Efficacy of Verbenone for Protecting Ponderosa Pine Stands From Western Pine Beetle (Coleoptera: Curculionidae: Scolytinae) Attack in California

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ABSTRACT The western pine beetle, *Dendroctonus brevicomis* LeConte (Coleoptera: Curculionidae: Scolytinae), is a major cause of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., mortality in much of western North America. Currently, techniques for managing *D. brevicomis* infestations are limited. Verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) is an antiaggregation pheromone of several *Dendroctonus* spp., including *D. brevicomis*, and it has been registered as a biopesticide for control of mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and southern pine beetle, *Dendroctonus frontalis* Zimmermann. We evaluated the efficacy of a 5-g verbenone pouch [82%-(−); 50 mg/d] applied at 125 U/ha for protecting *P. ponderosa* stands (2 ha) from *D. brevicomis* attack over a 3-yr period. No significant differences in levels of *D. brevicomis*-caused tree mortality or the percentage of unsuccessfully attacked trees were found between verbenone-treated and untreated plots during each year or cumulatively over the 3-yr period. Laboratory analyses of release rates and chemical composition of volatiles emanating from verbenone pouches after field exposure found no deterioration of the active ingredient or physical malfunction of the release device. The mean release rate of pouches from all locations and exposure periods was 44.5 mg/d. In a trapping bioassay, the range of inhibition of the 5-g verbenone pouch was determined to be statistically constant 2 m from the release device. We discuss the implications of these and other results to the development of verbenone as a semiochemical-based tool for management of *D. brevicomis* infestations in *P. ponderosa* stands.

KEY WORDS antiaggregation pheromone, *Dendroctonus brevicomis*, pest management, *Pinus ponderosa*, stand protection

The western pine beetle, *Dendroctonus brevicomis* LeConte (Coleoptera: Curculionidae: Scolytinae), is a major cause of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., mortality in much of western North America and particularly in California (Furniss and Carolin 1977). Typically, this bark beetle prefers large diameter (>50 cm at 1.37 m in height) trees, but under certain conditions (e.g., during extended periods of drought) it can attack and kill apparently healthy trees of all ages and size classes. Currently, techniques for managing *D. brevicomis* infestations are limited to tree removals (thinning) that reduce stand density and presumably host susceptibility (Fettig et al. 2007), and/or the use of insecticides to protect individual trees (Fettig et al. 2006).

Orientation of *D. brevicomis* during flight is determined mostly by olfactory stimuli (Strom et al. 2001). Females colonize suitable hosts by landing on trees and tunneling through the outer bark and into the phloem and outer xylem where they rupture resin canals. Oleoresin exudes from the entrance hole and collects on the bark surface to form a pitch tube. During colonization females release *exo*-brevicomin, which in combination with the host monoterpene myrcene is attractive to conspecifics (Bedard et al. 1969). Frontalin, produced by males (Kinzer et al. 1969), enhances attraction and mass attack ensues (Wood 1972, Bedard et al. 1985). During the attack sequence, verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) is produced by auto-oxidation of the host monoterpene α-pinene via the intermediary compounds *cis*- and *trans*-verbenol (Hunt et al. 1989), by the beetles themselves (Byers et al. 1984), through degradation of host material by yeasts associated with *D. brevicomis* (see Leufvén et al. 1984 and Hunt and Borden 1990 for synthesis of verbenone by yeasts from related bark beetles), or a combination of these mechanisms. Attack density on the host is thought to be regulated by the ratio of verbenone to aggregation pheromones and host kairomones (Byers et al. 1984, Tilden and Bedard 1988).

Verbenone was first identified in male *D. brevicomis* by Renwick (1967) and was later demonstrated to elicit a negative response from tethered, flying *D.*

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brevicomis females (Hughes and Pitman 1970). Bedard et al. (1980a) showed that verbenone reduced the number of *D. brevicomis* trapped at a baited source. Trap catches were further reduced with higher release rates of verbenone (Bedard et al. 1980a,b; Tilden and Bedard 1988; Bertram and Paine 1994a), and by combining verbenone with ipsdienol (Paine and Hanlon 1991, Shea and Wentz 1994), the latter produced by male *D. brevicomis* (Byers 1982, Seybold et al. 1992) and sympatric *Ips* and *Dendroctonus* spp. (Borden 1985, Seybold et al. 2000). Shea (1990) and Shea and Wentz (1994) evaluated the effect of chirality on *D. brevicomis* response to verbenone reporting racemic and 97%-(−) were most effective in reducing attraction. Acetophenone, which reduces attraction in southern pine beetle, *Dendroctonus frontalis* Zimmermann (Sullivan 2005), also inhibits *D. brevicomis* attraction (Erbilgin et al. 2007, 2008). Verbenone (8–12 mg/d [25°C]) and 4-allylanisole, the latter a host volatile that has been demonstrated to inhibit aggregation in other *Dendroctonus* spp., did not affect the number of *D. brevicomis* caught in attractant-baited traps (Hayes and Strom 1994). In a more recent study, verbenone significantly reduced attraction of *D. brevicomis* to attractant-baited traps, but no difference was observed between 4 and 50 mg/d (30°C) release rates (Fettig et al. 2005). It is assumed that verbenone reduces intraspecific competition by altering adult behavior to minimize overcrowding of developing brood within the host (Byers and Wood 1980, Byers et al. 1984). Lindgren et al. (1996) proposed that verbenone is an indicator of host tissue quality and that its quantity is a function of microbial degradation. Verbenone was not detected in extracts of volatiles collected from logs cut from the main stem of *P. ponderosa* but was detected in extracts obtained by similar methods from several nonhost conifers (Shepherd et al. 2007).

