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Anaerobic soil disinfestation is an alternative to soil fumigation for control of some soilborne pathogens in strawberry production

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Alternatives to soil fumigation are needed for soilborne disease control. The aim of this study was to test anaerobic soil disinfestation (ASD) as an alternative to soil fumigation for control of critical soilborne pathogens in Californian strawberry production. Controlled environment experiments were conducted at 25 and 15 °C to test different materials as carbon sources for ASD using soil inoculated with *Verticillium dahliae*. Field trials were conducted in three locations comparing ASD with 20 Mg ha^{-1} rice bran (RB) against fumigated and untreated controls, steam, mustard seed meal and fish emulsion. In ASD-treated soils, temperature and extent of anaerobic conditions were critical for control of *V. dahliae*, but multiple carbon inputs reduced inoculum by 80–100%. In field trials, ASD with RB provided control of a number of pathogens, and in three of four trials produced marketable fruit yields equivalent to fumigation. Little weed control benefit from ASD was found. ASD with RB also induced changes in the soil microbiome that persisted through the growing season. When equivalent yields were obtained, net returns above harvest and treatment costs with ASD RB were 92–96% of those with bed fumigation based on average prices over the previous 5 years. ASD can be a viable alternative for control of some soilborne pathogens. Growers are adopting ASD in California strawberry production, but research to determine optimal soil temperatures, anaerobicity thresholds and carbon sources for effective control of specific pathogens is needed.

Keywords: anaerobic soil disinfestation, fish emulsion, mustard seed meal, steam, *Verticillium dahliae*

Introduction

California (CA) is the major producer of strawberries in the USA, accounting for 91% of total production in 2014 (CDFA, 2015). The highly successful commercial cropping of strawberry in CA is based upon an annual planting system that has evolved around pre-planting soil fumigation, historically with methyl bromide (MeBr) mixed with other pesticides, primarily for control of fungal pathogens including *Verticillium dahliae*, but also for weed and nematode control (Fennimore *et al.*, 2003). Although CA strawberry production was reliant on pre-planting fumigation with MeBr, its use has been phased out through the Montreal Protocol, and the 2015/16 season was the last that strawberry growers in CA could use MeBr (USDS, 2014). In response to the loss of MeBr, use of other soil fumigants has increased, most notably 1,3-dichloropropene (1,3-D) and chloropicrin (Pic). Both fumigants are heavily regulated, with township caps on 1,3-D restricting use in many growing areas (Carpenter *et al.*, 2001). Expansion of buffer zones and reduced application rates of Pic are also being proposed by the California Department of Pesticide Regulation. Furthermore, for both health (Gemmill *et al.*, 2013) and environmental reasons, the public is becoming less tolerant of fumigant use in agriculture in general. All of these factors underscore the critical need for effective nonfumigant alternatives for the control of soilborne diseases and pests in strawberry production systems.

A promising soilborne disease control alternative is anaerobic soil disinfestation (ASD), which has been adapted from the previously described methods of biological soil disinfestation and soil reductive sterilization (Goud *et al.*, 2004; Messiha *et al.*, 2007; Momma,

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2008) to create a treatment suitable for strawberry (Shennan *et al.*, 2014) and vegetable production systems (Butler *et al.*, 2012). A wide range of soilborne plant pathogens and plant parasitic nematodes has been controlled in a variety of crops using ASD (Shennan *et al.*, 2014; Rosskopf *et al.*, 2015). Implementation of ASD involves the addition of a labile carbon source followed by the generation of anaerobic conditions, first through application of water to fill soil pore space, and then covering the soil with plastic mulch to prevent oxygen exchange. Microbial growth is stimulated by the carbon addition and the microbial community rapidly shifts to one dominated by facultative and obligate anaerobes as oxygen is reduced and soil redox potential (Eh) declines. The exact mechanisms that lead to disease suppression with ASD are not clearly understood, but may involve production of organic acids and other biologically active volatiles (Hewavitharana *et al.*, 2014), and amplification of specific microbes with biocontrol activity (Momma *et al.*, 2013). Production of Fe^{2+} and Mn^{2+} under low Eh conditions may also have some disease suppressive properties (Momma *et al.*, 2013). In certain locations such as Florida (FL), where soil temperatures are high, solarization may also occur and have a synergistic effect with ASD in terms of controlling weeds (Butler *et al.*, 2012).

Another strategy being tested is the application of residues or by-products of plants from the Brassicaceae that contain glucosinolates, a class of secondary plant metabolites, which, upon hydrolysis, produce isothiocyanates found to possess pesticidal activity (Chellemi *et al.*, 2015). This process has been referred to as biofumigation because the pest control achieved is commonly attributed to the release of isothiocyanates that are related to the active chemistry of the fumigant metam sodium (methyl isothiocyanate). The spectrum of biological activity is, however, dependent upon the type of glucosinolates present in the plant tissue and the conditions under which hydrolysis takes place. Disease suppression has also been attained with Brassicaceae seed meal, a waste product of the oil extraction process. In some cases, suppression was observed without biologically active chemistries and irrespective of glucosinolate content, indicating possible suppression by changes induced in the soil biota (Mazzola *et al.*, 2001). Specific elements of the soil biological community have been shown to function in disease or weed control in response to Brassicaceae residue amendments, although the dominant mechanism functioning in disease suppression may vary from pathogen to pathogen (Weerakoon *et al.*, 2012). Brassicaceae seed meals, including those from various mustard seed meals (MSM), have demonstrated effective levels of pest suppression in various cropping systems including tree fruits and, specifically, apple (Mazzola & Brown, 2010), where an MSM formulation provided equivalent disease control and plant growth response to pre-plant fumigation with 1,3-D/Pic (Mazzola *et al.*, 2015). However, seed meal from certain Brassicaceae species may also stimulate specific soilborne pathogens as was shown for *Brassica napus* (*Pythium*; Mazzola *et al.*, 2001) and *Brassica juncea* (*Phytophthora*; Mazzola & Brown, 2010). Little is known about the potential for soilborne pathogen control in strawberry systems with incorporation of MSM.

Steam has been used for over 100 years to kill soilborne pathogens and weeds in potting soil and it is widely accepted that raising the soil temperature to 70 °C for 20 min kills pathogens and weeds (Chellemi *et al.*, 2015). This has led to the development of prototype steam machines that can be used to heat soil in a field situation. Steam applied to field soil that raised the temperature to 60 °C for 20 min resulted in weed control comparable to MeBr (Vidotto *et al.*, 2009). Preliminary data derived from a new bed steamer in CA indicates that it rapidly heats soil to a 36 cm depth at a cost of $13 522 per hectare broadcast compared to $8896 per hectare for MeBr applied broadcast, and previous work found that strawberry fruit yields from steam treated soils were similar to MeBr/Pic (Samtani *et al.*, 2012).

It has also been shown that lowered soil pH and volatile fatty acids (VFAs) including organic acids may play an important role in suppression of soil diseases (Chet & Baker, 1980). For example, the use of fish emulsion containing a large concentration of VFAs reduced the viability of *V. dahliae* microsclerotia by up to 99%. However, field evaluations of the efficacy of VFAs from fish emulsion for disease suppression are lacking.

The present investigation sought to refine the ASD process as a treatment to control *V. dahliae* and other pathogens through a combination of controlled environment experiments and on-farm trials in strawberry fields throughout coastal CA. It was hypothesized that soil temperature, extent of anaerobic conditions and type of carbon source would affect the efficacy of disease control with ASD; therefore (i) controlled environment studies were conducted to determine the effect of soil temperature, anaerobiosis and carbon input on the efficacy of ASD for control of *V. dahliae*; and (ii) a series of field experiments was completed to test the efficacy of ASD in comparison to soil fumigation and other nonfumigant alternatives (steam, MSM and fish emulsion) in terms of yield, weed and disease suppression, and economics. Further, the effect of different C sources, timing of treatment and length of treatment on the effectiveness of the ASD process for suppressing *V. dahliae* and other pathogens was evaluated, and the impact of ASD and other treatments on the structure of the soil microbial community assessed.

Materials and methods

Controlled environment studies

Initial studies considered the effect of plastic mulch material and soil type on ASD efficacy at two temperatures, 25 and 15 °C. A completely randomized factorial experiment with four replicates was conducted using PVC pots (15 cm diameter × 20 cm tall). Treatments were plastic mulch (no mulch, green or white/black (both 0.0318 mm standard polyethylene films), virtually

impermeable film (VIF 0.0318 mm embossed black), or pit tarp (0.203 mm black/white)) and soil type (Watsonville sandy clay loam (pH 6.8, SOM: 28 g kg^{-1}) or Moss Landing sandy loam (pH 7.5, SOM 7 g kg^{-1})). A 10 Mg ha^{-1} equivalent amount of wheat bran (C: 397, N 17.5 g/kg dry weight (DW)) was mixed with soil in each pot and 10 cm of water gradually applied to saturate the soil. Excess water was allowed to drain through holes in the bottom of the pots. After covering the soil surface with plastic, pots were placed in 25 °C incubators for 3 weeks. No additional water was added during incubation. Effect of treatments on viability of *V. dahliae* microsclerotia was evaluated by burying a nylon mesh bag of inoculum in each pot at the beginning of the experiment and retrieving them at the end to test inoculum viability by plating on a semiselective medium (NP-10) using the Anderson sampler dry sieve technique (Koike *et al.*, 1994). Pathogen inoculum consisted of 100 g of naturally infested soil (32 ± 11 (SEM) microsclerotia per g soil) taken from the UCSC farm. Soil redox potential (Eh) and temperature at 15 cm depth in each pot were monitored every 30 s by an oxidation-reduction potential (ORP) sensor (S500-CD-ORP; Sensorex Inc.) and a soil temperature sensor (107-L; Campbell Scientific Inc.), respectively, both connected to an automatic data logger (CR1000; Campbell Scientific Inc.). The experiment was then repeated twice at 15 °C. The ORP reading in mV was converted to Eh mV by adding 199 mV. To compare intensity of anaerobic conditions, the cumulative Eh mV hours under 200 mV were calculated for each pot using hourly averages of soil Eh. The value of 200 mV was selected as the threshold below which soil is considered as anaerobic at a soil pH of 6.58 (Butler *et al.*, 2012).

