact that variations in pH across soils are associated with other soil chemical and biological properties and vegetation, which is not the case for short term

acidification. This further indicates that ES oxidation within a soil is independent of short-term changes in chemical properties and bacterial population. In addition, we did not observe any link between the ITS abundance and ES oxidation in this soil, similar as the finding in our previous study.

The fact that ES oxidation was not affected by the decrease in *soxB* abundance brought about by acidification or the increase in ionic strength, suggests that the number of S-oxidising microorganisms did not limit the ES oxidation. Because gene abundance in the present study included dormant to highly active bacteria, it cannot directly reflect the real-time process of ES oxidation. Therefore, methods that can provide information on active bacterial population, e.g. RNA measurement, should be considered for future research. Also the bacterial community shifts induced by changes in pH or ionic strength did not affect the ES oxidation. Sulphur-oxidising bacteria cover a broad range of bacterial species and the role of one species might have been fulfilled by another species when chemical changes occurred.

This chapter highlights that temporary chemical changes do not affect ES oxidation under the conditions tested in this study. These findings imply that the slow oxidation of co-granulated ES is likely not related to chemical changes (induced by the oxidation process or by dissolution of macronutrient fertiliser ions) in the vicinity of the granule and this was further investigated in Chapter 6.

4 Oxidation of elemental sulphur in co-granulated fertiliser (Chapter 6)

Oxidation of ES, and bacterial abundance and community composition, were compared between a co-granulated fertiliser (diammonium phosphate (DAP) +ES (10%)) and powdered DAP+ES mixed through soil at the same rate. Application of fertiliser decreased the pH and

increased the ionic strength over time due to ES oxidation, nitrification and the dissolution of DAP. A greater pH decrease was observed for the powdered treatment than for the granular treatment, which could be attributed to more ES oxidation in the powdered treatment.

Alterations in soil pH and ionic strength due to the application of DAP+ES brought about a decrease in bacterial abundance and shifts in bacterial community composition. However, in agreement with the findings of Chapter 5, these alterations did not seem to affect ES oxidation and could not explain the difference in the oxidation rate between co-granulated and powdered ES. We found that the difference in the relative oxidation (%ES oxidised) corresponded to the difference in the surface area between the granular and the powdered ES. This suggests that ES oxidation in granules is limited by the low degree of dispersion in soil, which consequently reduces the effective surface area of the ES particles available for the colonisation by S-oxidising microorganisms. It was hypothesised that the performance of ES in granular fertiliser can be improved by increasing the contact between ES particles and the soil medium/microorganisms.

2 Conclusions

Three main conclusions can be drawn from this work, based on the findings in Chapters 3 to 6.

1. Elemental S oxidation varied across soils with different physico-chemical properties, and was significantly correlated with soil pH, organic C and the size of bacterial population. Sulphur-oxidising bacteria showed a wide distribution in taxonomy and physiology in the soils, but no relationship between bacterial community composition and ES oxidation was observed.

2. While soil pH and *soxB* abundance were significantly correlated with ES oxidation rate across soils, significant shifts in bacterial abundance and community composition brought about by temporary changes in pH or ionic strength within a soil did not alter ES oxidation. This indicates that long-term variation in chemical and biological soil properties have an effect on ES oxidation, but short-term changes do not.

3. The slower oxidation of co-granulated ES than powdered ES could not be related to changes in bacterial abundance or community composition, but corresponded to the reduction in effective surface area of ES particles available for soil microorganisms, indicating that the lower degree of ES dispersion causes the slower oxidation of co-granulated ES.

This thesis, first of all, highlights the use of functional gene abundance as an index of a soil's potential to oxidise ES across soils. However, the prediction is not perfect and soil properties (pH and organic C) allow predictions of ES oxidation with a similar degree of accuracy. Another main outcome of this work is that temporary changes in soil pH and ionic strength (brought about by ES oxidation or by dissolution of a macronutrient fertiliser carrier) do not affect the oxidation rate of the remaining ES. Furthermore, it was shown that the slower oxidation of co-granulated ES could be related to the decrease in available surface area of ES. This finding significantly contributes to the understanding of the oxidation of co-granulated ES, which is key to improve the effectiveness of granular ES purposefully.