Because of its behavioral activity, as demonstrated in numerous trapping bioassays (Borden 1997), verbenone has been evaluated as a tool for mitigating coniferous tree mortality due to bark beetle infestations. In western North America, efforts have concentrated on single tree (Borden et al. 2004, Kegley and Gibson 2004, Gillette et al. 2006) or small-scale (e.g., <4-ha) stand protection, primarily from mountain pine beetle, *Dendroctonus ponderosae* Hopkins, infestations (Amman et al. 1989, 1991; Bentz et al. 1989; Lindgren et al. 1989; Lister et al. 1990; Gibson et al. 1991; Shea et al. 1992; Shore et al. 1992; Lindgren and Borden 1993; Borden et al. 2003, 2004; Progar 2003, 2005; Kegley and Gibson 2004; Bentz et al. 2005; Negrón et al. 2006; Gillette et al. 2009). Results have been favorable, but inconsistent in some cases (Bentz et al. 1989; Lister et al. 1990; Gibson et al. 1991; Shea et al. 1992; Progar 2003, 2005; Negrón et al. 2006). Negative results have been linked to photoisomerization of verbenone to behaviorally inactive chrysanthenone (Kostyk et al. 1993); inconsistent or inadequate release (Bentz et al. 1989); rapid dispersal of verbenone (Gibson et al. 1991, Negrón et al. 2006), and limitations in the range of inhibition (Miller 2002), particularly when *D. ponderosae* populations were high (Progar 2003, 2005; Bentz et al. 2005). A lack of efficacy also may be due to the complexity of the host selection process, which involves other behavioral chemical signals produced by the host, by nonhosts, and by competing bark beetle species (Borden 1997, Shepherd et al. 2007). Generally, verbenone bubble cap (Bentz et al. 1989, Lister et al. 1990, Gibson et al. 1991, Negrón et al. 2006) and pouch (Gibson and Kegley 2004, Negrón et al. 2006) release devices have been ineffective for reducing *D. ponderosae* attacks in *P. ponderosa* stands.

Few publications are available on development of semiochemical-based tools for protecting *P. ponderosa* stands from *D. brevicomis* infestations. Bertram and Paine (1994b) reported that applications of verbenone and ipsdienol significantly reduced both numbers of *D. brevicomis* landing on *P. ponderosa* and densities of attacking beetles. In their study, paired treated (verbenone and ipsdienol) and untreated trees were baited with aggregation pheromones to stimulate mass attack, but tree mortality rates were not determined. Verbenone applied to the stem of individual *P. ponderosa* in a flake formulation was ineffective for preventing *D. brevicomis* attacks (Gillette et al. 2006). In Oregon, studies to determine the effectiveness of 5-g verbenone pouches and 7-g verbenone bags, within an integrated approach that also included infested tree removal (sanitation) and suppression trapping for protecting old-growth stands of *P. ponderosa*, were inconclusive (J. L. Hayes, personal communication). Fettig et al. (2008) demonstrated the successful application of verbenone, in combination with nonhost angiosperm volatiles, for protecting individual *P. ponderosa* from *D. brevicomis* attack and associated levels of tree mortality. To our knowledge, no studies have been published on the efficacy of verbenone for protecting small-scale *P. ponderosa* stands from *D. brevicomis* attack by using the metric of tree mortality to measure efficacy.

The primary objectives of this study were to determine the efficacy of the 5-g verbenone pouch for protection of small-scale (2-ha) *P. ponderosa* stands from *D. brevicomis* infestation in California; to determine the release rate gravimetrically and analyze the qualitative chemical content of field-exposed pouches; and to determine the effective range of inhibition of the 5-g verbenone pouch.

Materials and Methods

Stand Protection Study. In May 2002, study sites (blocks) were selected near McCloud, CA, McCloud Ranger District, Shasta-Trinity National Forest (41.35° N, 121.95° W; 1,150-m elevation) and Caldor, CA, Placerville Ranger District, Eldorado National Forest (38.62° N, 120.49° W; 1,359-m elevation) in areas with recent *D. brevicomis* activity (Table 1; USDA Forest Service 2002). Six square plots (2 ha) were established in each block. At McCloud, plots had a mean species composition of 95% *P. ponderosa*, with the remainder Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco; white fir, *Abies concolor* (Gord. & Glend.) Lindl. ex

Table 1. Pretreatment stand characteristics at McCloud, Shasta-Trinity National Forest (41.35° N, 121.95° W; 1,150-m elevation) and Caldor, Eldorado National Forest (38.62° N, 120.49° W; 1,359-m elevation), CA, 2002

Block	Plot	Treatment[a]	% crown cover	Mean dbh ± SEM[b]	Basal area (m^2/ha)	Trees per ha	% *P. ponderosa*	No. *D. brevicomis*-infested trees[c]
McCloud	1	Control	80	36.8 ± 1.0	46.6	362	90	21
	2	Verbenone	51	55.9 ± 2.0	30.6	113	100	3
	3	Verbenone	67	50.8 ± 1.8	33.4	151	99	8
	4	Control	47	43.9 ± 3.3	25.8	119	96	1
	5	Control	71	71.9 ± 1.8	36.9	88	100	0
	6	Verbenone	80	62.0 ± 3.6	52.5	132	84	0
Caldor	1	Control	73	41.1 ± 1.5	33.7	211	49	0
	2	Verbenone	67	37.6 ± 1.8	39.8	261	52	1
	3	Control	60	52.6 ± 2.8	40.3	144	64	0
	4	Verbenone	84	41.4 ± 1.8	64.2	335	28	12
	5	Verbenone	80	55.1 ± 1.8	46.7	176	57	2
	6	Control	78	33.3 ± 0.8	49.3	468	46	0

[a] Control, untreated control. Verbenone, 5-g verbenone pouches [82%-(−), 97% chemical purity, 50 mg/d at 30°C] applied at 125 pouches per ha.

[b] dbh, diameter at breast height (1.37 m) in centimeters.

[c] Based on presence of successful attacks (i.e., oxidized phloem material present in pitch tubes or points of attack containing dry frass) in late May 2002 on each 2-ha experimental plot.

Hildebr.; incense cedar, *Calocedrus decurrens* (Torr.) Florin; and sugar pine, *Pinus lambertiana* Dougl. (Table 1). At Caldor, plots had a mean species composition of 49% *P. ponderosa*, with the remainder *Ps. Menziesii; A. concolor; C. decurrens; P. lambertiana;* and California black oak, *Quercus kelloggii* Newb. (Table 1). *P. ponderosa* was the only host of *D. brevicomis* present in these stands. Temperatures were monitored on two plots within each block with dataloggers placed ≈2 m in height at the northern aspect of one *P. ponderosa* (HOBO model H08-001-02, Onset Computer Corp., Bourne, MA).

