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Endothall and 2,4-D activity in milfoil hybrid (*Myriophyllum spicatum* × *M. sibiricum*) when applied alone and in combination

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Abstract

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is an invasive aquatic plant that can hybridize with the native northern watermilfoil (*M. sibiricum* Kom.). These milfoil hybrids (*M. spicatum* × *M. sibiricum)* are becoming more prevalent in many lakes where the invasive and the native *milfoil* co-occur. Hybrid plants are more vigorous than either parent with a faster growth rate and lower sensitivity to some herbicides. The aquatic herbicides endothall and 2,4-D provide two effective modes of action for management of the hybrids. For more than a decade, these two herbicides have been used in combination as an effective control option and a resistance management strategy. How this combination impacts herbicide movement and efficacy is unknown. Therefore, the objective of this research was to determine the activity of endothall and 2,4-D combined compared with activity applied alone. Absorption and translocation of endothall, 2,4-D, and the combination was determined in hybrid plants over a 96-h time course. Endothall accumulation was not impacted when these herbicides were applied in combination; however, 2,4-D accumulation increased by 80%, relative to when 2,4-D was applied alone. Endothall translocation from shoots to roots decreased by almost 50% when applied in combination with 2,4-D (alone = 16.7% ± 2.6%; combination = 9.2% ± 1.2%). Shoot-to-root translocation of 2,4-D also decreased when the two herbicides were applied in combination (24.8% ± 2.6% when applied alone to only 3.93% ± 0.4% when in the presence of endothall). This research demonstrates that combining herbicides can significantly impact herbicide activity in plants. Future research is needed to determine whether this reduced translocation negatively impacts operational effectiveness when these herbicides are applied in combination.

Introduction

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is a widespread invasive aquatic plant species across the United States. Its management is one of the most extensive and expensive among aquatic invasive plants (Gettys et al. 2020; Pimentel 2009). *Myriophyllum spicatum* can hybridize with native northern watermilfoil (*M. sibiricum* Kom.), and many populations originally identified as invasive *M. spicatum* were later confirmed as milfoil hybrids (*M. spicatum* × *M. sibiricum*) (Moody and Les 2002; Sturtevant et al. 2009). *Myriophyllum spicatum* × *M. sibiricum* infestations can rapidly displace native plant communities, resulting in dense monotypic mats of vegetation that reduce light penetration. A severe *M. spicatum* × *M. sibiricum* infestation can negatively affect water quality, altering native aquatic habitats; reducing native fish and macroinvertebrate diversity; and impairing recreational uses of the water, such as fishing, boating, and swimming (Madsen et al. 1991; Newroth 1985; Schultz and Dibble 2012; Smith and Barko 1990).

Myriophyllum spicatum × *M. sibiricum* grows more aggressively than either parent and requires intensive management (Glisson and Larkin 2021; Taylor et al. 2017; Thum and McNair 2018), and while there are several control strategies for aquatic invasive plants, herbicides are one of the most important management options. The synthetic auxin, 2,4-dichlorophenoxy acetic acid (2,4-D) and the serine/threonine protein phosphatase inhibitor, 7-oxabicyclo(2.2.1) heptane-2,3-dicarboxylic acid (endothall) are herbicides typically used to manage *M. spicatum* × *M. sibiricum* (Getsinger et al. 1982; Netherland et al. 1991; Wersal et al. 2010). The herbicide 2,4-D is intensively used for *M. spicatum* × *M. sibiricum* control, as it is one of the least expensive management options and is selective toward this species. However, the extensive use of 2,4-D eventually selected for *M. spicatum* × *M. sibiricum* with reduced sensitivity to 2,4-D (Ortiz et al. 2022a). Similarly, endothall, a broad-spectrum herbicide, is widely used for large-scale and spot treatments of *M. spicatum* × *M. sibiricum*. These two herbicides are also often used in combination at low concentrations to improve selective control of milfoil species and curlyleaf pondweed (*Potamogeton crispus* L.) in a single treatment event (Skogerboe and Getsinger 2006).

Combining herbicides with different modes of action (MOAs) or using herbicide rotations is widely recommended to delay the development of herbicide resistance (Beckie and Reboud 2009). In terrestrial studies and modeling simulations, mixtures were the most effective measure for delaying resistance (Beckie and Reboud 2009; Busi et al. 2020; Evans et al. 2018).

