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Enzyme activities and microbial biomass in agricultural soils fumigated with methyl bromide alternatives

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ENZYME ACTIVITIES IN AGRICULTURL SOILS FUMIGATED WITH METHYL BROMIDE ALTERNATIVES

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Abstract

Pre-plant fumigation of agricultural soils with a combination of methyl bromide (MeBr) and chloropicrin (CP) to control nematodes, soil-borne pathogens and weeds has been a common practice in strawberry (*Fragaria* X *ananassa* Duchesne) production since the 1960s. MeBr will be phased out by 2005, but little is known about the impacts of alternative fumigants on soil microbial processes. We investigated the response of microbial biomass and enzyme activities in soils fumigated over two years with MeBr + CP and the alternatives propargyl bromide (PrBr), InLine, Midas and CP. Results were compared to control soils, which were not fumigated for the last 4 to 5 years for Watsonville and Oxnard, respectively, but had a 10 year history of MeBr + CP fumigation (history soils). Soil samples (0-15cm) were taken from two sites in the coastal areas of California, USA, in Watsonville and Oxnard, at peak strawberry production after two years of repeated application. In addition to the soil enzymes, the activities of purified reference enzymes of β-glucosidase, acid phosphatase and arylsulfatase were assayed before and after fumigation with MeBr + CP and alternative biocides. At the Oxnard site, microbial respiration significantly decreased in soils fumigated with MeBr + CP ($P = 0.036$), while microbial biomass C and N showed no response to fumigation at both sites. These results may indicate that fumigation promotes the growth of resistant species or that soil microorganisms had recovered at the time of sampling. Repeated soil fumigation with MeBr + CP significantly decreased the activities of β-glucosidase and acid phosphatase at the Watsonville site, and dehydrogenase activity at the Oxnard site. Although, enzyme activities in soils fumigated with PrBr, InLine, Midas and CP were lower compared to the control soil, effects were, in general, not significant. Fumigation with MeBr + CP and alternatives reduced the activities of purified reference enzymes by 13, 76 and 28% for acid

phosphatase, β-glucosidase and arylsulfatase, respectively. Mean enzyme protein concentrations in fumigated agricultural soils were 2.93, 0.105, and 2.95 mg protein kg^{-1} soil for acid phosphatase, β-glucosidase and arylsulfatase, respectively, all lower than in control soils. Organic matter turnover and nutrient cycling, and thus, the long-term productivity of agricultural soils seem unaffected in soils repeatedly fumigated with PrBr, InLine, Midas and CP.

Keywords: Methyl bromide alternatives, Agricultural soils, Acid phosphatase, β-Glucosidase, Arylsulfatase, Enzyme protein concentrations

1. Introduction

Methyl bromide (CH_3Br, MeBr) is a soil fumigant of environmental concern because of its high potential to deplete stratospheric ozone (Wofsy et al., 1975; Prather et al., 1984). A treaty signed by 160 nations of the United Nations Environment Program (UNEP) regulates the stepwise decrease of MeBr consumption to a complete phase-out by January 2005 for developed countries and by 2015 for developing countries (US Department of Agriculture, 2000). The stringent regulations limiting the use of MeBr prior to its complete phase-out stimulated the search for alternative fumigants because soil fumigation remains a central tool in strawberry production.

For the past 45 years, preplant fumigation of agricultural soils with a combination of MeBr and chloropicrin (CP) has been a reliable and effective tool to control soilborne pathogens, nematodes and weeds in many vegetable, fruit, nuts and nursery crops worldwide. The irritant compound CP is added to the odorless MeBr as a warning agent to reduce the risk of accidents during soil fumigation and because of the synergistic biocidal effect of these two chemicals on soil pathogens (Wilhelm et al., 1961). The elimination of MeBr could severely impact growers and farmers in the United States and the Mediterranean region (Ajwa et al., 2003; Haar et al., 2003). In continuous strawberry (*Fragaria x ananassa Duch*.) production systems, the soil may host many deleterious nematodes and pathogens such as *Phytophtora cactorum*, *P. fragariae*, *Verticillium dahliae* and *Colletotrichum acutatum*. In California, where 80% of US strawberries are

grown, MeBr + CP combinations effectively control wilt disease (*Verticillium dahliae* Kleb.), thus playing a crucial role in commercial strawberry production.

Currently, there are several available alternatives to MeBr, including an emulsifiable concentrate of CP (CP-EC) and 1,3-dichloropropene (1,3-D). Applied alone, CP has high biocidal activity against fungal pathogens but is not as effective as MeBr against weeds and nematodes. Another viable alternative is 1,3-dichloropropene (1,3-D), which is an effective nematicide but has relatively low activity against fungi and weeds (Noling and Becker, 1994). To broaden its biocidal activity, 1,3-D can be combined with chloropicrin as found in various combinations such as InLine (61% 1,3-D and 35% CP). In addition, several experimental chemical alternatives are being studied for their efficacies against pathogens and pests. Iodomethane (Midas) can be as effective as MeBr, and it is not as likely to deplete ozone because Midas is photolyzed before it reaches the stratosphere (Solomon et al., 1994). The dilution of Midas with CP can decrease costs for this fumigant and increase efficacy due to synergy with CP (Hutchinson et al., 2000). Another experimental chemical alternative is propargyl bromide (PrBr), which was developed during the 1960s. Although, PrBr demonstrated potential as a viable MeBr replacement it was never registered due to its highly explosive character (Yates and Gan, 1998). With the development of a stabilized formulation of PrBr, research interest in this compound as a soil fumigant has increased recently.