Subsequent experiments examined the effect of different carbon sources on ASD-induced suppression of *V. dahliae* and included wheat bran, rice bran (C: 464, N: 24.8 g/kg DW), grape pomace (C: 473, N: 21.4 g/kg DW), onion skin waste (C: 397, N: 17.5 g/kg DW), MSM (Biofence; Triumph Italia; C: 436, N: 52.2) and ethanol (Momma *et al.*, 2013). The treatments were replicated four times in a randomized complete block design. Plastic pots (7.5 cm diameter × 20 cm tall) were packed with sandy loam soil from Moss Landing, CA, and mixed with each carbon source at a rate of 10 Mg ha^{-1} equivalent of dry solid material, and ethanol at 10 cm equivalent of liquid (10 mL L^{-1} ethanol). ASD conditions were applied as described above using standard green plastic mulch. Experiments were conducted in controlled environment growth chambers using a day/night temperature regime of 25/15 °C to simulate conditions in soil under plastic during autumn in the central CA coastal region. The effect of treatments on survival of *V. dahliae* was examined as described above. Soil Eh was continuously monitored at 15 cm depth over the 3 week ASD treatment period.

Field trials

Four field trials were conducted in coastal CA: Castroville and Watsonville in the 2010/11 season and Watsonville and Santa Maria in the 2011/12 season. For all trials, typical fertility, pest, and irrigation management practices for conventional strawberries in the region (Bolda *et al.*, 2010; Dara *et al.*, 2010) were employed unless otherwise stated.

Castroville site (2010/11)

In autumn 2010, an on-farm trial was established at Castroville, Monterey County, in a field with clay loam soil (pH: 6.8, SOM: 30 g kg^{-1}) with moderate *V. dahliae* populations (11 microsclerotia per g soil) A split-plot experiment was conducted with time of year (September and October) as main plots, and four ASD treatments (that varied in C source and length of treatment), an untreated control (UTC) and Pic-Clor 60 fumigation as subplots (Table 1). Each treatment was replicated four times with each plot 1.2 m wide × 12 m long. For all the ASD treatments, RB and MSM were mixed from 0 to 15 cm depth in premade beds using a hand-push rototiller. After drip tape and plastic mulch installation over the beds, water was intermittently drip-irrigated to all ASD plots (see Table 1). The amount of irrigation water was adjusted according to the degree of anaerobiosis development in the ASD plots measured using ORP sensors at 15 cm depth in the centre of the main strawberry root zone. Bed fumigation with Pic-Clor 60 EC at a rate of 337 kg ha^{-1} was conducted on 24 September. Strawberry planting, cultivar Albion, was done 2 December, at a density of 53 820 plants per hectare, and yield of marketable fruit was evaluated twice weekly from 21 April until 28 September from 20 premarked plants per plot.

Watsonville site (2010/11)

A field trial was established in a sandy-loam field (pH: 6.7, SOM: 14.0 g kg^{-1}) at Watsonville, Santa Cruz County, a site with no detectable *V. dahliae*. Treatments included ASD RB (20 Mg ha^{-1}), MSM (pelleted MPT Mustard Products & Technologies Inc., 3.3 Mg ha^{-1}), steam, ASD RB + MSM (RB 16.7 Mg ha^{-1} + MSM 3.3 Mg ha^{-1}), steam + MSM (3.3 Mg ha^{-1}), Pic-Clor 60 fumigation, and UTC arranged in completely randomized block design with four replicates. Each plot was a 1.3 m wide × 12 m long bed. MSM was shank applied at 15 cm depth of beds (two rows per bed) on 7 October 2010, and RB was applied to the bed surface and incorporated from 0 to 15 cm depth by a hand-push rototiller. After reshaping the beds and applying standard green plastic mulch, water was drip irrigated intermittently in ASD and ASD + MSM plots (Table 1). Steam was applied by spike injection from a stationary steam generator for sufficient time to raise the soil temperature to 70 °C for 20 min on 13–14 October 2010. The spike injectors were mounted on 15 cm diameter polypropylene mesh hoses (Syn-Tex). The spike injector hoses were connected to the steam generator (Sioux) operating at a pressure of 7 to 12 psi. Pic-Clor 60 EC was applied to beds on 15 October 2010 at a rate of 337 kg ha^{-1}. Holes were cut through the plastic on 18 November, and the site was planted (cv. Albion) on 22 November at a density of 49 421 plants per hectare. Weed densities were measured in 2.3 m^2 areas covered with clear plastic on 15 December, 21 January, 23 February and 6 April. Yield of marketable fruit was assessed twice weekly from 28 premarked plants per plot, from 18 April to 15 September.

Watsonville site (2011/12)

The same trial as above was repeated in the 2011/12 season at the Watsonville site, except that for MSM treatments, powdered 'Strawberry Mix' from Farm Fuel Inc. was used at 3.3 Mg ha^{-1}, because it was confirmed that this product releases more allyl isothiocyanates (AITC) than the pelleted form (M. Mazzola, data not shown). Treatments were applied as previously, except for amounts of irrigation during ASD (Table 1). Steam was applied on 18–20 October as described previously. Bed fumigation with Pic-Clor 60 EC was conducted on 3 November 2011 at a rate of 337 kg ha^{-1}. Planting holes were cut on 17 November and strawberry cv. Albion was transplanted at 49 421 plants per hectare on 21 November. Weed densities were measured on 17 January, 8 March and 24 April from 1.9 m^2 sample areas

Table 1 Description of treatments applied in each field study and the cumulative soil redox potential (Eh; mV h below 200 mV) and soil temperature at 15 cm depth during anaerobic soil disinfestation (ASD) treatment.

Site/soil type	Date ASD applied	Treatment period (weeks)[a]	Amount of water (cm)	C source (Mg ha^{-1})	Cumulative Eh mV h below 200 mV	Mean soil temperature (range) (°C)
Castroville/Pacheco clay loam	2010-09-16	3	7.5 (LF)[b]	ASD1: RB 20	45 200[c]	23.5 (17.0–29.0)
	2010-09-16	3	7.5 (LF)	ASD2: RB 17.8 + MSM 2.2	49 100	23.4 (16.7–28.2)
	2010-09-16	6	15 (LF)	ASD3: RB 20	33 600[c]	21.4 (14.6–28.6)
	2010-09-16	6	15 (LF)	ASD4: RB 17.8 + MSM 2.2	56 600[c]	21.4 (14.3–30.1)
	2010-10-16	3	12.5 (LF)	ASD1: RB 20	59 700	19.7 (15.4–25.9)
	2010-10-16	3	12.5 (LF)	ASD2: RB 17.8 + MSM 2.2	50 400	20.2 (16.0–26.4)
	2010-10-16	6	12.5 (LF)	ASD3: RB 20	66 300	17.2 (8.4–26.9)
	2010-10-16	6	12.5 (LF)	ASD4: RB 17.8 + MSM 2.2	147 000	16.9 (7.9–25.2)
Watsonville/Elder sandy loam	2010-10-08	4.1	6.4 (HF)	ASD: RB 20	78 200	19.0 (14.4–26.2)
	2010-10-08	4.1	6.4 (HF)	ASD: RB 16.7 + MSM 3.3	135 000	18.9 (14.2–26.2)
	2011-10-14	4.4	4.5 (HF)	ASD: RB 20	43 300	20.8 (13.2–28.8)
	2011-10-14	4.4	4.5 (HF)	ASD: RB 16.7 + MSM 3.3	115 000	20.2 (13.3–27.6)
Santa Maria/Sorrento sandy loam	2011-09-16	4.6	7.5 (HF)	ASD: RB 20	94 500	23.0 (17.0–29.3)
	2011-09-26	4.6	7.5 (HF)	ASD: RB 16.7 + MSM 3.3	88 600	23.2 (18.6–28.5)
	2011-09-26	4.6	7.5 (HF)	ASD: RB 20 + FE	115 000	22.9 (17.8–28.7)

RB, rice bran; MSM, mustard seed meal; FE, fish emulsion.
[a]Period that soil Eh and temperature was monitored.
[b]Two lines of low flow (LF, 250 L h^{-1} per 100 m) or high flow (HF, 500 L h^{-1} per 100 m) drip tapes per bed were used.
[c]Due to malfunction of the ORP sensors, only 1 or 2 repeats of Eh data were used.

with clear plastic mulch at each plot. Yield of marketable fruit from 35 plants per plot was monitored twice weekly from 24 April until 12 September.

Santa Maria site (2011/12)

A randomized block experiment was established in a Sorrento sandy-loam soil (pH: 8.0, SOM: 12.3 g kg^{-1}) with no major soilborne disease pressure in Santa Maria, CA. There were four replicates and treatments were: UTC, ASD RB, fish emulsion (FE; True Organic 402 acidified by sulphuric acid (20 mL L^{-1}) pH 4.8), ASD RB + MSM (MSM 'Strawberry Mix' from Farm Fuel Inc.), ASD RB + FE, and Pic-Clor 60 fumigation. Each plot was a 1.6 m wide × 11 m long bed. Rice bran and MSM were applied on top of the beds and incorporated to 15 cm depth by a bed shaper-attached rototiller. After applying 0.0318 mm VIF black mulch, ASD RB and ASD RB + MSM plots were intermittently drip irrigated (Table 1), and 0.75 cm of acidified FE diluted 1:50 with water was applied to FE and ASD + FE plots on 30 September and 15 October. ASD + FE plots were intermittently drip irrigated with an additional 6 cm of water. Bed fumigation with Pic-Clor 60 EC was conducted on 7 October at a rate of 269 kg ha^{-1}. Holes were cut through the plastic mulch on 11 November and strawberry cv. PS-4634 transplanted at a density of 75 120 plants per hectare on 15 November. Approximately twice monthly (12 times in total), 140 L ha^{-1} of acidified FE diluted 1:50 with water were applied to FE and ASD + FE plots from 31 January until 21 June. Yield of marketable fruit from 40 plant sample areas per plot was monitored twice weekly from 23 March until 8 August.

Soil Eh and temperature monitoring during ASD

In the ASD plots of all field trials, soil Eh and temperature at 15 cm depth were monitored every 30 s during ASD treatment using a monitoring system with an ORP and soil temperature sensor in each plot and a data logger as described in the controlled environment experiments. Cumulative Eh mV h below 200 mV and soil temperatures achieved during ASD treatment for each field trial are summarized in Table 1.

Root and crown sampling and pathological tests

At the Watsonville (2011/12) and Santa Maria trials, three entire plants per plot were removed at early harvest (2 April) and the root and crown portions were bagged in sealable plastic bags, placed on ice on site and transported to the laboratory for pathogen evaluation.