3 Suggestions for future research

To address key issues raised in this study, and to further our knowledge on ES oxidation in soil as well as to improve ES granular fertilisers, the following suggestions for future research are made:

1. Elemental S oxidation can be well predicted by functional gene abundance under steady environmental conditions. However, DNA-based methods can only provide the potential rate of a biological process, but not the information on the dynamics of active populations of S-oxidising bacteria and their linkage to ES oxidation. Future research should include RNA or enzyme measurements in the study of ES oxidation in soil, which have been utilised in assessing other biochemical processes e.g. nitrification in the environment. In addition, being aware of different functional groups of bacteria that regulate S oxidation would help gain better insights into ES transformation from a zero-valence element to a six-valence ion, and into the biological drivers of this process as influenced by management across soils. Therefore, new primers utilising the latest *soxB* sequence dataset to cover S-oxidising bacteria from diverse taxonomic groups need to be developed to get a more complete picture of S-oxidising bacterial communities. Finally, the contribution of S-oxidising fungi was not included in this work, and it is recommend to identify the species and to quantify the abundance of S-oxidising fungi in soil, e.g. by developing primers for functional genes in S-oxidising fungi, in future research.

2. From this work, it is confirmed that the slow oxidation of ES in granular form is due to poor dispersion of ES particles in the granule, which limits the exposure of ES particles to soil microorganisms. Therefore, if faster oxidation is sought, formulation of ES fertilisers should aim to improve the dispersion of ES granules once added to soil, e.g. by decreasing granule

size, and hence increasing granule numbers at a given rate, by inoculating S-oxidisers into granules, or by encouraging the granule to disperse completely after application to soil. (Note that despite some granulated ES fertilisers being formulated with bentonite to encourage this dispersive behaviour, dispersion may not always be effective (Degryse et al., 2015; Janzen and Karamanos, 1991)). In order to keep S-oxidisers active after application of the fertiliser to soil, efforts should be made to keep environmental conditions suitable for microbial growth both inside and outside the granule.

3. This research provides information on predicting the oxidation of powdered ES mixed through soils, as was also reported by many previous studies, but the models developed for prediction of powdered ES oxidation in soil are not suitable for ES in granular fertilisers. Hence, to predict the oxidation of granular ES, a model needs to be developed by taking into consideration the granule size, ES content, nature of ES carriers (soluble or insoluble), etc.

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Appendices

Appendix 1: Modification of elemental sulphur measurement in soil

Introduction

This appendix describes the experimental work carried out to improve the measurement of ES by chloroform extraction. Determination of ES in soil was initially carried out following the method developed by Watkinson et al. (1987). Briefly, the procedures for their method were: 1) extracting ES from soil with chloroform and water; 2) separating chloroform from water by filtering; and 3) determining ES concentration using high performance liquid chromatography. Given the use of filtration for phase separation is labour-intensive and may cause volatilisation losses of chloroform, modification was made by using high-speed centrifugation (4600 g for 20 min) to separate chloroform from water. After centrifugation, a clear separation between chloroform and water was observed, with the chloroform under the water phase. With the modified separation procedure, ES recovery from soil was tested with different water:soil (W:S) ratios or different mixing techniques. A chloroform:soil ratio of 2/1 l kg^{-1} was used for all ES extractions.

Water:soil ratio tests

Water was added to disperse soil aggregates in which ES could be occluded. A small amount of water does not effectively disperse soil aggregates, whereas a large amount of water prevents the contact between chloroform and soil ES. Hence, we first tested ES recovery at different W:S ratios.

The ES recovery ranged between 89–100% at W:S ratios between 0.75 and 1.00 l kg^{-1}, whereas the recovery varied greatly when the ratios were less than 0.75 l kg^{-1} (Fig. A1). Therefore, further testing of ES recovery in additional soils with different textures was performed at W:S

ratios of 75% and 100% (Fig. A2). The ES recovery varied from 85–102% at the W:S ratio (with an outlier of 60%) of 0.75 l kg^{-1}, and 84–114% at W:S ratio of 1.00 l kg^{-1}.