In 2002–2004, 5-g verbenone pouches [82%-(−), 97% chemical purity, 50 mg/d at 30°C, 1.5-mil plastic membrane thickness; Pherotech (now Contech Inc.) International Inc., Delta, BC, Canada] were hand-applied in a 9.1- by 9.1-m grid (125 pouches/ha) at a height of ≈2 m to the north side of the nearest tree or stapled to the top of 1.5 m tall wooden stakes if the nearest tree was >2 m from the grid point. Pouches were generally applied on 1 June of each year, replaced on 1 July, and removed on 31 July. In all cases, the treatment interval was 61 d/yr. Three uninfested and apparently healthy *P. ponderosa* were baited each year near each plot center with a single *D. brevicomis* tree bait (Pherotech International Inc.) consisting of the aggregation pheromone components *exo*-brevicomin (racemic, 97% chemical purity, 3 mg/d at 24°C) and frontalin (racemic, 98% chemical purity, 3 mg/d at 24°C), and the host volatile myrcene (90% chemical purity, 18 mg/d at 24°C). In these areas, *D. brevicomis* is active from April through November with peak flight activity occurring in June and July (Fettig et al. 2004a). Therefore, it was necessary to conduct a complete census of each plot in late May of each year of the study for trees that had been attacked by *D. brevicomis* external to the treatment interval. These trees were excluded from subsequent analyses. At the end of the treatment interval each year, plots were examined again for *D. brevicomis*-attacked and/or killed trees. In all cases, the bole of each tree was examined for *D. brevicomis* attacks (i.e., oxidized phloem material present in pitch tubes or entrance holes containing dry frass). Tree mortality was estimated based on presence of crown fade within 10 mo of attack.

The experimental design was a randomized complete block with two blocks and three replicates/treatment/block (df = 1, 9). For each year and cumulatively over the 3-yr period, a two-way analysis of variance (ANOVA) (treatment and block) ($\alpha = 0.05$) was performed on the percentage of *P. ponderosa* killed by *D. brevicomis* and the percentage of *P. ponderosa* containing *D. brevicomis* attacks, but which did not die as a result of those attacks (i.e., unsuccessfully attacked) (JMP version 3.2.6, SAS Institute, Cary, NC). Data on unsuccessfully attacked trees were analyzed because significant increases in their proportion within verbenone-treated plots could indicate avoidance by *D. brevicomis* to levels below critical thresholds necessary to overcome host tree defenses. A test of normality was performed and arcsine square root (angular) transformations were used when data deviated significantly from a normal distribution.

Laboratory Analyses of Release Rate and Chemical Composition. The performance of verbenone pouches from the stand protection study was analyzed by two means in the laboratory. Unexposed and field-exposed pouches were analyzed gravimetrically to estimate release rate. Volatiles from a subset of these pouches were collected and analyzed for chemical composition and the chemical purity of verbenone by gas chromatography-mass spectrometry (GC-MS).

One verbenone pouch was randomly removed and immediately replaced from each field plot on a weekly basis during each treatment interval in 2002. Pouches were placed in individually marked Mylar bags, placed in a cooler on blue ice, and transported to the laboratory where they were immediately stored at −20 and then −80°C before analyses. A subset of pouches collected from both study blocks (McCloud-21 pouches, Caldor-24 pouches) and 12 pouches that had been stored in a freezer since purchased in April 2002

[at −20°C (spring 2002 until fall 2003) and −80°C (fall 2003 until 30 June 2006)] were randomly selected and weighed individually on 30 June 2006 and suspended in the dark in a Precision Low Temperature Incubator (model FU199JRW2, Precision Scientific Inc., Chicago, IL) at 30°C for 5 d to equilibrate the devices from freezer storage. Airflow from an internal fan in the incubator compartment was 129.6 and 31.3 cm/s at 15 and 30 cm from the fan inlet, respectively. Pouches were only exposed to light for a short period (≈1 h) when being weighed during each sample date. After the equilibration period, pouches were weighed again and then incubated and weighed every 24–72 h for 56 d, resulting in 32 sample dates. The experimental design was completely randomized ($df = 4, 52$). A test of normality was performed and square root transformations were used when data deviated significantly from a normal distribution. A one-way ANOVA ($\alpha = 0.05$) was performed on the weight of unexposed and field-exposed (1, 2, 3, and 4 wk) pouches during each sample date (SigmaStat version 2.0, SPSS Inc., Chicago, IL). If a significant treatment effect was detected, Tukey's multiple comparison test (Tukey's honestly significant difference [HSD]) was used for separation of treatment means.

After 56 d of incubation, three groups of five pouches (one unexposed and four field-exposed within each group) were randomly selected for volatile entrapment. Group 1 was processed (i.e., volatiles were collected) on 30–31 August 2006, group 2 was processed on 31 August–1 September 2006, and group 3 was processed on 1–2 September 2006. All field-exposed pouches had been collected at McCloud during 7 June–2 August 2002.

To trap volatiles for qualitative chemical analysis, we scaled up and modified the aeration system described by Bartelt et al. (2004). Pouches were suspended individually in side-armed 500-ml Pyrex Erlenmeyer flasks whose main apertures were sealed with a no. 7 rubber stopper. Teflon tubing (0.95 cm in diameter PTFE; Cole Parmer Instrument Company, Vernon Hills, IL) was inserted into each flask through the center of each stopper to serve as an inlet into the flask. The tubing extended to within 1 cm of the bottom of the flask. This inlet tubing was connected to a glass column [(1.1 cm i.d./1.2 cm o.d. × 14.5 cm length, ground glass 10/19 joint) with constricted, open ends (0.4 cm i.d./0.6 cm o.d.)] containing ≈1 g of Porapak-Q (50/80 mesh size; product 20330-U, Sigma-Aldrich, St. Louis, MO). Porapak-Q is an absorbent resin with high affinity for organic volatiles (Byrne et al. 1975). Incoming air (30 ml/min) from a commercial gas cylinder was first prefiltered in bulk through activated charcoal (6–14 mesh, product 05-685A, Thermo Fisher Scientific, Waltham, MA) and then individually for each sample flask through the Porapak-Q columns. Five-centimeter-long pieces of the same Teflon tubing were attached to the glass sidearm of each flask and these outlet tubes were connected to a larger glass column [(2.8 cm i.d./3 cm o.d. × 31 cm length, ground glass 24/40 joint) with constricted, open ends (0.3 cm i.d./0.5 cm o.d.)] containing 7–8 g of Porapak-Q. All tubing and column junctions were sealed with Teflon tape and Duraseal Laboratory Stretch Film (PGC Scientifics, Gaithersburg, MD). Volatiles were collected from each of the 15 samples for 24 h. Based on hourly readings from a datalogger, laboratory conditions during the collection period were 1) 30–31 August 2006, 23.2 ± 0.1°C (22.5–24.0°C), 43.0 ± 0.8% RH (35.7–50.0%); 2) 31 August–1 September 2006, 23.3 ± 0.1°C (22.4–24.3°C), 43.4 ± 0.5% RH (39.4–47.1%); and 3) 1–2 September 2006, 23.4 ± 0.1°C (22.7–23.9°C), 44.9 ± 0.3% RH (42.6–47.5%) (all expressed as mean ± SEM [range]).