Root colonization

A composite root sample was obtained from each of the four replicate plots per treatment. Fungi were isolated by washing roots with tap water and plating 100 randomly selected segments (0.5–1.0 cm in length) on 1.5% water agar amended with ampicillin (100 μg mL^{-1}). *Pythium* spp. were isolated by plating root segments on a semiselective agar medium (*Pythium* semiselective medium, PSSM; Mazzola *et al.*, 2001). After 72 h of incubation at 20–23 °C, root segments were examined using a light microscope (Olympus BH2 series), and sporulating fungi were identified to genus. *Fusarium* spp., *Pythium* spp. and *Rhizoctonia* spp. were identified by DNA sequence analysis of the internal transcribed spacer (ITS) region as previously described (Mazzola & Brown, 2010). For each sequence, a BLAST search was performed on GenBank to identify the most closely related species (*Fusarium* and *Pythium*) and anastomosis groups (*Rhizoctonia*). Roots were examined visually for galling, indicative of infestation by *Meloidogyne* spp., and gall-inducing nematodes were identified to species by sequence analysis of the rDNA ITS region.

Soil sampling and *Verticillium* analysis

At the Castroville trial, soil samples were obtained from all plots to test for viable *V. dahliae* microsclerotia before and immediately after ASD treatment. Ten to 20 cores (2 cm diameter) from 0 to 15 cm depth were taken and bulked for each plot, air dried for 4 weeks, then assayed for *V. dahliae* as described previously using the method of Koike *et al.* (1994). At the Watsonville (2011/12) and Santa Maria trials, 20 soil cores were collected as above and bulked for each plot at pre-treatment (Watsonville: 12 October, Santa Maria: 22 September), post-treatment (17 November, 24 October), and early harvest (2 May, 2 April). Soils were bagged in a sealable plastic bag, placed on ice and transported immediately to the laboratory for microbial analysis.

Soil microbial analysis

The effect of soil treatments on total culturable bacteria and fungal populations was assessed. Two soil samples were processed from each treatment plot. Soil suspensions were prepared by adding 5 g soil to 50 mL sterile distilled water and vortexing for 60 s. Serial dilutions of the suspension were plated onto 1/10th-strength tryptic soy agar (TSA; Difco Laboratories), 1/10th-strength potato dextrose agar (PDA) amended with 100 μg mL^{-1} ampicillin, PSSM agar (Mazzola *et al.*, 2001), 1/10th-strength starch-casein agar, and King's medium B (KMB) agar amended with ampicillin (100 μg mL^{-1}), chloramphenicol (13 μg mL^{-1}) and cycloheximide (75 μg mL^{-1}) (KMB+) for quantification of total culturable bacteria, total culturable fungi, *Pythium* spp., *Streptomyces* spp. and fluorescent *Pseudomonas* spp., respectively. Plates were incubated at 25 °C and colonies counted daily over a 1-week period. For *Streptomyces* spp., colonies exhibiting growth characteristics representative of this genus were subjected to microscopic examination (×100) for confirmation of identity. *Fusarium* spp. were enumerated by examining microscopically all fungal colonies emerging on PDA.

Real-time quantitative polymerase chain reaction (qPCR) was used to quantify the presence of *V. dahliae* and *Macrophomina phaseolina* in the Watsonville and Santa Maria strawberry field soils. DNA was extracted from composite soil samples obtained from each replicate plot using an Ultra-Clean DNA Isolation kit (MoBio Laboratories) according to the manufacturer's instructions. Quantitative PCRs were conducted in a total volume of 10 μL containing 1 μL of a 1:100 dilution of DNA from root or soil extractions, 1 μL of a 2 pM primer pair solution, 3 μL SYBR Green PCR Master Mix (Applied Biosystems), and 5 μL Nanopure water. Dilutions of purified *M. phaseolina* isolate 07-3 or *V. dahliae* isolate 484 DNA were prepared to generate concentrations from 1 ng μL^{-1} to 1 fg μL^{-1} for use in deriving a standard curve. Each set of qPCRs included three replicates of each soil DNA sample, purified target fungal DNA dilution and the no template control. Quantitative PCR analysis was conducted using a StepOnePlus Real Time PCR System (Applied Biosystems) with conditions consisting of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 58 °C. The primers VetBtF and VetBtR (Atallah *et al.*, 2007) were used in amplification reactions for detection of *V. dahliae*. The *M. phaseolina* primers were modified from those previously published (Babu *et al.*, 2007). The published primer pair (MpKFI and MpKRI) in reactions with purified DNA extracted from *M. phaseolina* 07-3 recovered from strawberry repeatedly failed to yield any amplification product. Sequence analysis of the ITS region from isolate 07-3 indicated a mismatch at the 5′ end between the published MpKRI primer and the strawberry *M. phaseolina* isolate. The modified primer MpRm (5′-GCTCCGAAGCGAGGTGTATT-3′) used in conjunction with MpKFI effectively amplified *M. phaseolina* DNA and this primer pair was used in all subsequent qPCR to quantify the fungal pathogen in soil.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis was used to determine whether changes in fungal and bacterial community composition occurred in response to soil treatments. DNA extraction, amplification, digestion and fragment analysis was conducted as described by Weerakoon *et al.* (2012). For assessment of the bacterial community, primers targeting the bacterial 16S rDNA (8f and 1406r; Amann *et al.*, 1995) were used in PCR and resulting products were digested using the restriction enzyme *Hae*III prior to fragment analysis.

Economic analysis

Effect of soil treatments on gross returns, harvest costs and net returns above harvest costs was estimated based on sample cost studies of strawberry production on the central coast region of CA (Bolda *et al.*, 2010), in which a typical farm size, farming practices and conventional market prices in the area are assumed. Yields and cultural costs data, including steam treatment, amendment addition and incorporation, plus additional irrigation costs for ASD, were used to estimate the expected income, costs and net returns for a commercial size farm adopting the practices of each of the research plots. Harvest costs were adjusted according to yields and were calculated at \$1.32 kg^{-1}. One strawberry crop per year at a value of \$2.50 kg^{-1} was assumed. Prices for strawberries vary across locations and years: here, the 2014 price in the Central Coast region was used. Other costs such as planting, fertility inputs and other pesticides used were not included in the analysis because they did not vary across treatments within each study location. Steam costs were calculated based on fuel used, labour and capital expenditure for the machine assuming a 5-year depreciation. For the machine used in the field trials the cost was \$25 243 ha^{-1}; however, recent improvements have reduced the cost of steam application to \$13 448 ha^{-1} and this number was used in the analysis presented. ASD costs were calculated based on material costs including shipping, material incorporation and additional irrigation. Because fumigation costs and ASD material costs vary from year to year, net returns were also calculated for selected treatments across a range of cost scenarios. For the main analysis RB and MSM costs reflected the average price for the material and delivery over the previous 5 years (\$268 Mg^{-1} for RB and \$1451 Mg^{-1} for MSM) and fumigation costs were based on the average cost of bed fumigation over the previous 4 years for the growers surveyed (\$3642 ha^{-1}). Some, however, use broadcast fumigation at an average cost of \$8568 ha^{-1}, so net returns are also compared with this scenario.

Statistical analysis

Data from controlled environment and field trials were analysed for treatment effects with ANOVA using STATISTIX v. 10 (Analytical Software). To satisfy normality and homogeneity of variance assumptions, log transformation was performed when needed. Protected LSD at $\alpha = 0.05$ (Ott & Longnecker, 2001) was used for separation of the means.

To determine differences in bacterial and fungal community composition, peak presence/absence T-RFLP data were subjected

to principal coordinate analysis using the Jaccard similarity measure. All analyses were performed using the PAST v. 2.14 software package (Hammer *et al.*, 2001).

Results

Controlled environment studies

Strong to moderate anaerobic conditions (Eh −200 to 100 mV) developed within 1 week in all treatments except for the no plastic mulch control at 25 °C. Figure 1a shows a typical time course for development of anaerobic conditions. Strong anaerobic conditions were attained with ASD regardless of tarp type, and greatly reduced the number of viable *V. dahliae* microsclerotia relative to the no plastic control ($P < 0.001$), with no difference among the plastic mulch types and soil types in number of surviving microsclerotia (data not shown). Therefore, all data were combined to examine the relationship between cumulative level of anaerobic conditions, measured as Eh mV h below 200 mV (see Fig. 1b for typical changes of cumulative Eh mV h under 200 mV), and survival of microsclerotia of *V. dahliae* at soil temperatures of either 25 or 15 °C. At the lower temperature ASD did not reduce the number of viable microsclerotia irrespective of how many mV h of Eh below 200 mV were accumulated (Fig. 1c). In contrast, at 25 °C there was a clear threshold of cumulative Eh below 200 mV of 50 000 mV h, above which there was consistent reduction in viable microsclerotia; this clearly illustrated that both temperature and anaerobic conditions impact disease suppression by ASD for this pathogen. Thus, the timing of application of ASD in the field should ensure soil temperatures are sufficiently high for disease suppression. A wide variety of different C-sources for ASD were found to be equally effective for suppressing *V. dahliae*, reducing numbers of viable microsclerotia by between 81% and 100% (Fig. 1d).