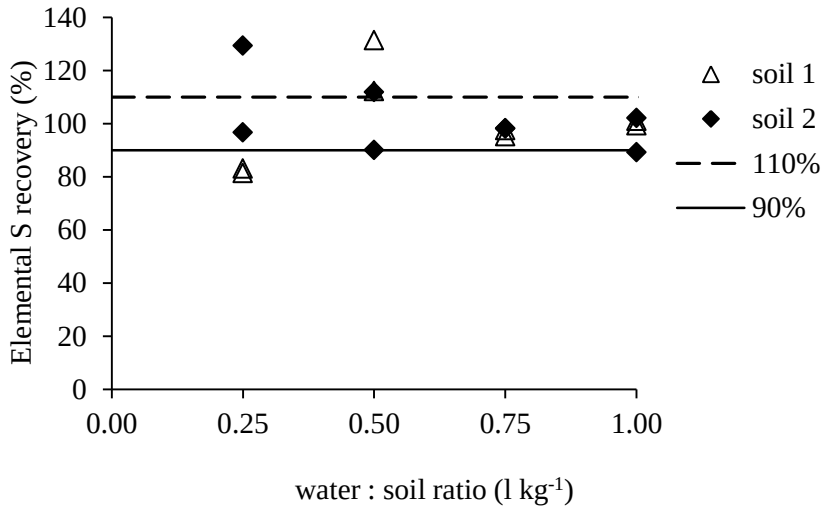

Fig. A1. Elemental S recovery from a clay soil (soil 1) and a loam soil (soil 2) at different water:soil ratios. Two grams of air-dry soil were weighed into a centrifuge tube and ES was mixed into the soil at a rate of 1000 mg kg^{-1}. Water was added to the mixture at ratios of 0.25, 0.5, 0.75 and 1.00 (l kg^{-1}), and soils were incubated overnight before extraction.

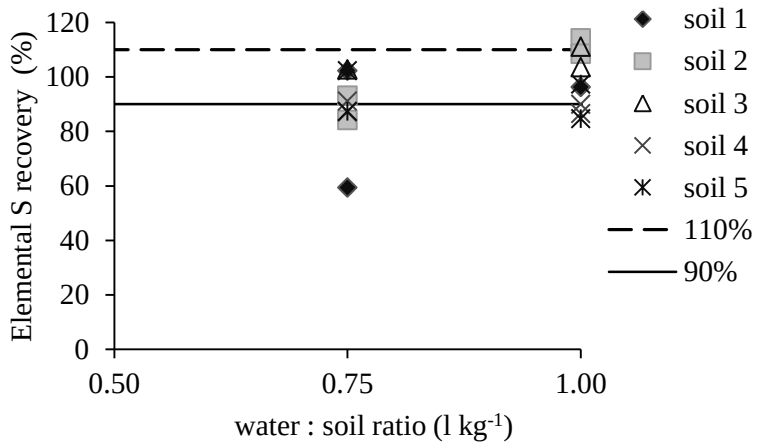

Fig. A2. Elemental S recovery from five soils with different soil textures at water:soil ratios of 0.75 and 1.00 l kg^{-1}. Two grams of air-dry soil were weighed into a centrifuge tube and ES was mixed into the soil at a rate of 1000 mg kg^{-1}. The textures for soils 1 to 5 are sand, sandy clay, loam, clay and clay, respectively.

Based on these experiments, a W:S ratio of 1.00 l kg^{-1} was selected. A final test was carried out for confirmation (Fig. A3).