After the collection period, the ends of the 15 sample columns were immediately sealed with Teflon tape and Duraseal and then stored at −80°C before extraction. Each Porapak-Q sample was extracted in the glass column with ≈50 ml of pentane and then again with ≈50 ml of dichloromethane (Fisher, Pasadena, CA). Both pentane and dichloromethane extracts were collected in 50-ml brown glass sample bottles and an internal standard (82.3 μg of 4-decanone in 1 ml of pentane) (99.9% chemical purity; product 19,467-0, Sigma Aldrich) was added. The contents of each bottle were transferred to a 250- or 500-ml concentration flask with a 10-ml graduated receptacle. A boiling chip was added and the extract was concentrated to ≈4 ml by Kuderna-Danish evaporative concentration (Kontes Glass, Vineland, NJ) in a 50°C water bath. The flask was then rinsed and added to the concentrate. The combined solution (total volume, ≈8 ml) was transferred to a sealed vial and stored at −80°C for later analysis.

The extracts were analyzed by GC-flame ionization detector (FID) with an Agilent 6890N gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a DB-Wax capillary column [60 m × 0.25 mm [i.d.] × 0.25-μm film thickness]. The carrier gas (He) had a flow rate of 31 cm/s, and the GC was temperature programmed from 50°C (2 min) to 220°C (30 min) at 2°C/min. The injector and detector were set at 200 and 230°C, respectively.

Standard verbenone [catalog no. P8020-87, lot no. 2007015-0004; 90.8%-(*S*)-(−), 98.8% chemical purity] was purchased (Bedoukian Research Inc., Danbury, CT) and chemical purity was verified by GC analysis. Verbenone was identified in the extracts initially by GC-FID through comparison of the largest peak in the samples with the retention time of the authentic standard. Identification of verbenone was further confirmed from three of the samples (one unexposed, one field-exposed collected on 7 June 2002, and a second on 2 August 2002) by GC-MS. For this analysis, an Agilent 6890 GC was fitted with an HP-1MS capillary column [30 m × 0.25 mm (i.d.) with 0.25-μm film thickness of crosslinked methyl siloxane] and coupled with an Agilent 5973 Mass Selective Detector. Injection (220°C) was splitless (0.7 min). The carrier gas (He) had a flow rate of 36 cm/s, and the GC was temperature programmed from 50° (0.7 min) to 250°C at 6°C/min with a final hold of 2 min. Electron impact mass spectrometry was performed with a scanning range of 40–400 at 70 eV. Compounds were identified

by comparison with known spectra from the Wiley6 and the National Institute of Standards and Technology (NIST98) libraries.

Range of Inhibition Trapping Study. In August 2006, a trapping bioassay was conducted on the Shasta-Trinity National Forest in an area adjacent (<1 km) to the McCloud block of the stand protection study. Ten blocks, consisting of six traps each, were separated by >30 m to avoid interference. A 5-g verbenone pouch was attached to the outside of an unbaited 16-unit multiple funnel trap used to create a visual stimulus (Strom et al. 2001) with its collection cup removed, and placed at the center of each block (center trap). One 16-unit multiple funnel trap baited with the *D. brevicomis* tree bait (Pherotech International Inc.) was then placed at 0.5, 1, 2, 4, and 9 m from the center trap at a randomly selected bearing of 0, 72, 144, 216 or 288°. Traps were hung on 3-m metal poles with collection cups ≈1 m above the ground. A three by 3-cm time-released insecticidal Prozap Pest Strip (2,2-dichlorovinyl dimethyl phosphate, Loveland Industries Inc., Greeley, CO) was placed in the collection cup to kill arriving insects and reduce damage or loss to predacious insects. Samples were collected, and each treatment was rerandomized daily between 0630 and 1000 hours to avoid disturbing traps during periods of peak *D. brevicomis* flight activity (Fettig et al. 2004b). Specimens were tallied and identified by using available keys (Wood 1982) and voucher specimens.

The experimental design was a randomized complete block with 50 replicates per distance (df = 4, 236). A test of normality was performed and square root transformations were used when data deviated significantly from a normal distribution. A two-way ANOVA (treatment and block) ($\alpha = 0.05$) was performed on the number of *D. brevicomis* caught per trap per day (SigmaStat version 2.0, SPSS Inc.). If a significant treatment effect was detected, Tukey's multiple comparison test (Tukey's HSD) was used for separation of treatment means.

Results

Stand Protection Study. There were no significant differences in the percentages of P. *ponderosa* killed by *D. brevicomis* between verbenone-treated and untreated plots in 2002 ($F_{1,9} = 0.71$; $P = 0.42$), 2003 ($F_{1,9} = 0.56$; $P = 0.48$), 2004 ($F_{1,9} = 0.53$; $P = 0.48$), or cumulatively over the 3-yr period ($F_{1,9} = 0.01$; $P = 0.98$) (Fig. 1). Although there was a consistent trend in that higher percentages of unsuccessfully attacked *P. ponderosa* occurred on verbenone-treated plots, no significant treatment effects were observed for this variable in 2002 ($F_{1,9} = 0.01$; $P = 0.95$), 2003 ($F_{1,9} = 0.01$; $P = 0.94$), 2004 ($F_{1,9} = 0.41$; $P = 0.54$), or cumulatively over the 3-yr period ($F_{1,9} = 0.03$; $P = 0.88$) (Fig. 2).

Laboratory Analyses of Release Rate and Chemical Composition. Gravimetric analysis of unexposed and field-exposed pouches showed a gradual decline in mass during the 56-d incubation period (Fig. 3A). Few significant differences were observed among unexposed pouches and those that had been field-exposed

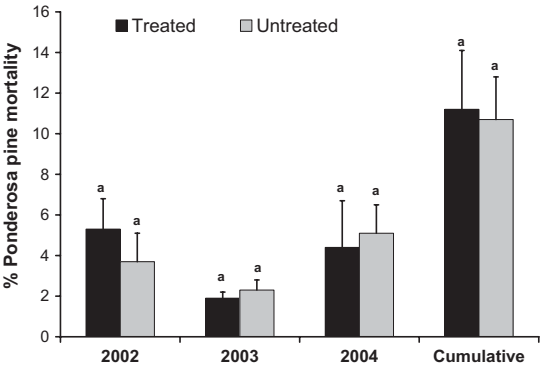

Fig. 1. Mean percentages (+ SEM) of ponderosa pines killed by western pine beetle in verbenone-treated and untreated plots in California. Means followed by the same letter within year and cumulatively (2002–2004) are not significantly different ($P > 0.05$; Tukey's HSD).