Field trials

Castroville field trial 2010/11

Anaerobic conditions were created in all ASD treatments across both application dates (Table 1), with both 3- and

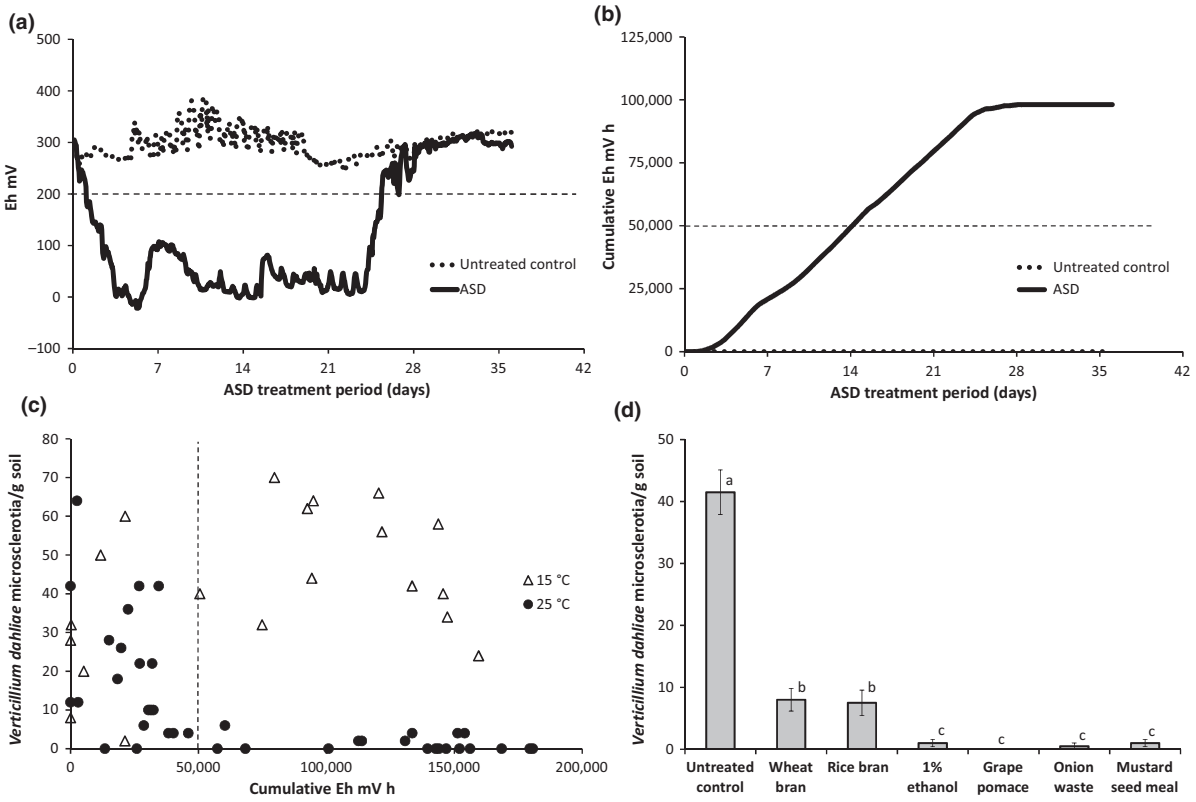

Figure 1 Effect of soil redox potential (Eh), soil temperature and carbon sources in anaerobic soil disinfestation (ASD) on the population of *Verticillium dahliae* microsclerotia in soil in controlled environment studies with strawberry. (a) An example of Eh dynamics during ASD; (b) cumulative Eh plots in mV h below 200 mV, the value of 200 mV was selected as the threshold below which soil is considered as anaerobic at a soil pH of 6.58; (c) plots of number of viable *V. dahliae* microsclerotia in soil following ASD against cumulative Eh in pots incubated at 15 or 25 °C, and (d) effect of carbon sources on reduction of *V. dahliae* microsclerotia in soil following ASD for a 3-week period. For (d), each bar indicates back-transformed mean ± SEM ($n = 4$) and bars with the same letter are not significantly different according to the protected-LSD test ($\alpha = 0.05$). Ethanol (1%) was applied at 10 cm depth and remaining sources were applied at 20 Mg dry weight ha^{-1} equivalent rate.

6-week treatment periods resulting in cumulative Eh close to, or exceeding, the 50 000 mV h below 200 mV threshold, with the exception of the September ASD RB 20 Mg ha^{-1} treatment (33 000 mV; Table 1). Regardless of variability in anaerobic conditions, ASD provided control of *V. dahliae*, equivalent to that of Pic-Clor 60 (Fig. 2b). Yields were equivalent among the various ASD treatments, with the September and October treatments being equally effective, despite the lower October soil temperature, and a 3-week treatment period was as effective as 6 weeks (Fig. 2a). Overall, three out of four of the ASD treatments improved yields relative to the UTC and Pic-Clor 60-treated plots, and the net returns were higher for the ASD plots than the fumigated plots across both treatment dates (Table 2). There was no additional benefit in terms of yield or *V. dahliae* suppression from including MSM as part of the C source for ASD.

Watsonville field trial 2010/11

In the sandy loam soil at the Watsonville site, both ASD RB and ASD RB + MSM plots developed strong anaerobic conditions, exceeding the 50 000 mV h threshold (Table 1). Soil temperature at 15 cm depth during ASD treatment averaged 19 °C with a range of 14.4–26.2 °C. *Verticillium dahliae* was not detected at this site, nonetheless yields in both the UTC and the MSM treatment plots were significantly lower than for the ASD, steam and Pic-Clor 60 plots (Fig. 3a), indicating that other pathogens may be important. MSM provided no benefit in terms of yield, and there was no difference in yield between the ASD RB and ASD RB + MSM treatments. While yields and gross revenues were comparable across treatments, except for UTC and MSM, net returns above treatment and harvest costs were highest for Pic-Clor followed by ASD RB + MSM (7% lower) and ASD RB (8% lower) and lowest for Steam + MSM (Table 2).

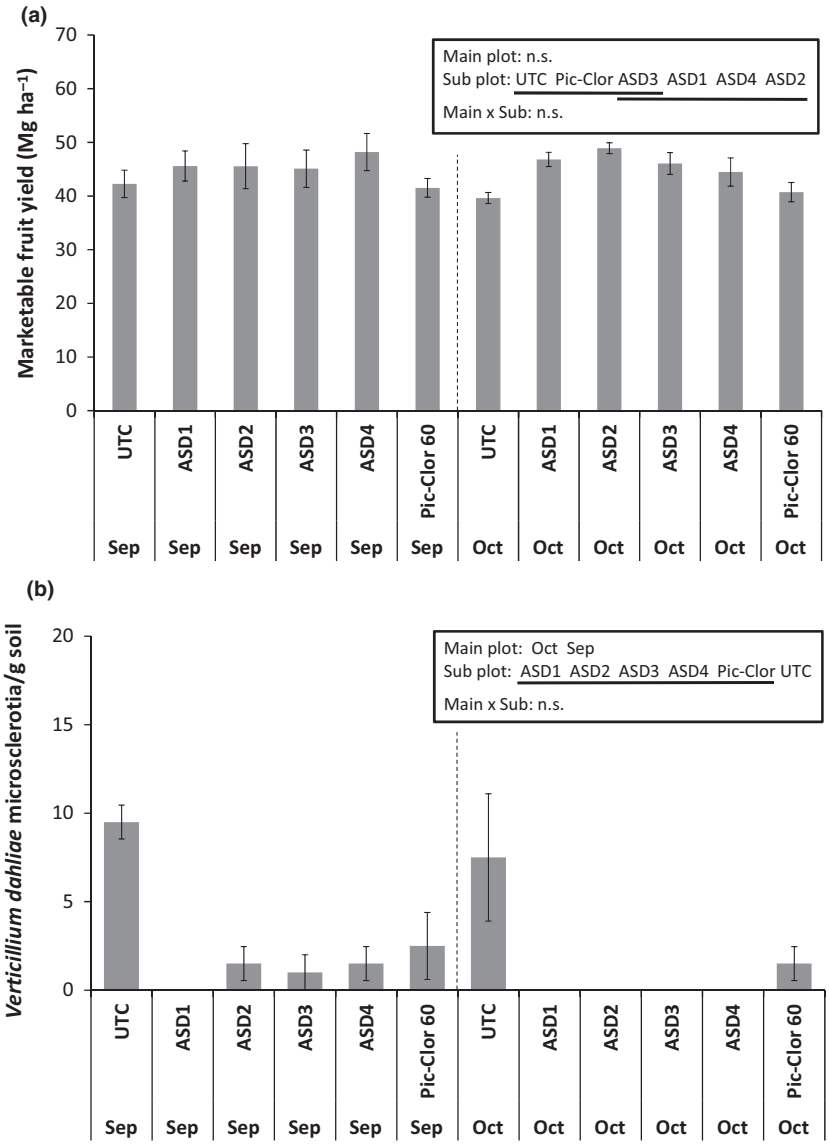

Figure 2 Effect of soil treatments on yield and the population of *Verticillium dahliae* in the soil in strawberry field trials at the Castroville site (2010/11). (a) Cumulative yield of marketable fruit and (b) post-treatment population of *V. dahliae* in soil from 0 to 15 cm depth. UTC: untreated control, ASD1: 3 weeks of anaerobic soil disinfestation (ASD) with rice bran (RB) 20 Mg ha^{-1}; ASD2: 3 weeks of ASD with RB 17.8 Mg ha^{-1} plus mustard seed meal (MSM) 2.2 Mg ha^{-1}; ASD3: 6 weeks of ASD with RB 20 Mg ha^{-1}; ASD4: 6 weeks of ASD with RB 17.8 Mg ha^{-1} plus MSM 2.2 Mg ha^{-1}; Pic-Clor 60: bed fumigation with Pic-Clor 60 at 337 kg ha^{-1}. For (a), each bar indicates mean ± SEM (n = 4). For (b), each bar indicates back-transformed mean ± SEM (n = 4). For (a) and (b), in top right box, treatments on the same line do not have significant difference according to protected-LSD test (α = 0.05).

Table 2 Treatment costs, revenue above harvest costs and net revenue above treatment and harvest costs for September and October treatments combined in the strawberry field trials at Castroville, Watsonville 2011 and 2012 and the Santa Maria 2012 trial.

Trial	Treatment	Treatment costs ($ ha^{-1})	Revenue above harvest costs ($ ha^{-1})	Net revenue above treatment and harvest costs ($ ha^{-1})
Castroville 2010/11	UTC[a]	54 072	102 738	48 665
	ASD1[b]	67 900	115 916	48 016
	ASD2[c]	72 484	118 504	46 020
	ASD3[d]	67 166	114 335	47 169
	ASD4[e]	71 394	116 246	44 852
	Pic-Clor 60	57 948	103 182	45 234
Watsonville 2010/11	UTC[a]	40 941	77 787	36 847
	MSM[f]	45 048	77 633	32 585
	Steam	76 264	119 419	43 154
	ASD RB[g]	69 913	119 928	50 015
	Steam + MSM[h]	87 225	124 781	37 555
	ASD RB + MSM[i]	76 995	127 261	50 266
	Pic-Clor 60	68 064	122 402	54 338
Watsonville 2011/12	UTC[a]	39 573	75 188	35 615
	MSM[f]	63 730	113 129	49 399
	Steam	71 469	110 308	38 839
	ASD RB[g]	73 653	127 033	53 380
	Steam + MSM[h]	96 709	142 800	46 091
	ASD RB + MSM[i]	84 490	141 503	57 012
	Pic-Clor 60	86 239	156 933	70 695
Santa Maria 2011/12	UTC[a]	100 604	191 148	90 544
	Fish emulsion	112 072	202 405	90 333
	ASD RB[g]	120 211	215 550	95 340
	ASD RB + fish emulsion	123 913	212 446	88 534
	ASD RB + MSM[i]	120 733	207 359	86 626
	Pic-Clor 60[j]	117 704	216 718	99 014

[a]Untreated control.
[b]Anaerobic soil disinfestation (ASD) 3 weeks with rice bran (RB) 20 Mg ha^{-1}.
[c]ASD 3 weeks with RB 17.8 Mg ha^{-1} plus mustard seed meal (MSM) 2.2 Mg ha^{-1}.
[d]ASD 6 weeks with RB 20 Mg ha^{-1}.
[e]ASD 6 weeks with RB 17.8 Mg ha^{-1} plus MSM 2.2 Mg ha^{-1}.
[f]MSM 3.3 Mg ha^{-1}.
[g]ASD with RB 20 Mg ha^{-1}.
[h]Steam plus MSM 3.3 Mg ha^{-1}.
[i]ASD with RB 16.7 Mg ha^{-1} plus MSM 3.3 Mg ha^{-1}.
[j]Pic-Clor 60, bed fumigation with Pic-Clor 60, 337 kg ha^{-1}.