Studies have been conducted to determine the biological degradation of various fumigants in soil (Miller et al., 1997; Gan et al., 1999, 2000) and their efficacies against soilborne pests and weeds relative to MeBr + CP combinations (Fennimore et al., 2003; Haar et al., 2003). Fumigants are among the pesticides with notable effects on soil microorganisms because of their broad biocidal activity (Domsch et al., 1983; Anderson, 1993). The high biocidal activity of fumigants may cause a "biological vacuum" and increase pathogen re-colonization. Kandeler et al. (1996) suggested that the composition of the microbial community strongly affects the potential of a soil for enzyme-mediated substrate catalysis. Consequently, changes in microbial diversity in fumigated soils may also reduce microbial functionality. Enzyme activities can be used as an index of microbial functional diversity (Nannipieri et al., 2002), although accumulated (adsorbed to

soil surfaces and free) enzymes may contribute considerably to the overall enzyme activity of a soil.

A semi-quantitative method to determine enzyme protein contents in soil based on the specific activities of reference enzymes and enzyme activity values of soils was reported by Klose and Tabatabai (1999, 2002a, b) in order to prove whether there is a direct correlation between the activity of any enzyme and its protein concentration in soil. This approach is based on the assumption that the compositions of the reference enzymes are similar to those in soils. Protein concentrations were suggested to serve as a suitable measure to quantify the effects of environmental changes, for example after application of pesticides, on soil biological properties (Klose and Tabatabai, 2002a).

The understanding of the impacts of pesticide fumigants on key biochemical reactions involved in organic matter degradation and soil nutrient dynamics (i.e., enzyme mediated processes) is important in order to evaluate the ecological significance of fumigation on the soil system. The toxicity of fumigants is related to (i) their interference with respiratory enzymes, including pyruvate dehydrogenase, (ii) their ability to chelate metal cations such as Cu, (iii) the inhibition by the unchelated ion, and (iv) toxic degradation products such as methyl isothiocyanate (MITC) (Corbett et al., 1984; Staub et al., 1995). MeBr can be degraded in soils by the following three pathways (Shorter et al., 1995; Yagi et al., 1995; Ou et al., 1997): a) chemical hydrolysis to form methanol and bromide, b) methylation to soil organic matter and release of bromide ion, and c) microbial oxidation to form formaldehyde and bromide ion. Biological hydrolysis and other microbial processes involving enzymatic processes are also likely to contribute to the degradation of MeBr in soil (Ou, 1997).

Microbial respiration, nitrification potential, and dehydrogenase and arylsulfatase activities were inhibited by MeBr + CP and the alternatives PrBr, InLine, Midas and CP-EC one week after soil fumigation (Schutter et al., 2001; and submitted for publication). After 30 weeks, there was no difference in microbial biomass and activities between the treatments studied, with the exception of lower acid phosphatase and arylsulfatase activities in fumigated soils. These results indicate that there are short- and long-term differences in the response of various microbial and enzymatic processes to MeBr + CP and alternative fumigants and thus, of the various functions of the soil biota in

ecosystems. A limitation of this study is that it was conducted for a maximum of 37 weeks; it remains unknown if MeBr + CP and alternative fumigants have longer-term impacts on soil biochemical processes under field conditions after multiple applications.

The objective of this study was to evaluate the effect of repeated soil fumigation with MeBr + CP and two registered (InLine, CP-EC) and two non-registered (PrBr, Midas) alternative fumigants on microbial biomass and respiration, the activities of dehydrogenase, acid phosphatase, β-glucosidase and arylsulfatase, and enzyme protein concentrations in soils. Furthermore, the effect of these fumigants was evaluated on dry proteins containing β-glucosidase, acid phosphatase and arylsulfatase in the absence of immobilizing or protecting constituents of soil (i.e., mineral and humic colloids).

The selected alternative fumigants represent the actual formulations that likely will be used by growers for strawberry production. Dehydrogenase activities (EC 1.1.) were selected because they reflect the total oxidative activities of soil microorganisms and are important in oxidizing soil organic matter. Acid phosphatase (EC 3.1.3.2) catalyzes the hydrolysis of a variety of organic phosphomonoesters and is therefore important in soil organic P mineralization and plant nutrition. The enzyme β-glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of cellobiose, and thus plays a major role in the initial phases of the decomposition of organic C compounds. Arylsulfatase (EC 3.1.6.1) is believed to be partly responsible for S cycling in soils as it participates in the process whereby organic sulfate esters are mineralized and made available for plants.

The first aim of the present study was to test whether soil fumigation with these four potential pesticides will alter important soil functions that, in turn, will affect the long-term productivity of agricultural soils. The second aim of this study was to evaluate the effects of soil fumigation on the activities of enzyme proteins, which may be present in the soil as free enzymes and not protected by clay-humus complexes. Free enzymes are likely to be more sensitive to environmental factors as intracellular or adsorbed enzymes, which are protected by the cell envelope or by clay-humic complexes.

2. Materials and methods

2.1. Site description and treatment application

Field studies were conducted in California, USA, in the central region in Watsonville ($121^0$50'W, $36^0$54'N) and in the southern region in Oxnard ($119^0$123'W, $34^0$146'N) in 2000 and 2001. Both sites are located in intensive strawberry production areas of California. Soil at both locations had not been fumigated for the past 2 and 3 years prior to this experiment for Watsonville and Oxnard site, respectively. However, before that soil at both sites had been fumigated routinely with MeBr + CP for the past 10 years. The soil in Watsonville is classified as an Elder sandy loam (coarse-loamy, mixed, thermic, Cumulic Haploxeroll). The soil in Oxnard is classified as a Hueneme sandy loam (coarse-loamy, mixed, calcareous, thermic, Aquic Xerofluvents). The past 50-year average annual precipitation is 582 mm and 385 mm at Watsonville and Oxnard, respectively. The average annual maximum and minimum temperature at Watsonville is 19.5ºC and 10.7ºC, respectively. Corresponding values for Oxnard are 21.2ºC and 10.7ºC.