for 1, 2, 3, or 4 wk [Figs. 3A, I (day 12), 3A-II; 19 of 32 sample dates; $P > 0.07$, all cases]. During days 2–9 and 13, unexposed pouches weighed significantly more than those that were field-exposed for 4 wk (Fig. 3A, I; $P < 0.02$, all cases). On day 2, unexposed pouches also weighed significantly more than those field-exposed for 3 wk and pouches field-exposed for 1 wk weighed significantly more than those field-exposed for 4 wk ($F_{4,52} = 5.63$; $P < 0.001$). During days 48–56, pouches that had been field-exposed for 4 wk weighed significantly more than unexposed pouches (Fig. 3A, III; $P < 0.02$, all cases). No other significant differences were observed. The mean release rates of unexposed and field-exposed (4 wk) pouches ranged from 132.5 ± 5.3 to 21.3 ± 1.0 and 88.5 ± 5.0 to 9.1 ± 4.7 mg/d, respectively, during the 56-d period (Fig. 3B). The mean release rate of all pouches was 44.5 mg/d.

GC-FID analysis of the Porapak-Q extracts of volatiles trapped from a subset of unexposed and field-exposed pouches from McCloud revealed one major

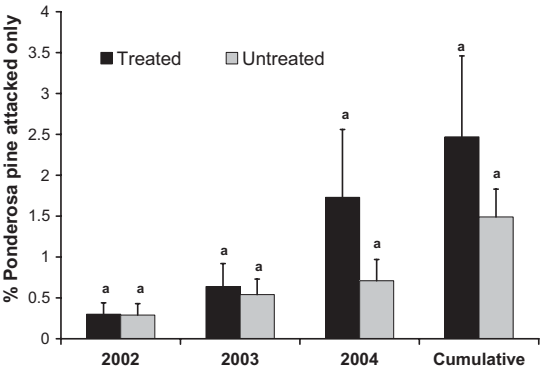

Fig. 2. Mean percentages (+ SEM) of ponderosa pines attacked, but not killed (i.e., unsuccessfully attacked), by western pine beetle in verbenone-treated and untreated plots in California. Means followed by the same letter within year and cumulatively (2002–2004) are not significantly different ($P > 0.05$; Tukey's HSD).

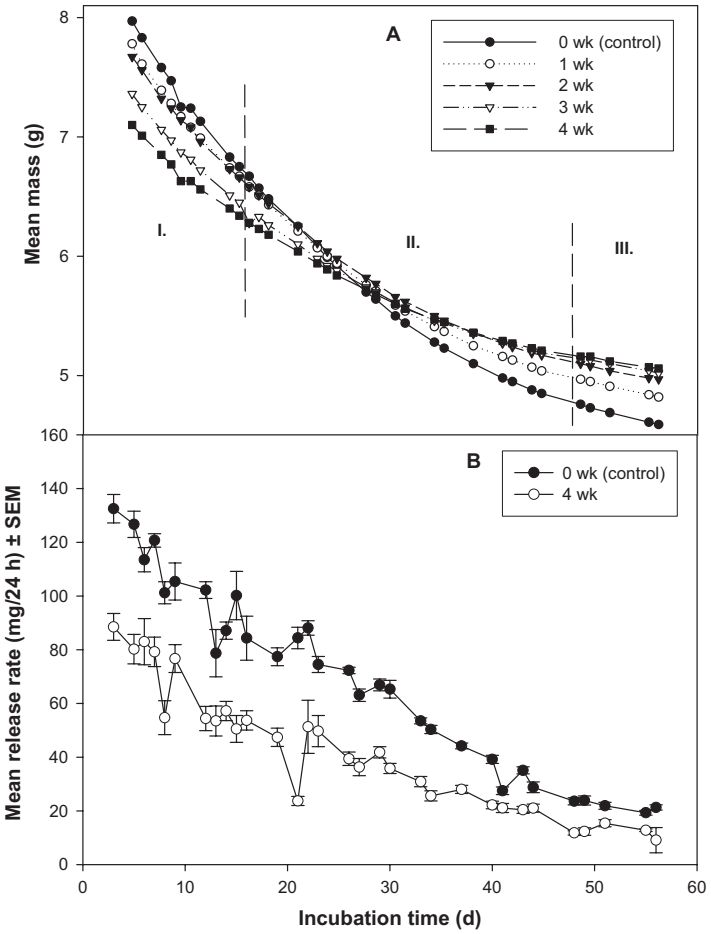

Fig. 3. (A) Mass of verbenone pouches that were unexposed ($n = 12$) or field-exposed for 1 ($n = 12$), 2 ($n = 9$), 3 ($n = 12$), or 4 ($n = 12$) wk at McCloud, Shasta-Trinity National Forest and Caldor, Eldorado National Forest, CA, 2002. (B) Release rate curves for verbenone pouches that were unexposed ($n = 12$) and field-exposed at both sites for 4 wk ($n = 12$). Release rates were determined gravimetrically at 30°C.

peak with a retention time of ≈45.5 min (data not shown). This peak coeluted with the authentic standard of verbenone and represented from 89 to 94% of the total peak area in the GC chromatograms of pentane extracts (Table 2). GC-MS analysis of this peak confirmed that it was verbenone (Fig. 4). The majority of verbenone (92.03 ± 2.80% [mean ± SEM], $n = 14$ extracts; range, 75.0–99.9%) eluted in the pentane and not the methylene chloride extract of Porapak-Q. GC-MS analysis of a sample from an unexposed pouch and from a pouch harvested on 2 August 2002 from McCloud (field-exposed for 2 wk) showed that the pentane extracts were identical in chemical composition (Fig. 4). There was no evidence of chrysanthenone in these extracts, but related compounds (three matching the mass spectrum of filifolone) and several monoterpenes and bicyclic monoterpene alcohols were present. GC-MS analysis of an extract from a pouch harvested on 7 June 2002 from McCloud (field-exposed for 1 wk) gave nearly identical results (data not shown).

Range of Inhibition Trapping Study. In total, 6,555 D. *brevicomis* were captured in multiple funnel traps. Overall, the ratio of males to females was 0.81. There was no significant treatment × gender interaction ($F_{4,\ 472} = 0.71$; $P > 0.58$); therefore, results pertain equally to both male and female responses. A significant treatment effect was observed ($F_{4,236} = 15.68$; $P < 0.001$). Significantly more *D. brevicomis* were collected four and 9 m from the center trap than at 0.5 m (Table 3). No significant differences were observed among captures at 0.5, 1, or 2 m (Table 3).