Watsonville field trial 2011/12

Less water was applied to the ASD RB plots in 2011, only 4.5 cm as compared to 6.4 cm in 2010 (Table 1) to test if water use could be reduced during ASD. However, anaerobic conditions in the ASD RB-treated soil failed to reach the threshold of 50 000 mV h, unlike ASD RB + MSM, and yields were lower in ASD RB compared to the ASD RB + MSM, steam and Pic-Clor 60 plots (Fig. 3b). Steam treatment performed less well than in the previous year in the absence of MSM, and was the only treatment with a high incidence of *Meloidogyne hapla* (data not shown), with 58% of plants exhibiting root galling compared to only 8% in the UTC. In contrast to the previous year, MSM provided a modest yield increase over the UTC, and the ASD RB + MSM treatment led to a higher yield than ASD with RB only. A different MSM source was used than in 2010/11 and was in a powdered rather than pelleted form, which was apparently more effective. The stimulation of yield in the ASD RB + MSM plots may also have been related to better anaerobic conditions being achieved in these plots. Overall, the Pic-Clor treatment produced the highest net returns above harvest and treatment costs, and the ASD + MSM treatment was the best alternative with a 20% lower net return (Table 2).

Neither *M. phaseolina* nor *V. dahliae* were detected by qPCR in roots of plants or soils sampled in April 2012 from this site, but analysis of root samples indicated the presence of a variety of pathogens in this field that were suppressed to varying degrees by the different treatments (Fig. 4a–d). Specifically, *Pythium* spp, *Cylindrocarpon* spp., *Rhizoctonia* spp. and *Fusarium* spp. were isolated from approximately 10%, 20%, 20% and 10%, respectively, of the roots in the UTC. All isolates of *Rhizoctonia* spp. were identified as *Rhizoctonia fragariae* anastomosis group (AG) A, which is a known pathogen

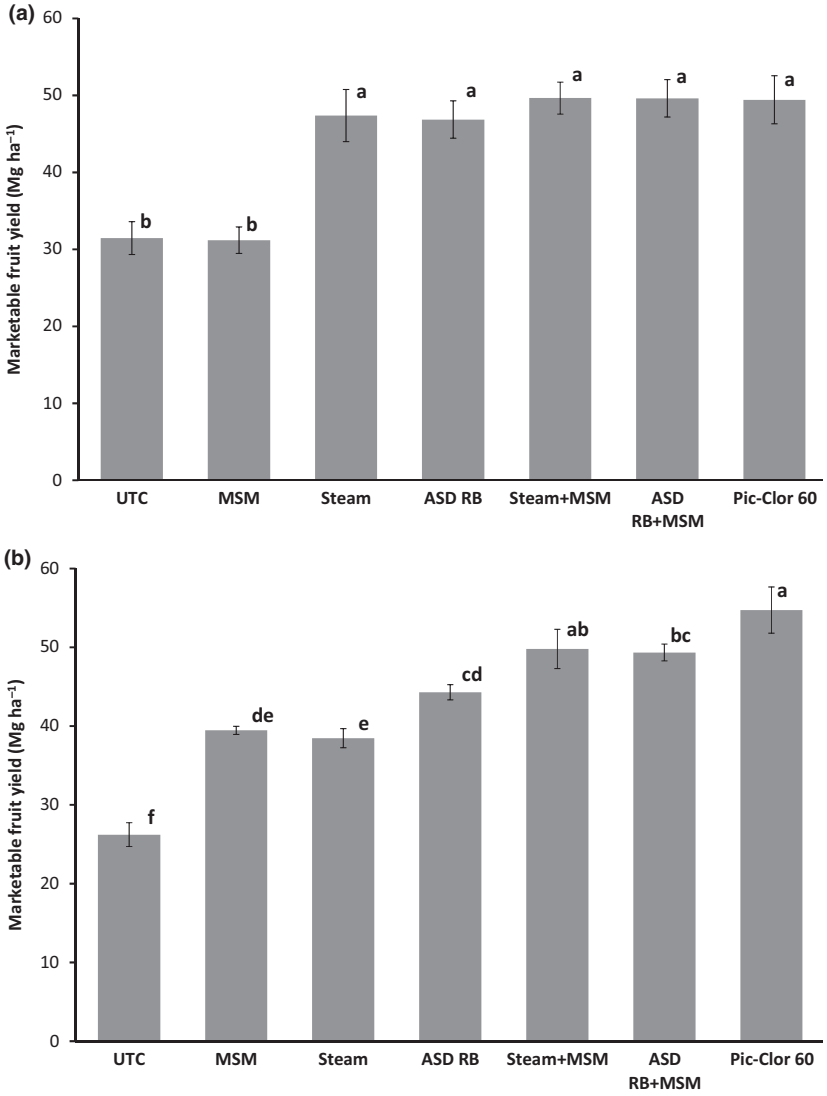

Figure 3 Effect of soil treatments on cumulative yield of marketable fruit in strawberry field trials at the Watsonville site in (a) 2011 and (b) 2012. UTC: untreated control; MSM: mustard seed meal 3.3 Mg ha^{-1}; ASD RB: anaerobic soil disinfestation with rice bran 20 Mg ha^{-1}; Steam + MSM: steam plus MSM 3.3 Mg ha^{-1}; ASD RB + MSM: ASD with RB 16.7 Mg ha^{-1} plus MSM 3.3 Mg ha^{-1}; Pic-Clor 60: bed fumigation with Pic-Clor 60 at 337 kg ha^{-1}. For (a) and (b), each bar indicates mean ± SEM (n = 4) and bars with the same letter are not significantly different according to the protected-LSD test (α = 0.05).

of strawberry (Martin, 1988). All *Pythium* isolates recovered were either *P. ultimum* or *P. sylvaticum*, both of which are significant pathogens of strawberry, and while Pic-Clor 60 and steam eliminated *Pythium*, it was greatly reduced but not eliminated by ASD RB and ASD RB + MSM (Fig. 4b). *Cylindrocarpon* exhibits great variation in virulence among isolates, but is often considered unimportant as a plant pathogen. However, studies have shown that this fungus can act in concert with *Pythium* spp. to cause damage greater than either pathogen alone (Tewoldemedhin *et al.*, 2011). Both genera were present in abundance in the control treatment, but, while *Cylindrocarpon* was not controlled effectively by Pic-Clor 60 or steam, in the ASD treatments both were significantly reduced (Fig. 4a,b). In contrast, *Rhizoctonia* was most effectively reduced by MSM alone, ASD RB + MSM and by ASD RB (Fig. 4c). None of the treatments reduced recovery of *Fusarium* spp. from strawberry roots by more than 55%, and treatments containing MSM resulted in increased recovery of this fungus relative to the UTC (Fig. 4d). *Fusarium* spp. recovered from strawberry roots were identified as *F. oxysporum* (77%) or *F. equiseti* (23%), the former a significant pathogen of strawberry and the latter known to promote plant growth.

Santa Maria field trial 2011/12

Soil temperatures were warmer in the Santa Maria trial during ASD application than in Watsonville and similar to September 3-week treatments in Castroville (Table 1), leading to strong anaerobic conditions (88 600 to 115 000 mV h) developing in ASD RB, ASD RB + FE, and ASD RB + MSM plots. Yields were high for all treatments including UTC; nonetheless, all ASD treatments resulted in significantly higher yields that were equivalent to Pic-Clor 60 (Fig. 5a). The application of FE alone resulted in yields intermediate between UTC and ASD, and there was no synergistic benefit from combining ASD with FE application. In terms of economic return, Pic-Clor 60 had the highest net returns (Table 2)