Because resistance is often target-site specific, treating weed populations, terrestrial or aquatic, with two or more different MOAs will delay or negate resistance. In this approach, an individual weed resistant to one MOA is controlled by the other herbicide acting at a different site of action (Busi et al. 2020).

In addition to delaying or preventing herbicide resistance, tank mixes or premixed herbicide combinations can reduce application costs and may work synergistically to improve treatment efficacy (Barbieri et al. 2023). Although this is an effective and popular strategy in terrestrial systems, herbicides must be compatible with each other. In some cases, herbicides may be antagonistic (e.g., ACCase with 2,4-D) resulting in reduced weed control. In other cases, incompatibility occurs if the mixture forms a precipitate or a gel. This occurs when certain formulations of endothall and 2,4-D are combined. Chinook® is a premix formulation combining endothall and 2,4-D developed to alleviate this problem, affording applicators the advantage of two different MOAs.

To date, only 15 activity ingredients encompassing nine MOAs are registered for aquatic use (Ortiz et al. 2020); consequently, when resistance develops in aquatic weeds, the options for alternative herbicide MOAs are limited. The use of herbicide mixtures is being implemented in aquatic weed management practices; however, there is limited information on herbicide activity when applied in combination. There are examples of both herbicide antagonism and synergism in terrestrial weed management, and the same could occur in aquatic systems (Kyser et al. 2021; Wersal and Madsen 2010, 2012). To better understand herbicide activity when used in combination, we investigated the activity of endothall and 2,4-D applied alone and in combination. More specifically, the objectives of this research were to determine absorption and translocation patterns in *M. spicatum* × *M. sibiricum* when these herbicides were applied alone and in combination.

Materials and Methods

Plant Material

Plant shoot fragments of confirmed hybrid *M. spicatum* × *M. sibiricum* (Patterson et al. 2017) were obtained from Hayden Lake, ID, in 2015. Uniform plant material was obtained by propagating 10-cm apical sections of these plants in 16 cm by 12 cm by 6 cm (1,152-cm^3) plastic pots filled with soil known to be pesticide-free for 6 yr before collection (Colorado State University Organic Research Farm). Each pot received 2 g of slow-release fertilizer (Osmocote Smart Release® Plant Food 15-9-12, Scotts, 14111 Scottslawn Road, Marysville, OH 43040) and covered with a 1-cm layer of washed sand before planting of 6 apical shoots per pot. Pots were then placed in 1.2 m by 1 m by 0.9 m (1,041 L) plastic tanks and grown in dechlorinated tap water under greenhouse conditions. The photoperiod was 14:10-h light:dark, supplemental lighting was provided with 400-W sodium-halide light bulbs, and the greenhouse temperature was set at 24 C during the day and 18 C at night.

When apical shoots reached 15 to 18 cm in length (approximately 2 wk after propagation), plants with well-developed roots of similar size were selected for absorption and translocation experiments. Roots were cleaned with tap water and transplanted into 15-ml plastic tubes (15-ml conical centrifuge tubes, Thermo Fisher Scientific, 81 Wyman Street, Waltham, MA 02451). The tubes were filled with unwashed silica sand and sealed at the base of the shoot with a low melting point eicosane wax (Eicosane 99%, ACROS Organics, 81 Wyman Street, Waltham, MA 02451) to isolate the root system from water column (Frank and Hodgson 1964; Ortiz et al. 2019). Plants were transferred to 4-L plastic tanks (22.7-cm tall by 17-cm diameter) filled with dechlorinated water for a 24-h acclimatization to the laboratory environment before application of radiolabeled herbicides.