Discussion

There are several possible reasons for the lack of efficacy observed in this study. We consider each in reference to the development of verbenone as a semiochemical-based tool for management of *D. brevicomis* infestations in *P. ponderosa* stands.

Stand Structure and Composition. In much of western North America, P. *contorta* stands tend to have

Table 2. Analysis of verbenone released from unexposed or field-exposed 5-g verbenone pouches collected at McCloud, Shasta-Trinity National Forest, CA, 2002

Sample	Chemical purity (%)[a]	Wt loss (mg)[b]
Unexposed[c,g]	92.31 ± 0.06	7
Unexposed[d]	93.82 ± 0.04	22
Unexposed[e]	93.58 ± 0.00	8
Exposed-7 June 2002[d,g]	92.03 ± 0.24	21
Exposed-15 June 2002[e]	90.43 ± 0.01	10
Exposed-15 June 2002[c]	93.61 ± 0.01	9
Exposed-22 June 2002[e]	93.04 ± 0.05	9
Exposed-22 June 2002[c,f]	NA	10
Exposed-27 June 2002[d]	92.70 ± 0.20	14
Exposed-6 July 2002[d]	88.91 ± 0.11	15
Exposed-13 July 2002[e]	92.36 ± 0.05	15
Exposed-13 July 2002[c]	92.46 ± 0.04	5
Exposed-20 July 2002[c]	92.40 ± 0.03	3
Exposed-2 Aug. 2002[e]	92.63 ± 0.07	14
Exposed-2 Aug. 2002[d,g]	92.13 ± 0.04	12

[a] Determined by GC-FID analysis of the pentane extract of Porapak-Q (mean ± SEM of three injections).

[b] Determined gravimetrically before and after volatile entrapment period (after ≫56 d of incubation at 30°C).

[c] Volatiles collected 31 August–1 September 2006 (23.3°C).

[d] Volatiles collected 30–31 August 2006 (23.2°C).

[e] Volatiles collected 1–2 September 2006 (23.4°C).

[f] This sample was lost due to an accident in the laboratory. The Porapak-Q extract was not analyzed, but the device was weighed before and after aeration.

[g] These pentane extracts were also analyzed by GC-MS for compound identification.

greater stem densities and canopy cover than *P. ponderosa* stands. Negrón et al. (2006) provided an excellent discussion of stand factors and microclimatic effects that may help explain why verbenone is generally effective for management of *D. ponderosae* in *P. contorta* (Amman et al. 1989, 1991; Lindgren et al. 1989; Bentz et al. 2005) but not *P. ponderosa* (Bentz et al. 1989, Lister et al. 1990, Gibson et al. 1991, Gibson and Kegley 2004, Negrón et al. 2006) stands. In our view, many of the same principles that attribute the effectiveness of thinning for preventing bark beetle infestations to reductions in host finding (Fettig et al. 2007) help explain the lack of success in using verbenone as a semiochemical-based tool in *P. ponderosa* stands. Semiochemicals released from any given point source diffuse outward passively into a three dimensional airspace. Concentrations rapidly decrease with increasing distance from the point source. Some authors have reported that inversions occur in the stem zone immediately beneath the canopy, which can create a chamber of stable air (Chapman 1967, Fares et al. 1980), thus affecting pheromone distributions and concentrations within forests. These inversions tend to be stronger and longer lasting in dense stands (Fares et al. 1980). Thistle et al. (2004) examined near-field canopy dispersion of a tracer gas (SF_6), as a surrogate for bark beetle pheromones, within the trunk space of trees. They showed that when surface layers are stable (e.g., during low wind velocities) the tracer plume remained concentrated and showed directional consistency due to suppression of turbulent mixing. Lower density stands (e.g., *P. ponderosa* compared with *P. contorta*) result in unstable layers and multidirectional traces (eddies) that dilute pheromone concentrations and could result in a reduction in beetle aggregation, thus influencing host finding and subsequent tree colonization. In the case of verbenone, these effects would negatively impact the performance of synthetic verbenone plumes created by multiple release devices within *P. ponderosa* stands.

In areas of direct sunlight, verbenone released into the airspace may be photoisomerized to chrysanthenone (Kostyk et al. 1993), a compound with no known behavioral effects on bark beetles. This process may be exacerbated by the Mediterranean climate typical of *P. ponderosa* forests in California. The white, UV-reflecting pouch release device and the addition of a cyasorb UV stabilizer that scavenges UV-generated radicals are thought to greatly reduce or eliminate the potential for photoisomerization of verbenone within the pouch (D. Wakarchuk, personal communication). Chrysanthenone was not detected in any of the volatile extracts from verbenone pouches examined in this study, but trace amounts of filifolone, a thermal or photo rearrangement product of (+)-chrysanthenone (Asfaw et al. 2001), were present in extracts from both unexposed and field-exposed pouches (Fig. 4). Therefore, there was only minor and indirect evidence of isomerization of verbenone to chrysanthenone in the release devices, and it is also possible that filifolone was present in the raw verbenone batch used to formulate the devices. However, our analyses do not address whether verbenone underwent photoisomerization once in the active airspace of our research plots.

The structure and composition of the two blocks in this study varied considerably (Table 1) potentially influencing verbenone plume distributions (as detailed above). Furthermore, the large proportion of nonhosts present at Caldor, compared with McCloud, presumably also present substantial physical and olfactory barriers to host finding. Despite this, we observed no differences in efficacy when data from each block were analyzed separately ($df = 1, 4$; $P > 0.23$, all cases).

Range of Inhibition. In our study, no significant differences occurred among trap captures at 0.5, 1, or 2 m from the 5-g verbenone pouch (Table 3). One limitation of our experimental design is that we are currently unable to determine where the break in inhibition occurs between 2 and 4 m. Conservatively, the optimal distribution of 5-g verbenone pouches (i.e., assuming inhibition was constant to 2 m) would require 625 pouches/ha, which is five-fold greater than used in the current study and likely uneconomical under most scenarios (≈$3,600/ha in 2008 US$, excluding labor). Some authors have suggested that many small, point-source releasers (e.g., impregnated beads or flakes) may provide for better dispersal of verbenone and would more appropriately simulate the natural release of verbenone from bark beetle-infested trees, perhaps yielding higher levels of efficacy compared with pouches or bubble caps (Gillette et al. 2006). However, two such attempts to protect