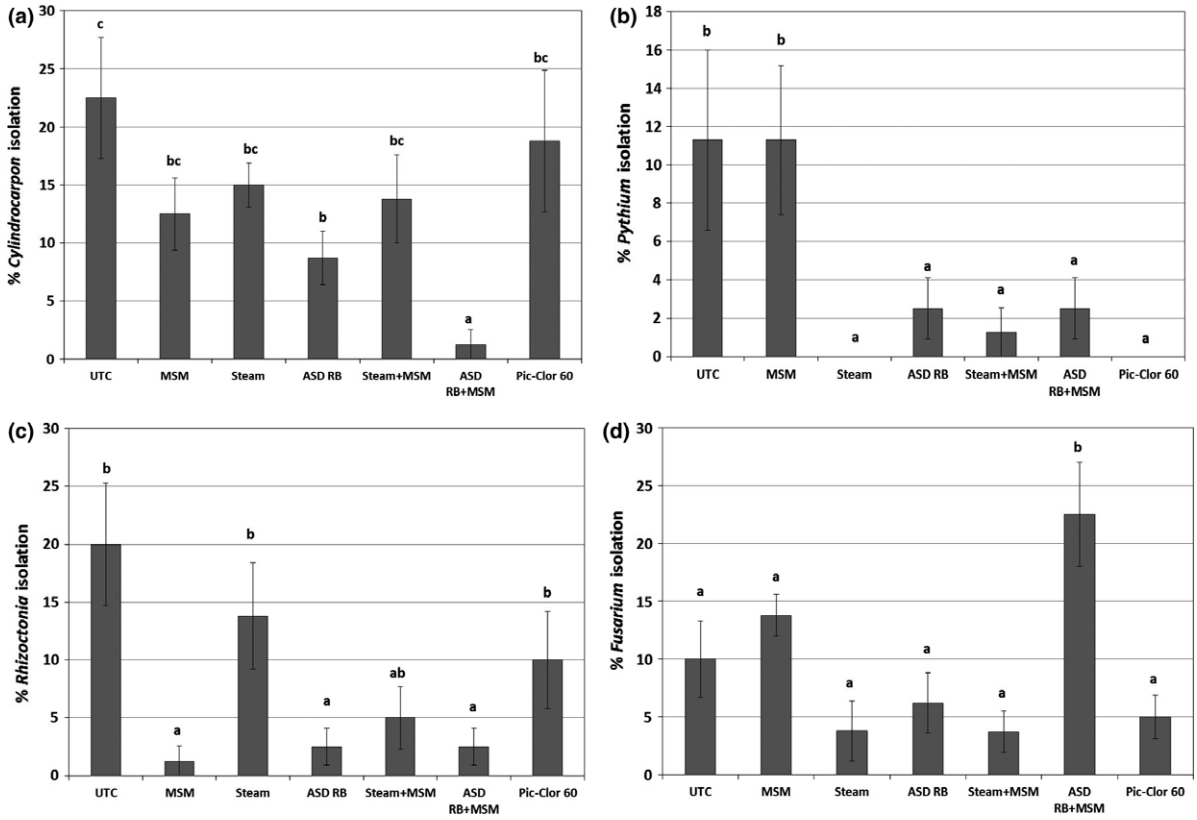

Figure 4 Effect of soil treatments on frequency of isolation of (a) *Cylindrocarpon* spp., (b) *Pythium* spp., (c) *Rhizoctonia* spp. and (d) *Fusarium* spp. from roots of strawberry at the Watsonville site in October 2012, expressed as percentage of root fragments analysed. UTC: untreated control; MSM: mustard seed meal 3.3 Mg ha^{-1}; ASD RB: anaerobic soil disinfestation with rice bran 20 Mg ha^{-1}; Steam + MSM: steam plus MSM 3.3 Mg ha^{-1}; ASD RB + MSM: ASD with RB 16.7 Mg ha^{-1} plus MSM 3.3 Mg ha^{-1}; Pic-Clor 60: bed fumigation with Pic-Clor 60 at 337 kg ha^{-1}. Each bar indicates mean ± SEM (n = 4) and bars with the same letter are not significantly different according to the Student–Newman–Keuls method.

while ASD RB returned about \$3600 ha^{-1} less, corresponding to 96.2% of the returns with bed fumigation.

Macrophomina phaseolina and *V. dahliae* were not detected by qPCR in roots of plants sampled in April 2012. *Fusarium* spp. were recovered from strawberry roots at a high frequency for all treatments; however, the highest recovery was from plants cultivated in fumigated soils (78% of root fragments colonized). Isolates were identified by DNA sequence analysis as *Fusarium solani*, *F. acuminatum* or *F. tabacinum* (*Plectosphaerella cucumerina*), with no isolates identified as *F. oxysporum*. Isolates of *R. fragariae*, representing AGs A and I, were recovered from strawberry roots in control and fumigated soils, but not in soils from ASD treatments. *Pythium* spp. were not recovered from strawberry roots grown in fumigated Santa Maria field soils but were isolated from 2.5% of root fragments from ASD treated soils. Isolates were identified as *P. megacarpum*, *P. spinosum*, *P. sylvaticum* and *P. violae*.

Soil microbial community composition

At the Watsonville and Santa Maria trials treatment-specific effects on fungal and bacterial community composition were detected based on analysis of T-RFLP data. At Watsonville, prior to treatment applications, there was a general randomness in relative similarity of the fungal community among plots, with no clustering of treatments. However, post-treatment application, the fungal community in fumigated soils were clearly distinct, and those in ASD and ASD + MSM soils were highly similar. The fungal community in nontreated soil and steamed soil were similar to each other, while the community detected in all MSM-treated plots were highly similar (Fig. 6a,b). At the end of the growing season, treatment effects on fungal community had largely broken down, reverting to a composition that appeared more similar to the untreated control. The exception was the clustering of fungal community similarity for ASD and ASD + MSM plots, which persisted (Fig. 6c). Treatments containing either ASD or MSM all possessed a greater number of fungal taxa and greater diversity than the untreated control (Table 3). Pic-Clor 60 fumigation significantly reduced soil fungal diversity. At the end of the growing season, the number of fungal taxa detected was diminished relative to that observed immediately after treatment application, but the ASD- and ASD + MSM-treated soils continued to possess a greater

number of taxa and greater diversity relative to the control.

As observed for fungi, prior to treatment applications there was a general randomness in the relative similarity of the bacterial community among plots, with no clustering of treatments. Post-treatment, clustering based on similarity of bacterial communities in the Watsonville soil was observed across broad soil treatments with the untreated control, steam and fumigation treatments forming one cluster and soils receiving an organic input comprising a second large cluster (Fig. 7a,b). In the Santa Maria soil, the effect of soil treatment on bacterial communities was less definitive. Bacterial communities from the untreated control and fish emulsion treatment appeared to form one similarity cluster. With the exception of one sample from the ASD + FE treatment, bacterial communities from all soil treatments containing ASD and the fumigation treatment formed a second cluster based on assessment of similarity (Fig. 7c,d).

Weed populations

Steam and Pic-Clor 60 treatments were equally effective at reducing weed populations at the Watsonville site across both years (Table 4). In contrast, MSM did not affect weed populations in either year, whereas ASD RB showed a modest reduction in 2010/11 but not in 2011/12, and ASD RB + MSM had no effect in 2010/11 but a modest impact in 2011/12.

Discussion

Compared to soil solarization, which requires a soil temperature of >40 °C at 5 cm depth for 4–6 weeks (Elmore *et al.*, 1997), ASD can provide effective control of

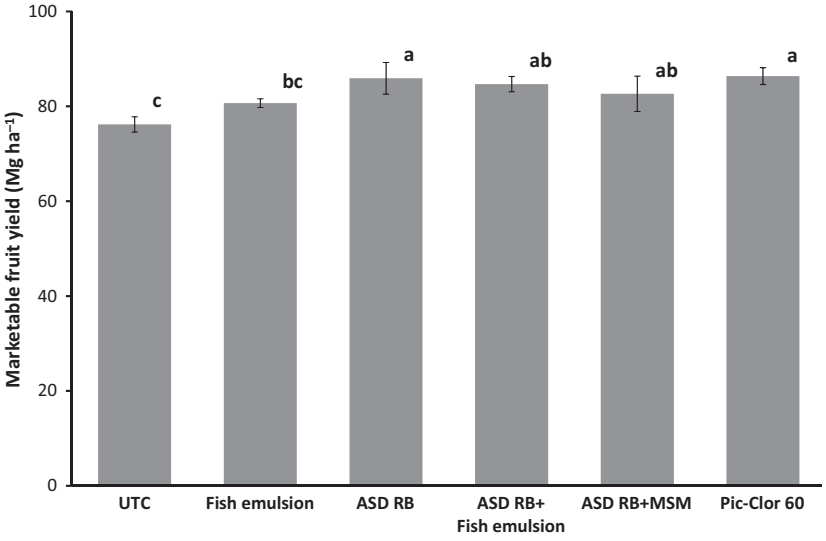

Figure 5 Effect of soil treatments on cumulative yield of marketable fruit in strawberry field trials at the Santa Maria site in 2012. UTC: untreated control; ASD RB: anaerobic soil disinfestation with rice bran 20 Mg ha^{-1}; ASD RB + Fish emulsion: ASD with RB plus acidified fish emulsion diluted 1:50 with water applied twice monthly at 140 L ha^{-1}; ASD RB + MSM: ASD with RB 16.7 Mg ha^{-1} plus mustard seed meal 3.3 Mg ha^{-1}; Pic-Clor 60: bed fumigation with Pic-Clor 60 at 337 kg ha^{-1}. Each bar indicates mean ± SEM (n = 4) and bars with the same letter do not have significant difference according to protected-LSD test (α = 0.05).

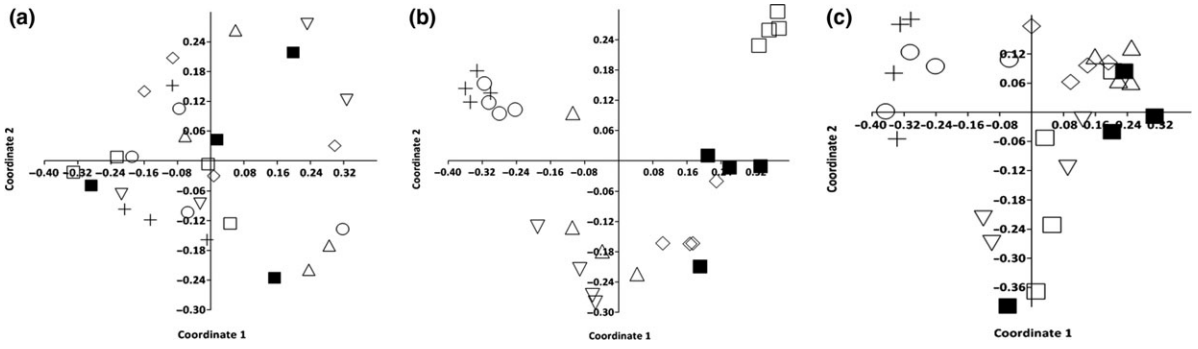

Figure 6 Similarity of fungal community composition among soil treatments prior to soil treatment (a), post-treatment application (b), and post-harvest (c) in strawberry field trials at the Watsonville site. Similarity was assessed by principal coordinate analysis of terminal-restriction fragment length polymorphism (T-RFLP) data obtained using DNA from soils sampled during the 2011/12 season. Analyses were conducted using the Jaccard similarity coefficient of profiles generated from digestions of amplified fungal DNA using primers specific for the ITS region of rDNA. Treatments: ◊ = untreated control; + = anaerobic soil disinfestation (ASD) with rice bran (RB) 20 Mg ha^{-1}; ○ = ASD with RB 16.7 Mg ha^{-1} plus mustard seed meal (MSM) 3.3 Mg ha^{-1}; ▽ = MSM 3.3 Mg ha^{-1}; □ = bed fumigation with Pic-Clor 60 337 kg ha^{-1}; ■ = steam; △ = steam plus MSM 3.3 Mg ha^{-1}.