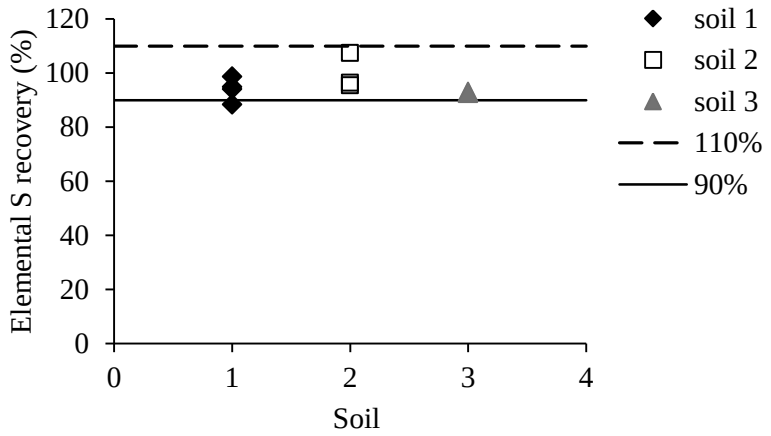

Fig. A3. Elemental S recovered from three soils at a water:soil ratio of 1.00 l kg^{-1}. Two grams of air-dry soil were weighed into a centrifuge tube and ES was mixed into the soil at a rate of 1000 mg kg^{-1}. Soils 1, 2 and 3 are the same as soils 1–3 in Fig. A2.

These results indicate that ES can be extracted effectively with a chloroform:soil ratio of 2 l kg^{-1} at a W:S ratio of 1.00 l kg^{-1}.

Homogeneity tests-mixing sulphur through soil

It is important that ES is mixed homogeneously through the soil at the commencement of an incubation study to avoid spatial variation in ES concentrations. These tests aimed at optimising the method to add ES to the soil, in order to achieve a homogeneous distribution of ES within the soil.

In the first test, ES was added dry to the soil, and this dry mixture was shaken end-over-end for various times (Fig. A4). Elemental S recovery ranged from 97–112% after shaking for > 16 h in these two soils, with an outlier after 27 h shaking (Fig. A4).

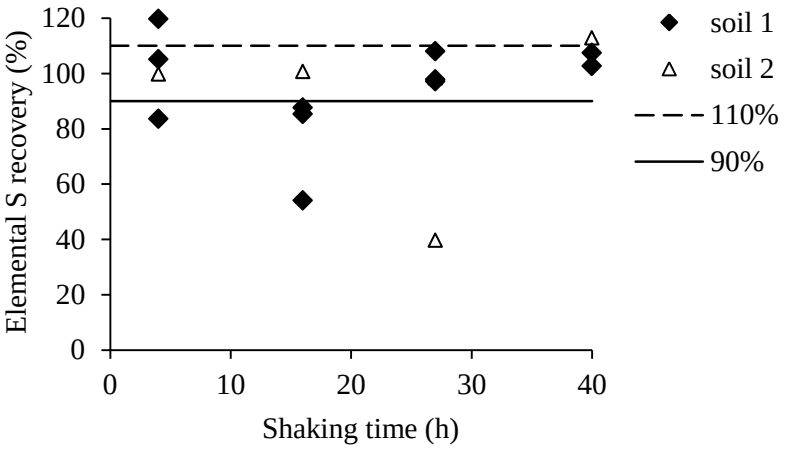

Fig. A4. Recovery of ES from silt and loam soils after shaking the soil-ES mixture for 4, 16, 27 or 40 h. Fifty g of dry soil were weighed into a container with1000 mg kg^{-1} ES powder incorporated.

In the following test, the homogeneity of ES added to a clay and a sandy soil was tested because it could be difficult to attain homogenous mixing in a soil with large aggregates. Low recovery of ES was obtained for the clay soil (Fig. A5). It was observed that ES powder adhered onto the wall of the container after shaking with the clay soil, which might be due to difficult mixing of dry ES powder in soils with large aggregates.

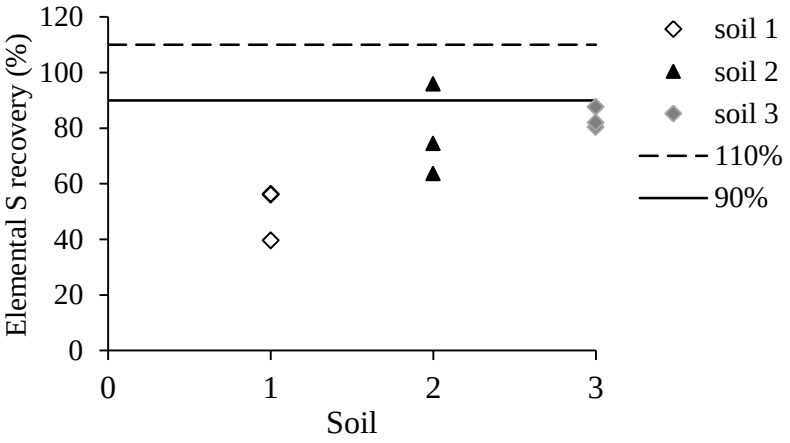

Fig. A5. Recovery of ES from soils after shaking the soil-ES mixture for 30 h on an end-over-end shaker. Soils 1, 2 and 3 are clay, sandy and loam soils, respectively. Fifty g of soil were weighed into a container with 1000 mg kg^{-1} ES applied.