Herbicide Exposure

Twelve 4-L glass beakers (25-cm tall by 15-cm diameter) were filled with 3.5 L of dechlorinated tap water (pH 6.8). Three beakers were treated with [^{14}C]endothall ring-labeled (56.6 mCi mmol^{-1} specific activity, Moravek Biochemicals, 577 Mercury Lane, Brea, CA 92821) combined with formulated dipotassium salt of endothall (Cascade®, United Phosphorus, 630 Freedom Business Center, Suite 402, King of Prussia, PA 19406) to achieve a final concentration of 0.75 mg L^{-1}. Three beakers were treated with [^{14}C]endothall combined with formulated, non-radiolabeled premix herbicide of endothall and 2,4-D (Chinook®, UPL OpenAg, 15401 Weston Parkway, Suite 150, Cary, NC 27513) to achieve final concentrations of 0.75 and 0.3 mg L^{-1} of endothall and 2,4-D, respectively. Three beakers were treated with [^{14}C]2,4-D ring-labeled (55 mCi mmol^{-1} specific activity, American Radiolabeled Chemicals, 101 Arc Drive, St Louis, MO 63146) combined with formulated 2,4-D (Clean Amine®, Loveland Products, 3005 Rocky Mountain Avenue, Loveland, CO 80538) to achieve a final concentration of 0.3 mg L^{-1}. Three remaining beakers were treated with [^{14}C]2,4-D combined with a formulated, non-radiolabeled premix herbicide of endothall and 2,4-D as previously described.

Radioactivity in each treatment tank was verified using liquid scintillation spectroscopy (LSS) (Packard 2500R, PerkinElmer, 940 Winter Street, Waltham, MA 02451), with [^{14}C]endothall-treated tanks containing 0.98 ± 0.03 μCi L^{-1} and [^{14}C]2,4-D-treated tanks containing 1.02 ± 0.04 μCi L^{-1}.

Each beaker contained six *M. spicatum* × *M. sibiricum* plants and one control tube with a toothpick to mimic a plant stem to assess the efficacy of the wax barrier. All plants were held in place using a round test tube rack (No-Wire Round Rack, Bel-Art Scienceware, 661 Route 23 South, Wayne, NJ 07470). Throughout the experiment, plants were maintained under controlled laboratory conditions at 22 C, subjected to a 14:10-h light:dark photoperiod, supplemented with LED grow lights (approximately 500 μmol m^{-2} s^{-1}) (LI-185B, Li-Cor, 4647 Superior Street, Lincoln, NE 68504). Beakers were stirred once a day, and total volume was adjusted daily. Plants were harvested at 6, 12, 24, 48, and 96 h after treatment (HAT). For each time point, three replicates were randomly selected from a different tank, rinsed four times with clean tap water, and divided into shoots and roots. Following separation, plant parts were dried at 60 C for a minimum of 48 h, and dry biomass was recorded for each plant part. Plant parts were combusted in a biological oxidizer (OX500, R.J. Harvey Instrument, 11 Jane Street, Tappan, NY 10983) for 2 min. The resulting $^{14}CO_2$ was captured using a ^{14}C trapping cocktail (OX161, R.J. Harvey Instrument). The efficiency of the oxidizer was greater than 98%. Following oxidation, radioactivity was quantified by LSS.

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Statistical Analysis

The study was repeated, and data collected from these experiments were analyzed using RStudio (v.1.4.1.1717, Posit Software, 250 Northern Avenue, Suite 410, Boston, MA 02210) and MS Excel and plotted with Prism 9 (GraphPad Software, 2365 Northside Drive, Suite 560, San Diego, CA 92108). Levene's test for homogeneity of variance ($\alpha = 0.05$ level of significance) was performed using the CAR package in R (v.4.0.0, R Foundation for Statistical Computing, Vienna, Austria) to confirm that data from repeated experiments could be combined. For all experiments, fresh weight was back-calculated from dry weight, considering 90% of water content, determined based on 10 *M. spicatum* × *M. sibiricum* plants. Absorption and translocation over time were analyzed using a nonlinear regression analysis to fit a hyperbolic function (Kniss et al. 2011).

Bioaccumulation of herbicides was estimated by calculating the plant concentration factor (PCF) using an equation adapted from de Carvalho et al. (2007) and can be defined as:

$$\mathrm{PCF} = \frac{\text{Herbicide concentration in plant (ng/g fresh biomass)}}{\text{Herbicide concentration in water (ng/ml)}} \qquad [1]$$

PCF is often used to compare herbicide absorption across different herbicide concentrations and aquatic plant species (Haug et al. 2021; Ortiz et al. 2019, 2022a, 2022b, 2022c; Vassios et al. 2017).