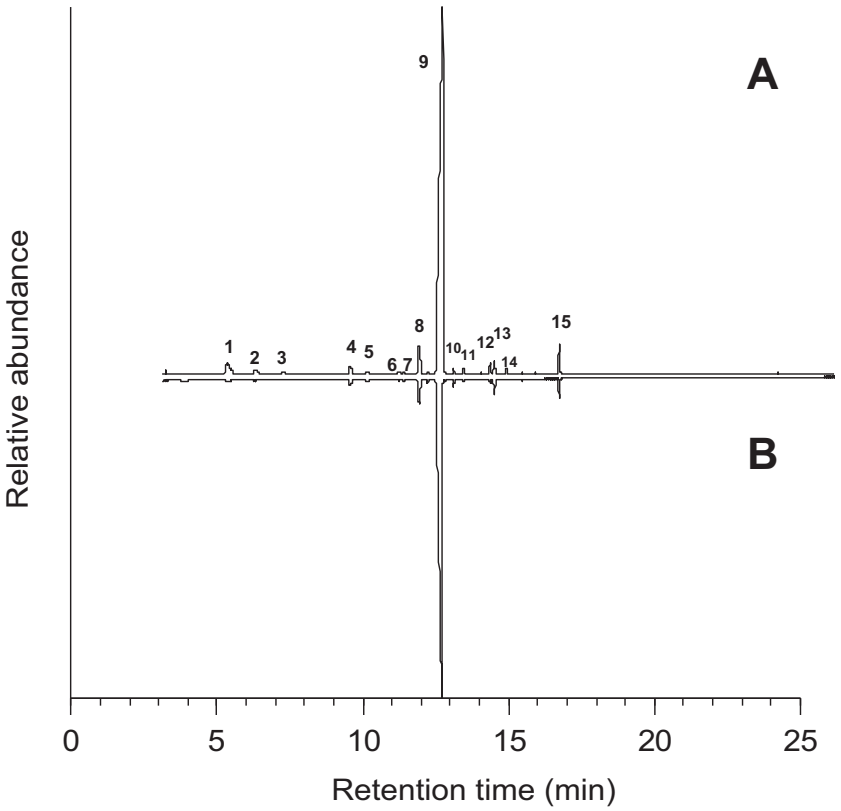

Fig. 4. Comparison of total ion chromatograms from GC-MS analysis of Porapak-Q trapped volatiles from a verbenone pouch that was (A) unexposed in the field with volatiles from a verbenone pouch that was (B) field-exposed for 2 wk (20 July–2 August 2002) at McCloud, Shasta-Trinity National Forest, CA. Compounds identified in the Porapak-Q extracts were identical in the two samples: α-pinene (1), β-pinene (2), 3-carene (3), filifolone (4), filifolone (5), *cis*-verbenol (6), filifolone (7), 4-decanone (8), verbenone (9), 4,7-dimethylbenzofuran (10), unknown (11), 3-ethylacetophenone (12), isopiperitenone (13), 4-ethylacetophenone (14), and piperitenone (15).

individual *P. ponderosa* from *D. brevicomis* attack were unsuccessful (Gillette et al. 2006).

Miller (2002) determined that verbenone bubble caps (14 mg/d at 24–28°C) inhibited *D. ponderosae* attraction to baited multiple funnel traps at a distance of <4 m in *P. contorta* stands in British Columbia. The similarity in results between the two studies is surprising given differences in semiochemical release rates, bark beetle species and stand structure and composition.

Table 3. Disruption of *D. brevicomis* attraction to multiple funnel traps baited with *exo*-brevicomin, frontalin, and myrcene at five distances from a single 5-g verbenone pouch, McCloud, Shasta-Trinity National Forest, CA, 2006

Distance (m)	N[a]	Mean ± SEM[b]
0.5	50	16.3 ± 1.5a
1	50	18.7 ± 1.6ab
2	50	21.2 ± 1.9ab
4	50	31.4 ± 4.0bc
9	50	43.5 ± 6.4c

[a] Number of replicates per treatment.
[b] Means followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD).

Levels of Inhibition. It is well established that verbenone reduces D. *brevicomis* attraction to attractant-baited traps (Bedard et al. 1980a,b; Tilden and Bedard 1988; Paine and Hanlon 1991; Bertram and Paine 1994a; Shea and Wentz 1994; Fettig et al. 2005), yet inhibition is not complete. In general, reductions in trap catches attributed to verbenone are greater for *D. ponderosae* than *D. brevicomis.* For example, Lindgren and Miller (2002) reported that levels of verbenone above 0.2 mg/d resulted in an almost complete shutdown of *D. ponderosae* attraction to its aggregation pheromone. Borden et al. (2004) reported that verbenone (1.8 mg/d) reduced catches of both male and female *D. ponderosae* by >97%. Fettig et al. (2005) reported that verbenone (4 mg/d) significantly reduced *D. brevicomis* attraction by 46.9%. In a second experiment, trap catches were reduced by 35.6 and 48.2% at 4 and 50 mg/d verbenone, respectively (Fettig et al. 2005).

The stronger negative effect of verbenone on captures of *D. ponderosae* in comparison with *D. brevicomis* could provide some explanation as to why verbenone is less effective for preventing *D. brevicomis* attack than *D. ponderosae* attack in forested stands.

However, it must be acknowledged that such comparisons are confounded by differences in the response of these species to their attractants used in trapping bioassays (i.e., the strength of an attractant presumably influences responses to other semiochemicals combined with it). For example, although Hayes and Strom (1994) reported that verbenone had no effect on *D. brevicomis* response to traps baited with its attractant (*exo*-brevicomin, frontalin, and myrcene; mean daily catch of baited controls, 104.9), a significant reduction in *D. brevicomis* trap catch was observed when traps containing verbenone were baited with *D. ponderosae* attractant (*exo*-brevicomin, *trans*-verbenol, and myrcene; mean daily catch of baited controls, 1.8). Although the latter result was obtained in a separate experiment (Hayes and Strom 1994), it is plausible that the efficacy of verbenone was enhanced in the presence of a less powerful attractant.