To assess whether better ES mixing could be obtained in clay soils when ES was mixed into wet soil, the difference between dry and wet mixing was examined (Fig. A6). To avoid ES losses by adhering onto container walls, mixing was carried out manually. For this wet mixing method, the soil and ES were first mixed dry, after which water was gradually added while mixing manually with a spatula. For the clay soil, water was gradually added and mixed until the point at which the soil was fully moistened but not sticky yet. The soil was further mixed for 10 minutes before the rest of the water was added to reach 70% of field capacity. For the sandy soil, all the water required (i.e. to reaching 70% field capacity) was added and thoroughly mixed. The soils were incubated overnight before performing ES extraction. This wet mixing method gave acceptable recoveries (Fig. A6) and was therefore selected as the ES addition method in the incubation studies described in this thesis. We used the mixing method for the clay soil (water added in two steps) for all non-sandy soils in the experiments.

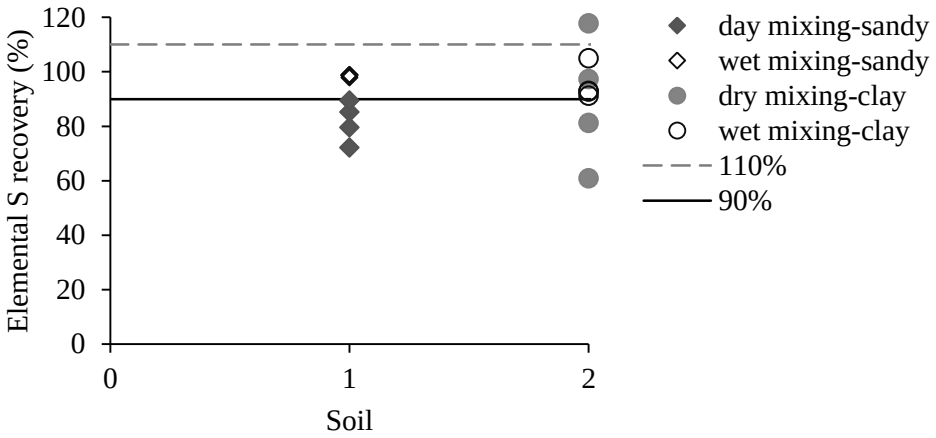

Fig. A6. Recovery of ES from a sandy (soil 1) and a clay (soil 2) soils when ES was mixed into dry or wet soil.

The performance of these modified procedures was confirmed from the ES recovery observed in samples from day 1 in our first experiment with ten soils (Fig. A7).

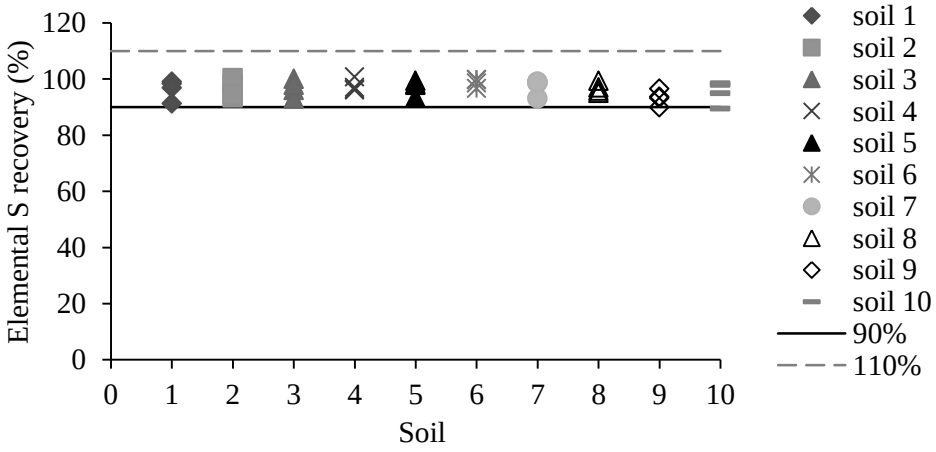

Fig. A7. Elemental S recovery from the ten soils studied (see Chapter 3).