The predicted absorption at 96 HAT (A_{96}) and the predicted time required for 90% of that absorption (t_{90}) were derived from the nonlinear regression equations of these analyses. The A_{96} value is a measure of the theoretical maximum absorption among different plant parts, plant species, and herbicides. The t_{90} value is a measure of the rate of absorption.

Results and Discussion

The eicosane wax barrier effectively isolated plant roots from the radiolabeled treatment solutions. At 96 HAT, only 0.029 ± 0.009 Bq ml^{-1} ($n = 6$) and 0.021 ± 0.008 Bq ml^{-1} ($n = 6$) of radioactivity was measured in the non-plant, control test tubes for [^{14}C]endothall and [^{14}C]2,4-D treatments, respectively. There was no detected radioactivity in 7 out of the 12 combined test tubes. This insignificant amount of radioactivity had no impact on the outcomes of this study.

Endothall absorption did not reach a maximum asymptote when applied alone or in the presence of 2,4-D (Figure 1). Although the asymptotic rise to max function is the most biologically relevant function to describe herbicide absorption (Kniss et al. 2011), previous research also demonstrated that endothall at 2 and 3 µg L^{-1} did not reach maximum asymptote in *M. spicatum* or hydrilla [*Hydrilla verticillata* (L. f.) Royle] at 192 HAT (Ortiz et al. 2019, 2022b).

Bioaccumulation of [^{14}C]endothall did not change in the presence of 2,4-D. At 96 HAT, the PCF_{96} was 12.0 ± 0.6 when applied alone and 13.2 ± 0.6 in the presence of 2,4-D (Figure 1). These values were not statistically different. Endothall bioaccumulation at 3 mg L^{-1} at 192 h was only 3.3 ± 0.4 in *M. spicatum* (Ortiz et al. 2019). The reason for greater herbicide bioaccumulation in this study is likely due to the difference in herbicide rate. The lower concentration may have allowed the plant to remain physiologically active, maintaining a stronger concentration gradient for a longer timer period. The increased growth rate of *M. spicatum* × *M. sibiricum* compared with *M. spicatum* also could have contributed the greater bioaccumulation.

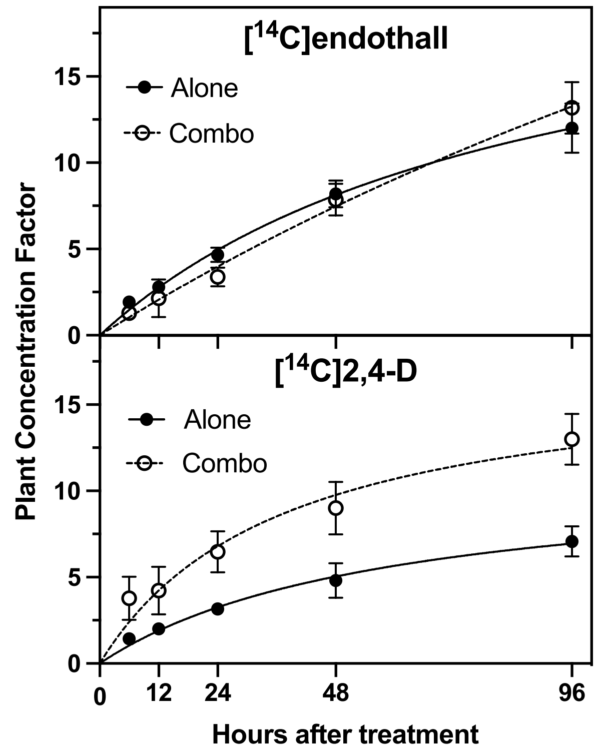

Figure 1. [^{14}C]endothall and [^{14}C]2,4-D bioaccumulation in *Myriophyllum spicatum* × *Myriophyllum sibiricum* over a 96-h time period expressed as plant concentration factor (PCF). Data presented are means and standard error of the mean ($n = 6$).

The *n*-octanol/water partition coefficient (log K_{ow}) of endothall is very similar to those of triclopyr and penoxsulam (−0.55, −0.45, and −0.35, respectively), which should translate to a similar PCF, but it varied greatly in *M. spicatum* and *H. verticillata* (Ortiz et al. 2019; Vassios et al. 2017). De Carvalho et al. (2007) demonstrated that in aquatic plants, herbicide bioaccumulation cannot be predicted when the herbicide's log K_{ow} values are <2 and increased herbicide accumulation does not necessarily correlate to better plant control (Ortiz et al. 2019).