Commercial agricultural practices for the area were followed (Calif. Strawberry Commission, 1999). The soil was tilled and beds were formed in Watsonville at 132 cm center-to-center (81 cm wide x 30 cm high) and in Oxnard at 173 cm center-to-center (122 cm wide x 30 cm high). Slow release fertilizer (27N-10P-12K) was applied to the beds at the rate of 400 kg ha^{-1} y^{-1}. A drip irrigation system was used consisting of two drip tapes (Netafilm Streamline 60; Netafilm, Fresno, Calif., USA), with emitters spaced 30 cm apart and an emitter flow rate of 0.87 l min^{-1} at 70 kPa, placed 10 cm (in Watsonville) and 30 cm (in Oxnard) from the bed center at a soil depth ranging from 2 to 5 cm. Fumigation treatments were randomized in a complete block design with four replicates per treatment at each site. Fumigants used, fumigant rates and application methods are summarized in Table 1. Each replicate consisted of three neighboring 15-m long beds. Soil at both sites was fumigated once a year in summer over a two-year period. Soil in Watsonville was fumigated on August 10, 2000 and September 27, 2001, the soil in Oxnard was fumigated on September 1, 2000 and August 24, 2001. At the time of fumigation, the average daily soil temperature within the raised bed ranged between 16 to 20ºC, and the average soil water content was less than 85% of field capacity (soil matric potentials ranged between -7.5 and -8.5 kPa). About 4 weeks after fumigation bareroot strawberry [*Fragaria* X *ananassa* Duchesne, variety "Diamante" (Watsonville) and "Camarosa" (Oxnard)] was transplanted in 2000 and 2001.

((Table 1))

2.2. Soil sampling and analysis of physical and chemical properties

Topsoil (0-15 cm) was collected in 2001 (i.e., the second year of fumigation treatment) on September 30 in Watsonville and on August 27 in Oxnard (e.g., 3 days after the second soil fumigation). Three subsamples, two near the drip tapes and one from the center of the bed, were collected from the middle bed of each treatment, and pooled to one sample (approximately 500 g dry soil). Soils were passed through a 2-mm mesh sieve and stored at 4ºC for microbial biomass determination and enzyme assays. Soil microbial analysis was completed within 3 weeks of sampling. A subsample of the sieved soil was air-dried for physical and chemical analysis.

Soil water content was determined by drying 10 g moist soil to constant mass at 105ºC. Measurements were carried out in duplicate. The particle-size distribution was measured on air-dried samples by the hydrometer method (Gee and Or, 1994). The pH was determined by a combination glass-electrode in 0.01 M $CaCl_2$ solution and in H_2O after 1 h (1:2.5 w/v). Total C and N contents were analysed on <180-µm samples with a Vario Max-ELEMENTAR CN-analyzer (D-63452 Hanau; Germany).

2.3. Soil microbial biomass, microbial respiration, and enzyme assays

Microbial biomass C and N were estimated by extracting 15-g oven-dry equivalents of field moist soils in 0.5 M K_2SO_4 (1:5 w/v) after chloroform fumigation (Brookes et al., 1985; Vance et al., 1987). Organic C and N concentrations in the extracts were determined with a CN analyzer (Shimadzu Model TOC-V/$_{CPH}$-TN) after acidification with one drop of 25% H_3PO_4 to remove any dissolved carbonate. Microbial biomass C and N were calculated using a k_{EC} of 0.45 (Wu et al., 1990) and a k_{EN} of 0.54 (Jenkinson, 1988).

Microbial respiration was determined on 15-g oven-dry equivalents of soil samples moistened with distilled H_2O to field capacity (e.g., 210 and 150 g H_2O kg^{-1} soil for Watsonville and Oxnard soils, respectively) incubated in 120 ml serum bottles fitted with rubber septa. Soil samples were conditioned at 22˚C for 72 h before respiration measurements. Headspace CO_2 concentrations were measured after incubation at 26˚C for

10 d with a Shimadzu GC-8A gas chromatograph (equipped with a 2 m PoropakQ column, operated at 70˚C) (Shimadzu Scientific Instruments Inc., Columbia, MD). Microbial respiration is expressed as a rate (mg CO_2-C kg^{-1} soil h^{-1}) after correcting for controls [e.g., CO_2 release from autoclaved (3 times for 30 min at 121˚C) soil].

Potential dehydrogenase activity was assayed by incubating 5 g moist soil amended with glucose [16 mg g^{-1} dry soil, finely ground and mixed with talcum powder (1:3 w/w)] with 5 ml of triphenyltetrazolium chloride (TTC) solution [0.8 %, dissolved in Tris buffer (0.1 M, pH 7.6)] at 30°C for 24 h. Controls contained only 5 ml Tris buffer (0.1 M, pH 7.6). Triphenyl formazan (TPF) produced was extracted with methanol and estimated colorimetrically (Thalmann, 1968). Results are expressed as mg of TPF released kg^{-1} soil 24 h^{-1}.

The activities of acid phosphatase, β-glucosidase and arylsulfatase were assayed on 1-g oven-dry equivalents of buffered soil solutions incubated for 1 h at 37°C after addition of the enzyme-specific substrate solution (Tabatabai, 1994). The product of all reactions, *p*-nitrophenol (PN), was measured colorimetrically (Tabatabai, 1994) and is expressed as mg of PN kg^{-1} soil h^{-1}.

2.4. Protein analysis

The reference enzyme proteins β-glucosidase (source: Almonds), acid phosphatase [source: Wheat germ (Type I)] and arylsulfatase [source: limpets, *Patella vulgate* (Type V)] tested, were obtained from Sigma (St. Louis, Mo.). A reference protein of dehydrogenase was not commercially available, and therefore, this enzyme was not included in protein analyses. The total protein concentration of these enzymes was determined by the Lowry method (Lowry et al., 1951). Because the reference enzymes contained proteins other than those of β-glucosidase, acid phosphatase and arylsulfatase, molecular weights and percentages of the specific enzyme proteins were estimated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Details of the methods were previously described (Klose and Tabatabai, 1999). The molecular weight and purity of the reference proteins are summarized in Table 2.

Prior to analyses, the enzyme proteins were fumigated with MeBr + CP and the four tested alternatives in microcosms at 25°C for 24 h. Enzyme assays were performed

on fumigated samples and the nonfumigated control within 30 min after fumigation as described in Table 2.