Table 3 Effect of treatments on number of fungal taxa and diversity of the community, determined by analysis of terminal restriction fragment length polymorphism data of DNA from soils of strawberry field trials at the Watsonville site, 2011/12

	Pre-treatment		Post-treatment		End of season	
Treatment[a]	No. of OTUs[b]	Shannon *H*[c]	No. of OTUs	Shannon *H*	No. of OTUs	Shannon *H*
UTC	99 ± 21	4.69 ± 0.32	143 ± 19	4.94 ± 0.13	79 ± 17	4.33 ± 0.21
MSM	81 ± 22	4.39 ± 0.28	261 ± 55	5.54 ± 0.19	97 ± 15	4.53 ± 0.14
Steam	93 ± 27	4.50 ± 0.27	115 ± 17	4.72 ± 0.15	67 ± 25	4.16 ± 0.21
ASD RB	103 ± 17	4.61 ± 0.18	490 ± 64	6.17 ± 0.14	143 ± 21	4.91 ± 0.14
Steam + MSM	114 ± 16	4.71 ± 0.14	243 ± 59	5.46 ± 0.21	76 ± 33	3.95 ± 0.39
ASD RB + MSM	93 ± 29	4.48 ± 0.33	340 ± 48	5.82 ± 0.13	129 ± 12	4.89 ± 0.11
Pic-Clor 60	98 ± 15	4.56 ± 0.16	80 ± 6	4.40 ± 0.12	79 ± 15	4.29 ± 0.20

Data are mean ± standard deviation.

[a]UTC, untreated control; MSM, mustard seed meal 3.3 Mg ha^{-1}; ASD RB, anaerobic soil disinfestation with rice bran 20 Mg ha^{-1}; steam + MSM, steam plus MSM 3.3 Mg ha^{-1}; ASD RB + MSM, ASD RB 16.7 Mg ha^{-1} plus MSM 3.3 Mg ha^{-1}; Pic-Clor 60, bed fumigation with Pic-Clor 60, 337 kg ha^{-1}.

[b]OTU, operational taxonomic unit.

[c]Shannon *H*, Shannon's diversity index.

certain pathogens at lower soil temperatures by creating anaerobic conditions in the soil. However, the present study clearly showed the importance of both the level of anaerobic condition and soil temperature for consistent ASD-induced control of *V. dahliae*. Over 50 000 cumulative mV h under 200 mV were required to consistently eliminate 80–100% of *V. dahliae* microsclerotia at 25 °C, but at 15 °C, the same or greater level of anaerobic condition did not result in effective pathogen control. Temperature and Eh thresholds during ASD for control of other pathogens are likely to be pathogen specific. Ebihara & Uematsu (2014) showed that under anaerobic conditions at 22.5 °C, *F. oxysporum* f. sp. *fragariae* and *Phytophthora cactorum* still grew slightly but *V. dahliae* did not grow. In field trials Yonemoto *et al.* (2006) found that *F. oxysporum* was consistently suppressed by ASD when 280–300 h of soil temperatures above 30 °C at 20 cm depth was achieved. Both results suggest that higher soil temperatures are needed for suppressing *F. oxysporum* with ASD than for *V. dahliae*. In FL, ASD using composted broiler litter and molasses, 50 mm of irrigation, and high soil temperatures was as effective as fumigation with 1,3-D for control of *F. oxysporum* and *M. phaseolina* (Rosskopf *et al.*, 2015). In CA, only modest control of either pathogen has been achieved to date, implying that soil temperature and/or C-source are critical for their control.

In the present study, the early season increases in strawberry yield observed with ASD may have been caused by the application of RB itself leading to enhanced fertility. Rice bran contains N 23 g kg^{-1}, P 18 g kg^{-1} and K 17 g kg^{-11} on a fresh weight basis. Therefore 20 Mg ha^{-1} of rice bran would provide 460, 360 and 340 kg ha^{-1} of N, P and K, respectively, which could increase fruit yield just by improving soil fertility in low disease pressure sites. Subsequent work has found that pre-plant fertilizer can be eliminated when rice bran is used as a carbon source, and that it is important to adjust fertility inputs to avoid excessive levels of nitrate post-ASD that are vulnerable to loss by leaching and denitrification. Nonetheless, the importance of adequate anaerobic conditions for disease suppression is indicated by both controlled environment studies, where disease suppression was not consistently observed in the absence of anaerobic conditions, and from the second year field trial at the Watsonville site. Here, the amount of water added was insufficient to reach the 50 000 mV h below 200 mV threshold and resulted in lower yields with ASD RB than with steam or fumigation. Optimizing water management to ensure good anaerobic conditions, while using as little water as possible, is important for water conservation and also to reduce potential leaching or denitrification losses of nitrate initially present in the soil.

Across the four field trials there was no consistent benefit of adding MSM to RB as a carbon source for ASD in terms of yields or suppression of *V. dahliae*. Furthermore, effects were similar between ASD RB and ASD RB + MSM on root infection by *Pythium* or *Rhizoctonia*; however, the addition of MSM increased suppression of *Cylindrocarpon*, but resulted in higher levels of *Fusarium* than either the UTC or ASD RB. While it is apparent that a number of different carbon sources are effective at controlling *V. dahliae* there may be some pathogens where the carbon source makes a difference to ASD efficacy (Butler *et al.*, 2012). Similarly, no synergistic effects were observed by adding FE during and after ASD. One of the mechanisms for achieving disease suppression with ASD is thought to be the production of organic acids (Momma, 2008), so adding more organic acids by FE application might be expected to provide additional disease control. However, application of organic acids alone has not achieved the same level of control as ASD (Rosskopf *et al.*, 2014). Disease pressure was also very low at the Santa Maria site, therefore no conclusions can be drawn about suppression with FE from this experiment, but repetition of these experiments in heavily infested fields would yield more information.

All soil treatments examined in these studies exhibited varying capacity to suppress strawberry root infections in a pathogen-specific manner. The volatiles produced

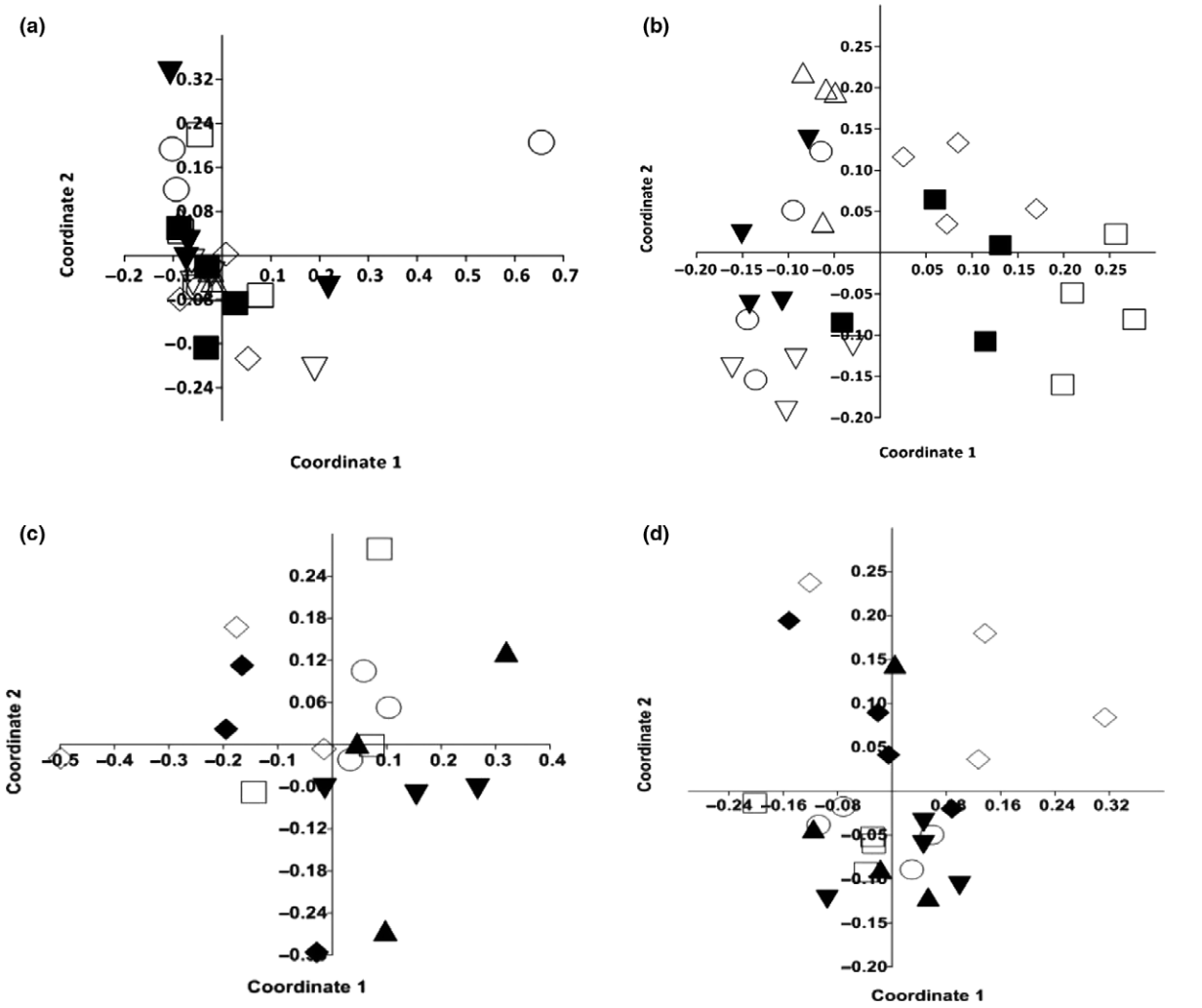

Figure 7 Similarity of bacterial community composition among soil treatments at the Watsonville and Santa Maria sites. Samples were collected prior to soil treatment ((a), Watsonville; (c), Santa Maria) and post-treatment ((b), Watsonville; (d), Santa, Maria) during the 2011/12 growing season. T-RFLP data obtained using DNA from soils sampled during the 2011/2012 season. Analyses were conducted using the Jaccard similarity coefficient of profiles generated from digestions of amplified bacterial DNA using primers specific for the 16S rRNA gene. Treatments: ◊ = untreated control; ▼ = anaerobic soil disinfestation (ASD) with rice bran (RB) 20 Mg ha^{-1}; ○ = ASD with RB 16.7 Mg ha^{-1} plus mustard seed meal (MSM) 3.3 Mg ha^{-1}; ▽ = MSM 3.3 Mg ha^{-1}; □ = bed fumigation with Pic-Clor 60 337 kg ha^{-1}; ■ = steam; △ = steam plus MSM 3.3 Mg ha^{-1}; ◆ = fish emulsion (diluted 1:50 with water), twice monthly 140 L ha^{-1}; ▲ = ASD with RB 20 Mg ha^{-1} plus fish emulsion (diluted 1:50 with water), twice monthly 140 L ha^{-1}.

during ASD with RB used as the C-source were shown to have differential effects on inhibition of several strawberry pathogens. For instance, the dominant volatile generated, 2-ethyl-1-hexanol, limited hyphal growth of *P. ultimum* and *Rhizoctonia solani* AG-5, but demonstrated no activity toward *F. oxysporum* f. sp. *fragariae* (Hewavitharana *et al.*, 2014). Modification of ASD by altering the carbon input resulted in generation of different volatile spectra with varying levels of inhibition towards targeted pathogen groups. Thus, adjusting the type of the carbon input during ASD could offer a way to target control of specific pathogens.