Population Density. Some authors have speculated that the efficacy of semiochemical-based tools varies with the population density of bark beetles. Progar (2003, 2005) examined the ability of verbenone to deter mass attack of *D. ponderosae* on *P. contorta* in campgrounds and administrative areas on the Sawtooth National Recreation Area, ID. Initially, verbenone was very effective in reducing attacks, but efficacy declined in subsequent year. The author hypothesized that the reduction in response to verbenone over time was related to both *D. ponderosae* population size and spatial scale (i.e., large numbers of beetles in a localized area with a declining proportion of preferred hosts). Similarly, Bentz et al. (2005) reported reductions in the efficacy of verbenone when >140 *P. contorta* were attacked per ha the previous year. At initiation of our study, more than twice as many trees were infested with *D. brevicomis* at McCloud than Caldor (Table 1). Furthermore, nine (of 54) baited trees survived at Caldor, whereas only one (of 54) survived at McCloud. Season-long trapping efforts in association with another study in 2004 resulted in collection of more *D. brevicomis* at McCloud (138,379) than Caldor (124,004) in nearby areas (C.J.F., unpublished data). These data suggest that there was a higher population density of *D. brevicomis* at McCloud than Caldor. In addition, forest health condition reports referenced several large spots of *D. brevicomis*-caused tree mortality on the McCloud Ranger District and in nearby stands during 2002–2004 but no elevated activity near Caldor (USDA Forest Service 2002, 2003, 2004). Despite this, we observed no differences in the efficacy of verbenone between the two blocks. Accordingly, we do not feel population density is a major factor in explaining the lack of efficacy observed in this study.

Ratio of Verbenone to Attractants. The response of bark beetles to verbenone in the presence of aggregation pheromones and host kairomones often depends on the ratio of verbenone to attractants (Byers et al. 1984, Tilden and Bedard 1988, Bertram and Paine 1994a, Miller et al. 1995, Pureswaran et al. 2000) and varies among individuals within a population (Borden et al. 1986, Bertram and Paine 1994b). Amman et al. (1989) suggested that baiting should be used to create a robust experiment and rigorous examination of efficacy before verbenone is adopted for operational use in *P. contorta.* Studies that have documented treatment efficacy often did not bait trees within experimental plots, which is typically unnecessary in *P. contorta* stands as sufficient numbers of trees come under attack by *D. ponderosae* by natural means in areas of active infestation. However, in *P. ponderosa* stands investigators risk having few trees attacked by *D. brevicomis* without baiting. In our experiment, baiting likely provided a rigorous evaluation of efficacy at the expense of detecting any subtle treatment effects.

Realism of Foraging Context. *Dendroctonus* spp. often colonize one or a few closely related host-tree species (Furniss and Carolin 1977). Dispersing beetles must therefore discriminate among different tree species as hosts are unevenly distributed spatially and temporally in many forests. Volatile stimuli associated with host and nonhost trees are important in mediating such behavioral responses (Byers 1995, Borden 1997, Graves et al. 2008). Failures regarding the efficacy of verbenone may be due, in part, to lack of a realistic foraging context (Seybold et al. 2000, Shepherd et al. 2007). That is, synthetic verbenone deployed alone without other beetle-derived or nonhost cues in appropriate quantities, may not provide foraging beetles with the desired misinformation about the stand in which they are searching. Shepherd et al. (2007) suggested future work involving verbenone and *D. brevicomis* should include other beetle-produced compounds in conjunction with verbenone and perhaps nonhost volatiles, such as acetophenone. To that end, the only successful applications of semiochemicals for reducing *D. brevicomis* attacks (Bertram and Paine 1994b; Fettig et al. 2008, 2009) and *D. brevicomis*-caused tree mortality (Fettig et al. 2008, 2009) combined verbenone with other semiochemicals.

Release Rates and Passive Release Devices. Several authors have speculated that previous failures in the efficacy of verbenone resulted from problems associated with passive release from bubble caps and pouches, which is controlled by ambient temperatures in conjunction with membrane composition and internal vapor pressure (Holsten et al. 2002). For example, Amman and Lindgren (1995) stated that weather factors, such as high temperatures, may cause verbenone to elute before beetle dispersal. Although this is an important concern in *P. contorta* stands where one application of verbenone is generally made per year, pouches in our study were replaced every 4 wk, which is more frequent than recommended by the manufacturer.

Holsten et al. (2003) detailed characteristics of an ideal pheromone release system: 1) release of consistent amounts of pheromone per unit time, 2) ability to release different pheromones, 3) ability to provide different release rates, 4) protection from environmental degradation, 5) release of all pheromone, and 6) time-specified release. They described beads, bubble caps, and pouches as first-order emitters whose

release rates generally decline over time, which was observed in our study (Fig. 3A and B). This is undesirable as pheromone plumes must be maintained during certain periods (e.g., during the flight activity period of *D. brevicomis* which extends for 5–6 mo in many areas) for effective treatment. Gibson and Kegley (2004) evaluated the release rate of verbenone pouches in *P. ponderosa* stands in Montana. Pouches were removed at ≈2-wk intervals. During the first 53 d, release rates ranged from 38 to 80 mg/d, but at 63 d no verbenone was released despite ≈1.9 g of verbenone remaining in the pouch (Gibson and Kegley 2004). Although concerns regarding passive release are important, once verbenone is released into the active airspace, distribution and concentration are mediated by microclimate and interaction with surfaces and aerosols in forest ecosystems, which are heavily influenced by stand structure and composition.

Our gravimetric and chemical analyses revealed few meaningful differences in the rates of release between unexposed and field-exposed pouches (Fig. 3A and B), and no adulteration of the chemical content of volatiles emanating from pouches (Fig. 4), both of which could have helped explain the lack of efficacy observed in this study. To that end, unexposed pouches maintained a target gravimetric release rate of >50 mg/d for 39 d (Fig. 3B; 5 d [equilibration incubation] + 34 d [analysis incubation]) at 30°C in the laboratory. During actual field exposure, temperatures were quite variable, ranging from −3.0 to 42°C and 3.0 to 34°C at McCloud and Caldor, respectively. Pouches that were field-exposed for 4 wk still maintained a release rate of >50 mg/d for 16 d in the laboratory (Fig. 3B). It seems field exposure may alter the pouch membrane as devices that were field-exposed for 4 wk had significantly higher release rates of verbenone than unexposed pouches during the last several days of the evaluation period (Fig. 3A, III).

In conclusion, there has been considerable interest in using verbenone as a tool for managing *D. brevicomis* infestations. However, our results suggest that using this semiochemical alone is not currently advisable. We believe lack of efficacy is primarily due to four factors: 1) levels of inhibition, as indicated by trapping studies evaluating verbenone, are low and much lower than for *D. ponderosae;* 2) a limited range of inhibition suggests larger numbers of pouches per unit area are required to achieve maximum efficacy than previously considered; 3) a single semiochemical is likely not sufficient to provide desired behavioral effects at the stand level; and 4) low stand densities and elevated temperature regimes result in unstable layers and multi-directional traces (eddies) that dilute synthetic verbenone plumes in *P. ponderosa* stands. Based on these results, we suggest future work should concentrate on the use of verbenone in combination with other semiochemicals at higher doses than considered previously. Furthermore, any semiochemical-based tools should be considered among all management techniques in an integrated approach.

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