((Table 2))

2.5. Data analysis

From the specific activity of the reference proteins and the activity values of the corresponding enzyme in the control and the fumigated soils, the enzyme protein concentrations [mg enzyme protein (kg soil)$^{-1}$] were calculated. Percentage change in activities due to fumigant treatment was calculated as [(A-B)/A] x 100, where A is the activity value of the control soil and B is the activity value of the treated soil. Enzyme activities were assayed for one control and in duplicate otherwise. All data were calculated on an oven-dry (105°C) basis and are given as arithmetic means of 4 field replicates for each site.

One-way analysis of variance (ANOVA) was used to assess treatment effects, and differences among means were calculated with Fisher's least significant differences (LSD) test, with SPSS (version 10.07 for Windows).

3. Results

3.1. Soil physical and chemical properties

The soil in Watsonville was characterized by a mean particle size distribution of 62% sand, 26% silt and 12% clay, pH of 7.75 (H_2O) and 7.08 (0.01 M $CaCl_2$) and 6 g kg^{-1} organic C. The soil in Oxnard revealed a particle size distribution of 60% sand, 28% silt and 12% clay, pH of 7.82 (H_2O) and 7.42 (0.01 M $CaCl_2$) and 7 g kg^{-1} organic C.

3.2 Microbial biomass and respiration

Microbial biomass C and N contents in the sandy loam soil at Watsonville ranged between 134 and 221 mg C kg^{-1}, and 15.4 and 23.0 mg N kg^{-1} soil (Table 3). Three days after the second consecutive fumigant application in 2001, there was a trend for higher microbial biomass C contents in plots receiving MeBr + CP or PrBr compared to other fumigants relative to the control (not fumigated for the last 4 years at the time of sampling), but this effect was not statistically significant. In the Oxnard soils, microbial

biomass C and N values were two-fold higher than in the Watsonville soils, ranging from 325 to 420 mg C kg^{-1}, and from 35.6 to 71.0 mg N kg^{-1} soil. At the Oxnard site, fumigation with MeBr + CP and the four alternative chemicals significantly affected microbial biomass N ($P = 0.050$), but not microbial biomass C. Fumigation reduced microbial biomass N contents relatively greater at the Oxnard site than at the Watsonville site.

At both sites, microbial respiration was lower in repeatedly fumigated soils compared to the control soil not been fumigated for the last 4 (Watsonville) to 5 (Oxnard) years. The treatment effect was significant for Oxnard ($P = 0.036$), but not for the Watsonville site. At the Watsonville site, respiration was the lowest in plots receiving MeBr + CP (89 mg CO_2-C kg^{-1} h^{-1}), followed by PrBr and InLine treatments (99 mg CO_2-C kg^{-1} h^{-1}) (Table 3). At the Oxnard site, microbial respiration was significantly lower in plots fumigated with MeBr + CP (106 mg CO_2-C kg^{-1} h^{-1}) compared to the control plots (167 mg CO_2-C kg^{-1} h^{-1}). InLine, Midas and PrBr treatments at Oxnard yielded intermediate respiration rates. Microbial respiration was higher in Oxnard soils than in Watsonville soils consistent with microbial biomass contents at the sites (Table 3).

((Table 3))

3.3. Soil Enzyme Activities

The responses of soil enzyme activities to repeated application of different fumigant pesticides varied among the fumigants and enzymes and by study sites. With exception of β-glucosidase at Oxnard site, enzyme activities were lower in soils fumigated with MeBr + CP and the four tested alternatives over two consecutive years when compared to a control soil not been fumigated for at least the past 4 years. At the Watsonville site, soil fumigation with MeBr alternatives reduced the activities of all four soil enzymes by 43, 30, 4, and 16% for dehydrogenase, β-glucosidase, acid phosphatase and arylsulfatase, respectively (Table 4) with one exception. Fumigation with PrBr resulted in an increase of acid phosphatase activity by 13% compared to the control soil. Corresponding values for MeBr + CP fumigated soils at the Watsonville site were 51, 52, 26, and 21%, respectively. The differences between enzyme activities in the control soil (not been fumigated over the past 4 years) and soils fumigated with alternative pesticides,

however, were not significant, with exception of β-glucosidase activity in the InLine plots which revealed significantly lower activity values (Fig. 1). Soil fumigation with MeBr + CP significantly reduced the activities of β-glucosidase and acid phosphatase ($P \leq 0.009$).

At the Oxnard site, fumigation with MeBr alternatives reduced the activities of soil enzymes by 42, 19, and 29% for dehydrogenase, acid phosphatase and arylsulfatase, respectively (Table 4). The β-glucosidase activity was higher in soils fumigated with PrBr, InLine and CP compared to the control, which has not been fumigated over the last 5 years. Soil fumigation with MeBr + CP decreased the activities of dehydrogenase, β-glucosidase, acid phosphatase and arylsulfatase by 68, 6, 8, and 72%, respectively. However, fumigation effects were only significant for dehydrogenase activities at the Oxnard site ($P = 0.008$) (Fig. 1). Recent soil fumigation with MeBr + CP, PrBr, InLine or Midas significantly reduced the dehydrogenase activity compared to the control soil. Soil dehydrogenase activities at the Oxnard site decreased in the order control > CP > PrBr > Midas > InLine > MeBr + CP (Fig. 1).