Appendix 2: Development of assay of quantitative polymerase chain reaction for *soxB* gene

Introduction

soxB gene is a functional gene involved in S oxidation, which has been applied to study the diversity and physiology of S-oxidising bacteria in the environment, but little information is available on its abundance. Hence, a new assay is needed to allow the quantification of *soxB* gene in soil. Five pairs of *soxB* primers were selected from Petri et al. (2001) (Table A1) for the development of the qPCR assay. The samples tested were DNA templates from soil with/without ES application history. A positive control (DNA extracted from an acid sulphide soil) was included in each PCR run to ensure the procedures were carried out properly, and a negative control (PCR reaction without any template) to examine contamination from the laboratory environment.

Table A1 Primer sequences of *soxB* gene.

Primer pair No.	Primers	Sequences	Base pair (ca.)
1	*soxB*1164F	TAYCGNCGNGGNAAYTT	240
	*soxB*1403R	TTRTCNGCNACRTCYTC	
2	*soxB*1164F	TAYCGNCGNGGNAAYTT	280
	*soxB*1446R	CATGTCNCCNCCRTGYTG	
3	*soxB*432F	GAYGGNGGNGAYACNTGG	260
	*soxB*693R	TANGGRAANGCYTGNCCGAT	
4	*soxB*693F	ATCGGNCARGCNTTYCCNTA	490
	*soxB*1164R	AARTTNCCNCGNCGRTA	
5	*soxB*693F	ATCGGNCARGCNTTYCCNTA	410
	*soxB*1403R	TTRTCNGCNACRTCYTC	

Development of PCR protocol for *soxB* amplification

For the quantification of gene abundance, a reaction protocol containing three steps is required to fit a Strategene MxPro3000 system. Annealing temperature determines the specificity and efficiency of gene amplification in PCR. Gradient PCR was firstly performed with positive control (DNA from an acid sulphate soil) to decide the annealing temperature for each primer set. Thereafter, we adjusted the composition of reaction system e.g. primer concentration and template concentration. Here we only show the final results of each primer as shown from gel imaging of PCR products using optimised protocols (Fig. A8).

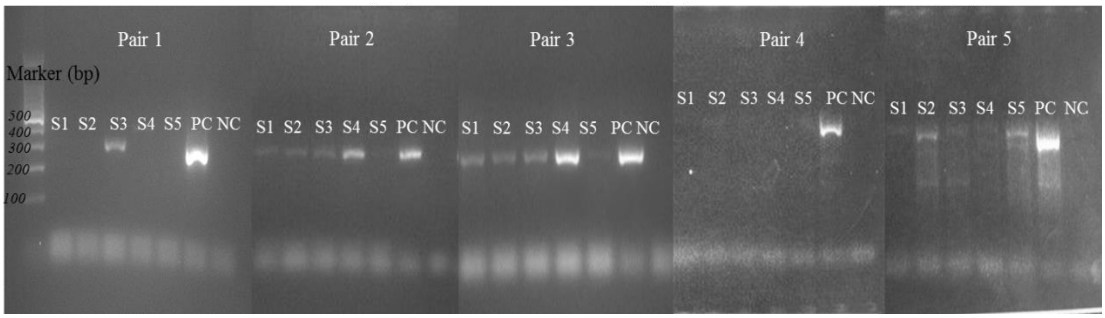

Fig. A8. PCR products (white bands under the samples labels) of *soxB* gene amplified by using primers listed in Table A1. S1–5 indicates DNA samples from soils 1–5, PC is the positive control and NC is the negative control.