The PCF_{96} for [^{14}C]2,4-D alone at 0.3 mg L^{-1} was 6.9 ± 0.3 (Figure 1). Previous research reported that 2,4-D bioaccumulation at 1 mg L^{-1} at 192 HAT was 5.7 ± 0.2 and 7.88 ± 0.2 for *M. spicatum* and *M. spicatum* × *M. sibiricum*, respectively (Ortiz et al. 2022a). When in the presence of 0.75 mg L^{-1} endothall, [^{14}C]2,4-D bioaccumulation in *M. spicatum* × *M. sibiricum* increased to 12.5 ± 0.6 (Table 1). Endothall caused a similar increase in foliar absorption of ethephon, another plant growth regulator, in red kidney bean (*Phaseolus vulgaris* L.) leaves (Sterrett et al. 1974).

Endothall absorption by *M. spicatum* × *M. sibiricum* at 96 HAT (A_{96}) was 63.3 ± 1.9 µg g^{-1} (Table 1), and it was not impacted when in combination with 2,4-D (74.0 ± 2.0 µg g^{-1}). In contrast, 2,4-D absorption increased significantly in the presence of endothall, 16.9 ± 1.2 µg g^{-1} and 36.7 ± 1.9 µg g^{-1}, alone and in combination with endothall, respectively (Table 1). Consistent with our findings, when applied at a higher rate of 1 ppm, 2,4-D's absorption by *M. spicatum* × *M. sibiricum* at 192 HAT was 75.1 ± 4.6 µg g^{-1} (Ortiz et al. 2022a).

Table 1. Predicted plant concentration factor 96 h after treatment (HAT) (PCF_{96}), herbicide absorption (μg g^{-1}) at 96 HAT (A_{96}), and the time in hours required to reach 90% of A_{96} (t_{90})[a]

Treatment	PCF_{96}	Plant part	A_{96}	t_{90}
			— μg g^{-1} —	— h —
[^{14}C]endothall	12.0 ± 0.6	Shoots	63.3 ± 1.9	78.3
		Roots	12.2 ± 2.1	75.0
[^{14}C]endothall + 2,4-D	13.2 ± 0.6	Shoots	74.0 ± 2.0	84.6
		Roots	7.9 ± 0.8	73.8
[^{14}C]2,4-D	6.9 ± 0.3	Shoots	16.9 ± 1.2	70.7
		Roots	5.6 ± 0.7	81.8
[^{14}C]2,4-D + endothall	12.5 ± 0.6	Shoots	36.7 ± 1.9	69.1
		Roots	1.3 ± 0.1	61.0

[a]Values represent the mean, and error terms represent the standard error of the mean ($n = 6$).