((Table 4))

Average dehydrogenase activities ranged between 21 and 38 mg TPF kg^{-1} soil 24 h^{-1} at Watsonville, and between 24 and 78 mg TPF kg^{-1} soil 24 h^{-1} at Oxnard for the MeBr + CP and control soils, respectively (Fig. 1). Average β-glucosidase activities varied between 17 and 33 mg PN kg^{-1} soil h^{-1} at the Watsonville site, and between 24 and 33 mg PN kg^{-1} soil h^{-1} at the Oxnard site. Acid phosphatase activities at the Watsonville site ranged from 38 (MeBr + CP) to 59 (PrBr) mg PN kg^{-1} soil h^{-1}, and from 39 (PrBr) to 58 (control) mg PN kg^{-1} soil h^{-1}. Arylsulfatase activity at Watsonville was comparably low (6.0 to 8.0 mg PN kg^{-1} soil h^{-1}) in the control soil and all recently fumigated soils compared to other enzyme activities. Similarly, at the Oxnard site, arylsulfatase activities varied within a small range (2.5 to 9.0 mg PN kg^{-1} soil h^{-1}). Arylsulfatase activity values in soils followed the order control > MeBr + CP > InLine > Midas > PrBr > CP for both sites.

((Figure 1))

3.4. Specific activities of reference enzymes and calculation of enzyme protein concentrations in soils

Fumigation of purified enzyme proteins for 24 h inhibited the activities of all three reference enzymes (Table 5). However, the response of the enzyme proteins to fumigation varied between the specific enzymes and the single pesticides tested. β-glucosidase showed the greatest fumigation effect with inhibition rates ranging from 98 to 35% (avg. = 76%). Fumigation reduced the specific arylsulfatase activity by 28% on average. Specific arylsulfatase activity was reduced the most by PrBr (54%) and the least by CP (1%). Specific acid phosphatase activity was inhibited the most by InLine (18%) and the least by CP (10%) (12% average of all fumigants studied).

((Table 5))

Assuming that the chemical structure and the composition of the enzyme proteins in the reference enzymes are similar to those in soils, the protein concentrations in the soils from Watsonville were, on average, 2.98, 0.095, and 2.99 mg protein kg^{-1} soil for acid phosphatase, β-glucosidase and arylsulfatase, respectively (Table 6). Corresponding values for the Oxnard site were 2.88, 0.114, and 2.91 mg protein kg^{-1} soil for acid phosphatase, β-glucosidase and arylsulfatase, respectively. In general, the enzyme protein concentrations at both sites were highest in the control soils, not been fumigated for the last 4 to 5 years, and lowest in soils fumigated with MeBr + CP. Among the alternative fumigants studied, plots that received InLine and Midas expressed, in general, the lowest enzyme protein concentrations.

((Table 6))

4. Discussion

Pesticide effects on soil microorganisms are difficult to evaluate because of the heterogeneous physical-chemical nature of soil, resulting in uncertainties about their distribution and fate within soil microsites. Previous studies on the effects of potential MeBr alternatives on the size, composition and activity of soil microorganisms are limited to one or a few fumigants, a relative short time period, and/or the laboratory (Miller et al., 1997; Macalady et al., 1998; Ibekwe et al., 2001). Recovery of microbial processes in the laboratory compared to the field may be reduced due to the absence of re-colonization by nonfumigated soils (Macalady et al., 1998). Furthermore, the effect of alternative fumigants on soil microbial processes was studied on soils with a 10-year history of

fumigation with MeBr + CP combinations (history soils) followed by a 2 to 3 year break prior to the initiation of these field experiments at Watsonville and Oxnard, respectively. Consequently, results obtained from these soils with a long-term fumigation history may not apply to soils previously not fumigated (non-history soils).

The results presented in this work are part of a longer study to evaluate application methods and efficacy of chemical MeBr alternatives to control weeds and pathogens in strawberry production systems in California, USA. The response of microbial performance to soil fumigation with InLine, CP, PrBr and Midas relative to the standard MeBr + CP application and a control soil (history soil; not fumigated for the last 2 or 3 years prior to this study for Watsonville and Oxnard, respectively) was determined at 1, 4, and 30 weeks after fumigation in 2000, the first year of the study. Fumigation initially (1 week after application) inhibited microbial respiration, nitrification potential, and activities of dehydrogenase, acid phosphatase and arylsulfatase (Schutter et al., 2001; and submitted for publication). After 30 weeks, microbial activities in fumigated and control soils were similar at both sites, with exception of acid phosphatase and arylsulfatase activities in selected treatments that remained lower in the fumigated soils. Soil microbial biomass C and β-glucosidase activity were not affected by fumigation with MeBr + CP and alternatives throughout the whole study period in the first year (Schutter et al., 2001; and submitted for publication).

This paper focused on the effects of repeated (over two consecutive years) soil fumigation with MeBr + CP, PrBr, InLine, Midas, and CP on the size and activity of soil microorganisms and hydrolytic enzymes, which control the degradation of organic substances and the rate at which nutrient elements become available for plants (Nannipieri, 1995).

Microbial respiration was significantly decreased in Oxnard soils fumigated with MeBr + CP, but not affected by the four selected alternative fumigants at both sites. In this study, microbial respiration showed a low sensitivity to detect changes in soil microbial activity due to repeated application of the standard MeBr + CP combination and alternative fumigants. This finding is in contrast with the high sensitivity of respiration measurements to treatment of soils with heavy metals and pesticides (Kandeler et al., 2000; Tu, 2003). Significant lower respiration rates in Oxnard soils fumigated with MeBr

+ CP compared to recently not fumigated control soils however, may indicate a decreased biological activity.

Soil fumigation had no significant effect on microbial biomass C, and the results for microbial biomass N were inconsistent over the two experimental locations. Therefore, the effects of soil fumigation on total microbial biomass content provided little information on possible changes in the size of microbial populations. The overall low response of microbial biomass and respiration to repeated soil fumigation may be related to selected effect on sensitive microbial populations and the growth of resistant species. The latter may feed on cell debris, leading to restructuring of soil microbial populations as indicated elsewhere (Macalady et al., 1998; Ibekwe et al., 2001; Orosco et al., 2001). Selected specialized bacteria may also use the fumigants as a source of carbon and energy, as documented for agricultural soils repeatedly subjected to MeBr fumigation (Miller et al., 1997).