The PCR products showed that primer pairs no. 2 (*soxB*1164F/1446R) and 3 (*soxB*432F/693R) can generate a single band with proper size, and therefore, these two pairs of primers were used in the subsequent qPCR test. The final reaction system and amplification program are listed in Table A2–A4.

Table A2 Composition of the PCR reaction system for amplification of *soxB* with primer pair 1164F/1446R.

	Initial concentration	Volume in each reaction (µl)	Concentration in each reaction
Buffer	10×	2	1×
Mg	25mM	0.4	0.5mM
dNTP's	40mM	0.2	0.4mM
P1 _F	50µM	0.4	1µM
P2_R	50µM	0.4	1µM
H_2O	-	14.4	-
Taq	5U/µl	0.2	0.05U
PCR mixture (µl)	-	18	-
DNA template	< 1 in 2	2	-
Total (µl)	-	20	-

Table A3 Composition of the PCR reaction system for amplification of *soxB* with primer pair 432F/963R.

	Initial concentration	Volume in each reaction (µl)	Concentration in each reaction
Buffer	10×	2	1×
dNTP's	40mM	0.2	0.4mM
P1 _F	50µM	0.6	1.5 µM
P2_R	50µM	0.6	1.5 µM
H_2O	-	14.4	-
Taq	5U/µl	0.2	0.05U
PCR mixture (µl)	-	18	-
DNA template	< 2 ng/ µl	2	-
Total (µl)	-	20	-

Table A4 PCR amplification conditions.

1164F/1446R			432F/693R		
Temperature	Time	Cycle no.	Temperature	Time	Cycle no.
95	10’	1	95	10’	1
95	40”		95	40”	
53	40”	30	54	40”	30
72	40”		72	40”	
72	10’	1	72	10’	1

Optimisation of qPCR protocol

The same reaction conditions were used for the qPCR test by using the Mxpro3000 system, but instead of using buffer dNTP’s and Taq separately, a SybrGreen Super mix (Bio-rad) was used for the quantification of amplified gene copies. The criteria of assessment of the protocol were: 1) the cumulative amplification should be in a S-shaped curve; and 2) the dissociation curve should have a single peak representing single sized products. Fig. A9 shows that the fluorescence signal from the amplications with 1164F/1446R was much weaker than that with 432F/693R, indicating that only a small quantity of *soxB* gene can be amplified with the primer set 1164F/1146R. Hence, we only used the primer set 432F/693R for further examination.

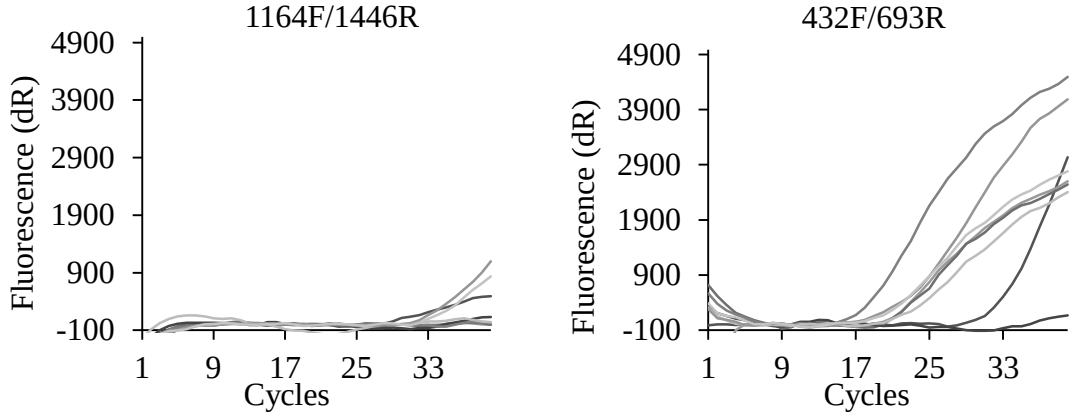

Fig. A9. Amplification curve of qPCR generated by two pairs of primers.

However, the dissociation curve generated with the selected primer set (432F/693B) revealed double peaks with DNA extracted from ES-amended soils (Fig. A10). According to Tourna et al. (2014), this is due to changes in bacterial community composition after ES addition, i.e. the proliferation of *Thiobacillus*-like bacteria. Similarly, double peaks on the dissociation curve in our study could also be due to shifts in bacterial community composition after the addition of ES.