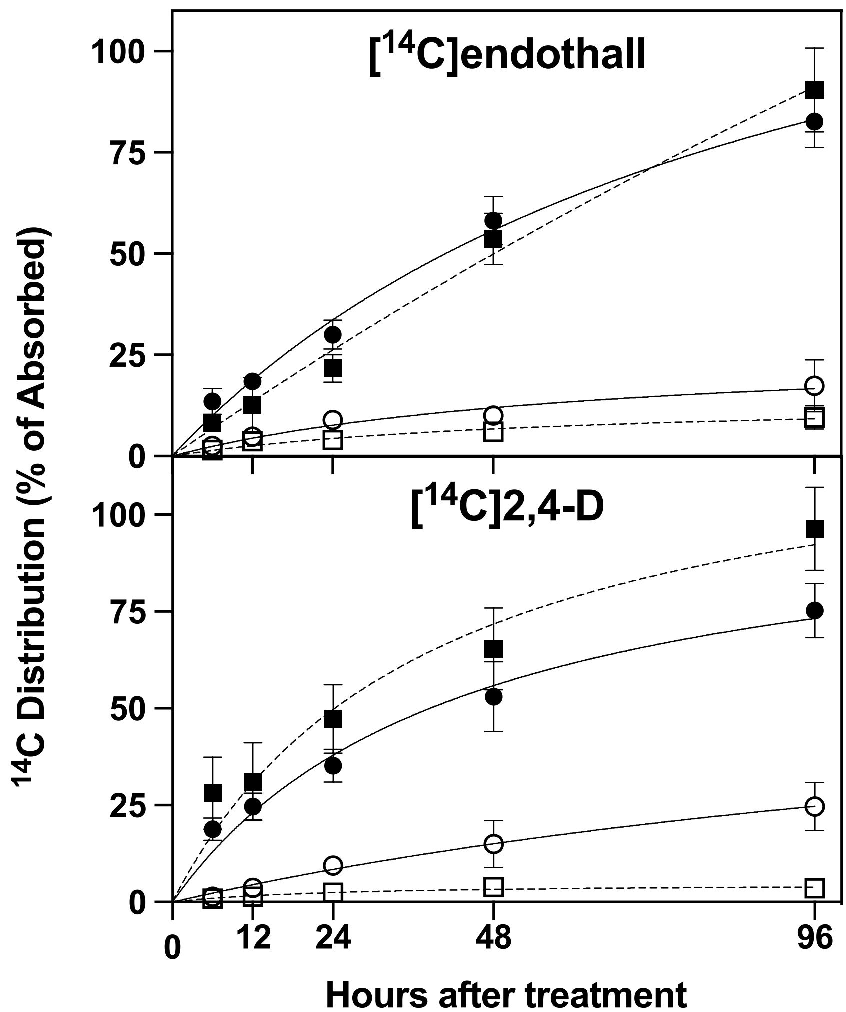

Figure 2. [^{14}C]herbicide distribution in plants over 96 h following exposure to [^{14}C]endothall or [^{14}C]2,4-D expressed as percentage of total herbicide absorbed. Filled circle, percentage of [^{14}C] alone in shoots; open circle, percentage of [^{14}C] alone in roots; filled square, percentage of [^{14}C]herbicide in combination with non-radiolabeled 2,4-D or endothall in shoots; open square, percentage of [^{14}C]herbicide in combination with non-radiolabeled 2,4-D or endothall in roots. Data presented are means, and error bars are the standard errors of the mean ($n = 6$).

Endothall shoot-to-root translocation, estimated by the presence of radioactivity, was 16.7% ± 2.6% when applied alone (Figure 2). This is approximately twice the amount of translocation previously reported for *M. spicatum* (Ortiz et al. 2019). The current study used a lower endothall concentration and the more aggressive *M. spicatum* × *M. sibiricum*, so these differences are not unexpected. The combination of endothall plus 2,4-D reduced endothall translocation by almost 50% (9.2% ± 1.2%). While this difference is statistically significant, it may not have any significant impact on the biological and operational usefulness of endothall.

Madsen et al. (2010) reported 100% control of *M. spicatum* in outdoor mesocosm treatments over a 4-wk period when endothall and 2,4-D were applied at higher rates of 1 and 0.5 ppm, respectively.

Shoot-to-root translocation of 2,4-D was 24.8% ± 2.6% when applied alone, but only 3.93% ± 0.4% when applied in combination with endothall (Figure 2). As previously mentioned, our research did not evaluate the efficacy of these herbicide interactions. Endothall also limits basipetal 2,4-D transport in detached bean leaves (Leonard and Glenn 1968). The reason for the decrease in 2,4-D translocation to plant roots may be attributed to the rapid induction of cell death by endothall (Bajsa et al. 2012), which limits the translocation of both herbicides. This contrasts with the delayed onset of cell death associated with 2,4-D (Grossmann 2010). Ortiz et al. (2022c) observed a higher concentration of endothall in the roots of *P. crispus* when exposed to 0.75 mg L^{-1} compared with 3 mg L^{-1} endothall. This discrepancy is attributed to the accelerated shutdown of the plant's functions at the higher herbicide concentration, preventing effective translocation.

In conclusion, the activities of endothall and 2,4-D are significantly impacted when applied in combination on *M. spicatum* × *M. sibiricum*. These differences included greater absorption of 2,4-D and reduced translocation to the roots for both herbicides. Future research needs to be conducted to determine whether this reduced translocation negatively affects the long-term effectiveness of this control strategy.

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Competing interests. No conflicts of interest have been declared.

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