The effect of soil fumigation on the activities of dehydrogenase, β-glucosidase, acid phosphatase and arylsulfatase varied among the soil enzymes and within the two study sites. At the Watsonville site, soil fumigation with alternative fumigants generally had no significant effect on the activities of the four soil enzymes studied over the two-year study period. Fumigation with MeBr + CP however severely affected the activities of β-glucosidase and acid phosphatase (i.e., reductions of 52 and 26%, respectively). These results suggest that biochemical reactions involved in organic matter degradation (hydrolysis of cellulose) and P mineralization were affected by fumigation to a greater extent than were those reactions reflecting the general oxidative capabilities of microbial communities or involved in S mineralization in soils. In contrast, at the Oxnard site, β-glucosidase and acid phosphatase activities were relatively stable towards repeated soil fumigation, but dehydrogenase activity was significantly decreased by MeBr + CP. The reasons for these site-related variations in the response of soil enzyme activities to soil fumigants remain unclear. The two study sites showed very similar soil physical and chemical properties, such as clay and organic C contents. Variations may have occurred in the actual soil moisture content and temperature at the time of fumigation, which were proved to be crucial for the efficacy of pesticide applications (Ajwa et al., 2002). The results also suggest that the four alternative fumigants had no longer-term impact on

enzyme reactions involved in organic matter turnover and nutrient cycling in soil. The inhibitory and/or activation effects of any compound in a soil matrix on enzyme activity are largely controlled by the reactivity of clay and humic colloids (Nannipieri, 1995).

The finding that MeBr + CP and the alternative fumigants led to a greater inhibition of the activities of the reference enzymes than that of soils suggests that free enzymes are more sensitive to soil fumigation than enzymes that are associated with the microbial biomass or enzymes adsorbed to clay or humic colloids. Ladd and Butler (1975) hypothesized that some enzymes are stabilized in the soil environment by complexes of organic and mineral colloids and therefore are partially protected from denaturation by fumigation. Similar results were observed for acid phosphatase, β-glucosidase and arylsulfatase in chloroform fumigated soils (Klose and Tabatabai, 1999, 2002a, b). Furthermore, reference enzymes were purified from one source for each protein, whereas soil enzymes derive from various sources leading to a set of isoenzymes [i.e., enzymes that catalyze the same reaction but may differ in origin, kinetic properties or amino acid sequencing (Dick, 1997)]. Different isoenzymes in the reference material and soil may also have contributed to variation in enzyme stability towards fumigation with different pesticides.

In order to show whether there is a direct relationship between the activity of any enzyme and its protein concentration in soil enzyme protein concentrations were calculated for acid phosphatase, β-glucosidase and arylsulfatase in the nonfumigated and fumigated soils. Specific enzyme protein concentrations were suggested to serve as a suitable measure to quantify the effects of environmental changes related to soil management, fertilization or pesticide application on soil biological properties (Klose and Tabatabai, 2002a). These numbers are indented to give an indication of enzyme protein concentrations in soils, not a precise measurement. Generally, lower enzyme protein concentrations in recently fumigated soils compared to control soils (history soil; without fumigation for the last 4 to 5 years at this sampling time for Watsonville and Oxnard, respectively) suggest that fumigation with MeBr + CP and the alternative biocides (i) denatured the accumulated fraction of this enzyme protein in soil or (ii) was lethal to that portion of microorganisms that is the major source of the specific enzymes studied. The response of enzyme protein concentrations, however, varied within the enzyme and

fumigant studied. Even though the arylsulfatase protein concentration was comparable high among the three soil enzymes, it showed the lowest activity values in soils. These results suggest that arylsulfatase has a lower catalytic activity than acid phosphatase or β-glucosidase or is associated with locations in soil different from those of the other two enzymes. Our results suggest that the activity rate of any enzyme does not necessarily correspond to the protein concentration of this enzyme in a soil.

In conclusion this study has shown that microbial and enzymatic processes were not affected by soil fumigation with the alternative pesticides propargyl bromide, InLine, Midas and chloropicrin in the longer term. Fumigation with the standard methyl bromide-chloropicrin combination significantly affected some enzymatic processes in soil. However, results were inconsistent over the two study sites. These findings imply that the application of alternative fumigants will not affect the longer-term productivity of agricultural soils because hydrolytic enzymes regulate the rate at which organic materials are degraded and become available for plants. Despite the importance of these findings for strawberry production systems with a history of soil fumigation as a pest control tool, results may not apply to soils previously not fumigated. Further studies should test whether soil fumigation with these alternatives is affecting (i) microbial and enzymatic processes relative to soils without fumigation history and (ii) other functional properties and the structural diversity of microbial communities.

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Figure Captions

Figure 1. Responses of enzyme activities to fumigation with MeBr + CP and alternative biocides in sandy loam soils at Watsonville and Oxnard, Calif., USA. The solid lines represent the median values, whiskers the 10^{th} and 90^{th} percentiles, and the boxes the 25^{th} and 75^{th} percentiles. Whiskers followed by different letters indicate that median values are significantly different at $P < 0.05$ according to the LSD test. Abbreviations: MeBr, methyl bromide; PrBr, propargyl bromide; InLine, 1,3-dichloropropene plus chloropicrin; Midas, Iodomethane plus chloropicrin; CP, chloropicrin.

Table 1. Fumigants used, vendor, rates and application method used at the two study sites in Watsonville and Oxnard, Calif., USA.

Soil fumigant[a]	Chemical structure	Percent a.c.[b]	Vendor	Rate kg $ha^{-1}y^{-1}$	Application method[c]
Control	NA[d]	NA	NA	0	drip
MeBr+CP	$CH_3Br + CCl_3NO_3$	67% + 33%	Tri-Cal Inc., Holister, Calif., USA	420	shank
PrBr	C_3H_3Br	66.5%	Albemarle Corporation Baton Rouge, Louisiana, USA	202	drip
InLine	$C_3H_4Cl_2 + CCl_3NO_3$	61% + 35%	DowAgroSciences, Redeck, North Carolina, USA	448	drip
Midas	$CH_3I + CCl_3NO_3$	50% + 50%	Arvesta Corporation, San Francisco, Calif., USA	448	drip
CP-EC	CCl_3NO_3	96%	Niklor Chemical Co., Long Beach, Calif., USA	224	drip

[a] Abbreviations: MeBr, Methyl bromide; CP, chloropicrin; EC, emulsifiable concentrate; InLine: mixture of 1,3-D, 1,3-dichloropropene and CP; Midas, iodomethane; PrBr, propargyl bromide.