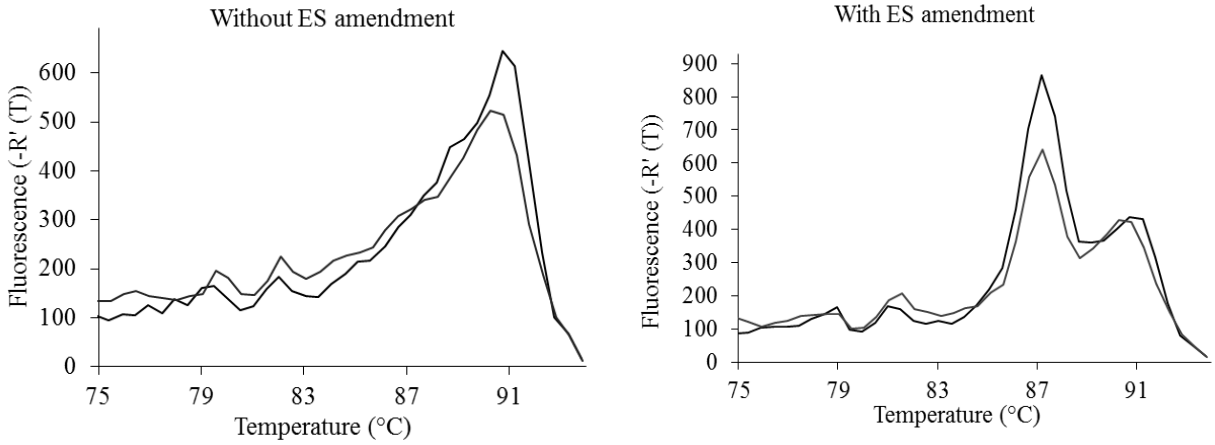

Fig. A10. Dissociation curve for soils with or without ES amendment.

The amplification efficiency for a *soxB* gene standard was tested with 432F/693R and generated an acceptable amplification curve, dissociation curve and amplification efficiency (Fig. A11).

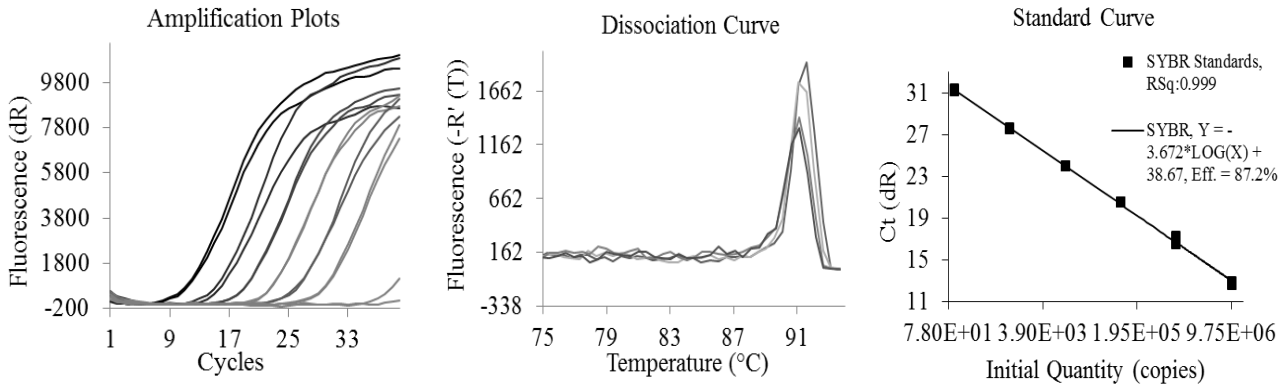

Fig. A11. Amplification curve and dissociation curve of qPCR standards.

Also the possibility of inhibition of the qPCR assay caused by humic substances was tested and described in Chapter 4.

References

Petri, R., L. Podgorsek, and J.F. Imhoff. 2001. Phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria. FEMS Microbiol. Lett. 197:171-178.

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