Although seed meal from *B. juncea* produces volatiles (e.g. allyl isothiocyanate, AITC) that exhibit significant inhibitory activity against the pathogens examined in the present trials (Hewavitharana *et al.*, 2014), it did not effectively suppress the incidence of *Fusarium* or *Pythium* spp. isolation from strawberry roots. This may have been due to failure to attain the volatile concentration threshold required for fungicidal activity, as the pelleted form of the seed meal used in these trials did not generate significant AITC in subsequent studies. In this environment, proliferation of *Pythium* spp. is expected as this oomycete has repeatedly shown the ability to use Brassicaceae seed meals as a growth substrate in the absence of AITC production (Mazzola *et al.*, 2009). Under conditions that amplified *Pythium* spp. density, ASD + MSM continued to suppress root infection by *Pythium* spp. to the same degree as that realized under ASD RB alone, suggesting that ASD should provide

effective control of *Pythium* spp. under a variety of conditions; this is significant due to the ubiquitous nature of this pathogen.

Soil treatments may achieve efficient disease control through various mechanisms. Steam controls disease through thermal inactivation of pathogen propagules (Schweigkofler *et al.*, 2014), MSM has been shown to suppress pathogens directly via chemical means as well as through generation of a disease suppressive soil microbiome (Mazzola *et al.*, 2015). Some studies reported no lasting effects on soil microbial communities in response to steam (Norberg *et al.*, 2001) whereas others report a more significant change (Tanaka *et al.*, 2003; Yamamoto *et al.*, 2008). Soil fumigants have been reported to alter microbial community diversity and abundance (Yamamoto *et al.*, 2008; Mazzola *et al.*, 2015); however, persistence of these effects appears to vary, with recovery to the nontreated system generally observed. In the present study, steam and Pic-Clor fumigation significantly reduced the number of fungal taxa detected in soils, while ASD and MSM treatments increased both number of fungal taxa and diversity. Qualitative shifts and enhanced diversity of the soil or rhizosphere microbiome is cited as a factor contributing to system resistance and a reduction in soilborne disease incidence (Klein *et al.*, 2013). These findings are in direct contrast to a previous study that employed a ground form of MSM, which resulted in a significant depression of fungal and bacterial diversity (Mazzola *et al.*, 2015).

Alteration of the soil microbiome has been proposed to play a role in disease suppression with both ASD (Momma *et al.*, 2013) and MSM (Mazzola *et al.*, 2015), although the functional microbial attributes have not been established and may differ depending upon application or site variables. With ASD, shifts in soil fungal and bacterial community profiles have consistently been associated with effective disease suppression, but it is not clear which specific elements of these communities function to suppress specific soilborne pathogens. Preliminary evidence indicates that amplification of the density of bacteroidetes in soil is at least a biological indicator of effective ASD disease suppression in strawberry, but the functional community may vary not only with the target pathogen but also the carbon input used. Changes in the soil microbiome induced by ASD treatment can be persistent, which may result in long-term system resistance to pathogen re-infestation (Goud *et al.*, 2004). In the present study, differences in soil fungal community composition induced by ASD RB were evident, even at the end of the strawberry production season.

ASD did not effectively suppress weed density in these trials, although ASD + MSM did reduce weed density relative to the control in the 2011/12 field trial. Control of grass weeds with ASD has been seen in FL, but control of nutsedge (*Cyperus* spp.) was more dependent on use of composted broiler litter and solarization than on anaerobic conditions (E. Rosskopf, unpublished data). The temperatures associated with ASD in FL generally exceed 30 °C during treatment periods (Butler *et al.*, 2012). The use of totally impermeable film with ASD in FL provided adequate weed control in some locations, but was inconsistent even with an increased quantity of carbon (Di Gioia *et al.*, 2016). The lower soil temperature during ASD in CA may explain the lack of weed control observed here, or addition of the composted broiler litter itself may lead to release of organic acids that inhibit weed seed germination (Ozores-Hampton, 1998) and enhance the ASD effect in FL. ASD with grass as a carbon input reduced weed biomass in an apple orchard replant site when applied in the autumn using a clear impermeable film (M. Mazzola, unpublished data). Soil temperature ranged from 16 to 23 °C during treatment suggesting that generation of herbicidal compounds during ASD with grass may not be limited by low soil temperatures.

In three of the four trials reported here, ASD with 20 Mg RB ha^{-1} as a carbon source gave similar or better net returns above harvest and treatment costs to fumigation with Pic-Clor, without making adjustments for potential saving on pre-plant fertilizer. In the remaining trial, yields were reduced with RB ASD relative to fumigation and hence net returns were also reduced. Subsequent work has found that pre-plant fertilizer can be eliminated when using 20 Mg ha^{-1} rice bran in autumn-applied ASD (C. Shennan and J. Muramoto, unpublished data), which represents an additional saving of around \$1000 ha^{-1} (Bolda *et al.*, 2010). Thus ASD with 20 Mg ha^{-1} RB can be an economically viable option for growers at the average prices seen over the past 5 years. Indeed, the commercial strawberry growing area in CA under ASD has risen rapidly to over 560 ha in 2016. Nonetheless, growers are concerned about the cost of rice bran and its future availability. Prices of rice bran and fumigation fluctuate from year to year and also depend upon the mode of fumigant application, i.e. whether it is applied as a bed treatment or a broadcast treatment. It is instructive to look at the effect of

Table 4 Effect of soil treatments on total weed density in strawberry field trials at Watsonville in the 2010/11 and 2011/12 seasons.

Treatment[a]	2010/11 (weed no. per m^2)	2011/12 (weed no. per m^2)
UTC	302 a	58 a
MSM	273 ab	54 a
ASD RB	214 b	49 ab
Steam	51 c	5 c
ASD RB + MSM	245 ab	36 b
Steam + MSM	40 c	6 c
Pic-Clor 60	40 c	2 c

Mean values within a column with the same letter are not significantly different according to Fisher's LSD test ($\alpha = 0.05$).

[a]UTC, untreated control; MSM, mustard seed meal 3.3 Mg ha^{-1}; ASD RB, anaerobic soil disinfestation with rice bran 20 Mg ha^{-1}; steam + MSM, steam plus MSM 3.3 Mg ha^{-1}; ASD RB + MSM, ASD RB 16.7 Mg ha^{-1} + MSM 3.3 Mg ha^{-1}; Pic-Clor 60, bed fumigation with Pic-Clor 60, 337 kg ha^{-1}.

Table 5 Net returns above harvest and treatment cost for anaerobic soil disinfestation using 20 Mg ha^{-1} rice bran calculated as a percentage of net returns from fumigation with Pic-Clor 60 for two locations where yields were similar across treatments, using a range of hypothetical costs of rice bran.

	Price of rice bran ($ Mg^{-1})			
Treatment	268	317	363	454
Bed fumigation @ $596 ha^{-1}				
Watsonville 2010/11	92.0	89.8	87.7	83.7
Santa Maria 2011/12	96.2	95.1	93.9	91.7
Broadcast fumigation @ $1402 ha^{-1}				
Watsonville 2010/11	101.0	98.7	96.5	92.0
Santa Maria 2011/12	101.0	10.00	98.8	96.4

Average cost of rice bran for the past 5 years was $268 Mg^{-1}. Costs of fumigation are estimated either for bed fumigation or for broadcast fumigation.

increased costs of RB on the ASD net returns relative to fumigation under both scenarios. Across a wide range of prices, ASD RB at 20 Mg ha^{-1} compares well with broadcast fumigation, with returns of 92–96% of fumigation even at a price of RB that is 69% higher than the average over the past 5 years (Table 5). However, in comparison with current costs of bed fumigation, returns above harvest and treatment costs are 84–92% of bed fumigation at the highest RB price used (Table 5).

Clearly, for widespread adoption of ASD it will be important to find a range of effective carbon sources to reduce reliance on a single material and potentially lower costs. Ideally, carbon sources would be found among agricultural waste products in each region, reducing costs of obtaining and shipping material. In Florida, for example, a double-filtered molasses is commercially available for use in ASD, which is waste from the local sugar industry that can be applied via drip lines (ACS, Terra Feed, LLC, FL). If liquid materials were as effective as the solid carbon sources now being used, this would simplify field management as the material could be applied through drip lines after the plastic has been installed. Similarly, from a nutrient management perspective, being able to reduce the amount of RB used or mixing it with a lower N material could reduce potential for nitrogen losses through leaching or denitrification. Experiments are underway to test a range of options including growing a summer cover crop prior to ASD as at least a partial carbon source.

In summary, ASD can provide control of a number of important soilborne pathogens in CA strawberry production, and the approach is already being adopted by commercial growers. Other strategies including steam, mustard seed meal addition and application of fish emulsion were either effective, but more expensive (steam), or less effective and uneconomical (MSM and fish emulsion). A range of carbon sources for ASD proved to be effective against *V. dahliae*, although current work suggests that other pathogens may require specific types of carbon sources to achieve adequate disease control. Temperature and cumulative anaerobic condition thresholds for efficacy of ASD appear to be pathogen specific. For example, *F. oxysporum* f. sp. *fragariae* requires much higher soil temperatures for ASD to be effective (Yonemoto *et al.*, 2006) than found for *V. dahliae* in the present study. Future research needs to elucidate the mechanisms important for suppression of different pathogens so that management recommendations can be tailored for specific conditions and pathogens present. Furthermore, to avoid unnecessary N losses via denitrification and leaching during and after ASD, it will be important to modify fertility management practices according to the carbon source used and the patterns of N mineralization observed.

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