[b] a.c.: Active component.

[c] Shank injection was at a soil depth of 20 cm and drip fumigation was applied in 50 l m^{-2} irrigation water.

[d] Not applicable.

Table 2. Sources, properties and assay conditions of the purified reference enzymes.

Purified enzyme	Source	Concentration		Molecular weight[a] (kDa)	Assay conditions[b]
		Total protein (%)	Enzyme protein (%)		
β-Glucosidase	Almonds	91.0	30.9	60	1 ml solution containing 0.05 mg solid material (0.0455 mg protein or 0.014 mg β-glucosidase protein) plus 3 ml MUB buffer (pH 5.0) plus 1 ml *p* -nitrophenyl-β-glucopyranoside solution
Acid phosphatase	Wheat germ	98.0	11.5	55	1 ml solution containing 0.002 mg solid material (9.8 µg protein or 1.13 µg acid phosphatase protein) plus 3 ml MUB buffer (pH 4.8) plus 1 ml *p* -nitrophenyl-phosphate solution
Arylsulfatase	Limpets (*Patella vulgata*)	77.0	20.0	41	1 mL solution containing 0.15 mg solid material (0.1155 mg protein or 0.023 mg sulfatase protein) plus 3 mL acetate buffer (pH 5.0) plus 1 mL of p-nitrophenyl-sulfate solution

[a] Molecular weights from Schomburg and Salzmann, 1991 (β-glucosidase and acid phosphatase), and Klose and Tabatabai, 1999 (arylsulfatase).

[b] Incubation at 37ºC for 30 min. Substrate concentration: 50 mM. Abbreviations: MUB: Modified universal buffer. The buffers and reagents were prepared as described by Tabatabai (1994).

Table 3. Microbial biomass C (Cmic) and N (Nmic), and microbial respiration in Watsonville and Oxnard soils (0-15 cm) fumigated with methyl bromide and alternative biocides.

		Cmic		Nmic		Microbial respiration	
		(mg kg^{-1} soil)				(mg CO_2-C kg^{-1} h^{-1})	
Sites	Treatment	Mean[a]	S_E[b]	Mean	S_E	Mean	S_E
Watsonville	Control	164	20	23.0	1.9	116	9
	MeBr+CP	199	60	21.5	2.4	89	11
	PrBr	221	52	15.4	1.7	99	9
	InLine	154	15	18.4	2.2	99	10
	Midas	134	43	19.1	2.2	100	10
	CP-EC	151	26	18.4	2.6	105	14
	P- value[c]	0.282		0.713		0.282	
	B[d]	0.372		0.170		0.372	
Oxnard							
	Control	402	24	71.0a	4.5	167ac	16
	MeBr+CP	368	60	35.6b	7.8	106b	20
	PrBr	382	38	38.4b	4.7	120bc	17
	InLine	325	78	41.0b	8.4	145c	19
	Midas	383	47	31.6b	9.8	148c	20
	CP-EC	420	78	51.2ab	4.3	156c	19
	P- value[c]	0.678		**0.050**		**0.036**	
	B[d]	0.181		0.694		0.741	

[a] Values shown are means of four replications (n = 4) for samples taken in 2001. Means followed by the same letter are not statistically different at $P = 0.05$ using the LSD test.

[b] Standard error.

[c] Significance level of treatment effects according to the ANOVA. Bold numbers indicate significance at $P = 0.05$.

[d] Observed power of the ANOVA to detect treatment differences at $P = 0.05$.

Table 4. Enzyme activities in Watsonville and Oxnard soils (0-15 cm) fumigated with methyl bromide and alternative biocides on enzyme activities (n = 4).

Site	Treatment	Changes in activity due to fumigation (%)[a]			
		Dehydrogenase	β–Glucosidase	Acid phosphatase	Arylsulfatase
Watsonville	MeBr+CP	- 51	- 52	- 26	- 21
	PrBr	- 45	- 18	+ 13	- 6
	InLine	- 36	- 37	- 5	- 29
	Midas	- 46	- 35	- 4	- 18
	CP-EC	- 44	- 29	- 2	- 11
	Avg. of alternatives	**- 43**	**- 30**	**- 4[b]**	**- 16**
Oxnard	MeBr+CP	- 68	- 6	- 8	- 72
	PrBr	- 39	+ 13	- 34	- 21
	InLine	- 63	+ 4	- 18	- 41
	Midas	- 51	- 19	- 9	- 37
	CP-EC	- 14	+ 8	- 15	- 18
	Avg. of alternatives	**- 42**	**+ 2**	**- 19**	**- 29**

[a] The percentages of change (+: increases, -: decreases) in activities due to fumigation were calculated from [(A-B)/A] x 100, where A is activity value of the control soil (history soil; not fumigated for the last 4 or 5 years) and B is the activity value of fumigated soil.

[b] Average was calculated without PrBr sample because of the opposite enzymatic response.

Table 5.Specific activities of reference enzymes fumigated with methyl bromide and alternative biocides.

	Specific activity of the enzyme proteins (mg PN mg^{-1} protein h^{-1})			Loss of activity due to fumigation (%)[a]		
Treatment	Acid phosphatase	β-Glucosidase	Sulfatase	Acid phosphatase	β-Glucosidase	Sulfatase
Control	812	260	2.2	-	-	-
MeBr+CP	733	12.8	1.3	10	95	43
PrBr	727	6.3	0.9	10	98	54
InLine	656	102	2.4	19	61	4
Midas	728	19.3	1.3	10	93	39
CP-EC	748	169	2.4	8	35	1

[a] Loss of activity due to fumigation were calculated from [(A-B)/A] x 100, where A is activity value of the nonfumigated enzyme protein and B is the activity value of fumigated enzyme protein.

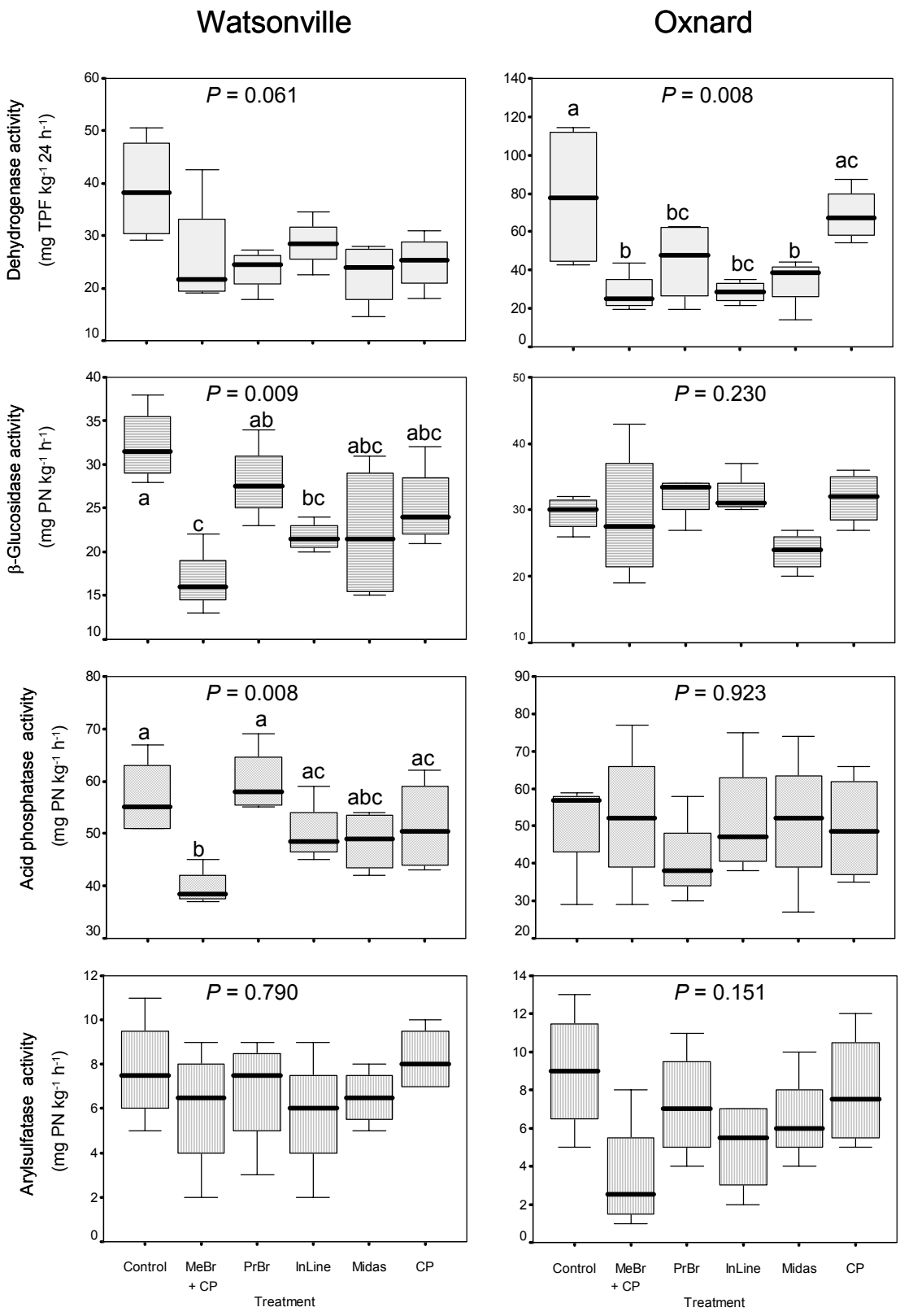
Table 6. Enzyme protein concentrations in Watsonville and Oxnard soils (0-15 cm) fumigated with methyl bromide and alternative biocides.

		Enzyme protein concentrations (mg protein kg^{-1} soil)		
Soil	Treatment	Acid phosphatase[a]	β–Glucosidase[b]	Sulfatase[c]
Watsonville	Control	3.32	0.124	3.45
	MeBr+CP	2.32	0.064	2.67
	PrBr	3.50	0.108	3.00
	InLine	2.93	0.088	2.56
	Midas	2.83	0.086	2.89
	CP-EC	3.00	0.097	3.34
	Average	**2.98**	**0.095**	**2.99**
Oxnard	Control	2.94	0.113	4.01
	MeBr+CP	3.06	0.113	1.56
	PrBr	2.39	0.123	3.23
	InLine	3.02	0.124	2.23
	Midas	2.99	0.091	2.89
	CP-EC	2.88	0.122	3.56
	Average	**2.88**	**0.114**	**2.91**

[a] 1 mg protein (Wheat germ, Type I) will release 17.16 mg PN h^{-1}

[b] 1 mg protein (Almonds) will release 260 mg PN h^{-1}

[c] 1 mg protein (*Patella vulgata*) will release 2.247 mg PN h^{-1}


Watsonville
Oxnard
Dehydrogenase activity (mg TPF kg-1 24 h-1)
β-Glucosidase activity (mg PN kg-1 h-1)
Acid phosphatase activity (mg PN kg-1 h-1)
Arylsulfatase activity (mg PN kg-1 h-1)
P = 0.061
P = 0.008
P = 0.009
P = 0.230
P = 0.008
P = 0.923
P = 0.790
P = 0.151
a
b
bc
bc
b
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a
c
ab
bc
abc
abc
a
b
a
ac
abc
ac
Control
MeBr + CP
PrBr
InLine
Midas
CP
Treatment