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Research Article

Efficacy, non-target impacts, and other considerations of unregistered fipronil-laced baits being used in multiple invasive ant eradication programs

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Abstract

We present three studies assessing the efficacy and non-target impacts of multiple unregistered forms of hydrogel ant baits, as well as some co-use with the granular bait Antoff, that were experimentally used targeting Argentine ant, *Linepithema humile,* and yellow crazy ant, *Anoplolepis gracilipes,* within natural/semi-natural environments in Australia. The three studies varied greatly in design and treatment regimens, and were each conducted to address real-time learning needs while attempting to understand how best to use these experimental baits within the spatial, temporal and logistic limitations of three eradication programs. All studies involved broadscale applications of numerous forms of the baits, with greatly varying treatment regimens, coupled with before-after sampling of ant communities, as well as other soil invertebrates in one study. All studies found the baits were highly efficacious against both species, more so for *A. gracilipes* than *L. humile*. Eradication is considered to have been achieved for *A. gracilipes* in one treatment area with a triple treatment regimen, but not using different treatment regimens in other areas. Six treatments conducted approximately one week apart did not eradicate *L. humile*. Few non-target impacts were found, predominantly occurring only when sampling was conducted within days of a treatment, or at the end of six treatments of a high application rate. Instead, non-target species richness and composition were most often more affected by spatial location or sample time than treatment. Any treatment effects were non-persistent after 6–18 months. Hydrogel baits are likely to have significant roles to play for ant management and eradication.

Key words: ants, invertebrates, food, lure, treatments, toxic

Introduction

As global trade increases so too does the number of species being accidentally transported to exotic locations (Essl et al. 2011; Seebens et al. 2017), many of which become problematic (Mack et al. 2000; Clavero and García-Berthou 2005). Many ant species have become widely dispersed

throughout the world (McGlynn 1999; Bertelsmeier et al. 2018), with a great variety of consequences (Holway et al. 2002; Gruber et al. 2022) and associated costs (Angulo et al. 2022), increasingly requiring management action for control or eradication (Hoffmann et al. 2016). Consequently, invasive ant management is also increasingly occurring within areas of conservation value, and so it is important that any baits used do not have significant non-target effects.

Many ant management products are available, but applications are primarily conducted using sprays or granular baits (Hoffmann et al. 2009). Both can be effective but also have severe limitations. Because sprays do not focus on any target species, they have excessive non-target impacts (Brown 1961; Markin et al. 1974; Summerlin et al. 1977), and therefore are not the most desirable treatment option in natural environments. Granule-based baits of varying matrix constituents (e.g. corn grit, fishmeal) will be consumed by species that forage for solid foods (Williams 1983; Krushelnycky and Reimer 1998; Montgomery et al. 2015), but will have low attractancy to species that prefer aqueous sugar (e.g. honeydew) (Baker et al. 1985; Samways 1985; Stanley and Robinson 2007). Consequently, there is not always a suitable or registered product available for treating some of the increasing number of species found outside of their native ranges within the increasing diversity of environmental situations they establish. As a result, new baits are constantly being developed and tested for efficacy and non-target impacts for ant eradication programs (Marr et al. 2003; Boser et al. 2014; Montgomery et al. 2015).

In 2002, a novel fishmeal-based bait was used for the first time to control yellow crazy ant, *Anoplolepis gracilipes,* on Christmas Island (Green et al. 2004). At the time this bait was called Presto Ant Bait (BASF, Brisbane), but it has subsequently changed ownership and had its physical form modified (water content and granule size) and is now called Antoff (Animal Control Technologies, Melbourne), but the product remains unregistered with Australia's chemical regulator. This bait has been the sole product used for the past 20 years for *A. gracilipes* management on Christmas Island, and has been the primary product used in multiple *A. gracilipes* eradication programs elsewhere around Australia for almost as long. However, despite its long term and widespread use, little has been published in the peer-reviewed literature of its non-target impacts (Stork et al. 2014) with most work remaining in unpublished reports (e.g. Marr et al. 2003; Stork et al. 2003; Hoffmann and Pettit 2017).

In 2012, the novel bait form of hydrogels was used for the first time against ants (Boser et al. 2014). Polyacrylamide hydrogels are superabsorbent polymers that can absorb up to around 350 times their weight in water, and in this instance the hydrogels had absorbed a sucrose solution containing a toxicant. The use of hydrogels for ant control was novel in that it provided a liquid food substance that can be imbibed by ants, but it is in a solid form which allows for ground or aerial dispersal. Active constituents delivered

in polyacrylamide hydrogels have proven to be highly efficacious against the Argentine ant, *Linepithema humile,* (Boser et al. 2014; Buczkowski et al. 2014a, b; Rust et al. 2015; Tay et al. 2017) and other invasive ant species (Peck et al. 2017; Sunamura et al. 2022), in numerous locations in both laboratory and field settings, but non-target impacts remain poorly explored and reported. Notably, the sugar in the hydrogels may make these baits more attractive to a wider variety of ants and other invertebrates than would occur with other solid baits, and therefore they may have greater non-target impacts.

Here we present three studies assessing the efficacy and non-target impacts of multiple unregistered forms of hydrogel ant baits, as well as some co-use with the granular bait Antoff, as they were experimentally used within three ant eradication programs targeting *L. humile* and *A. gracilipes* within natural/semi-natural environments in Australia. The three studies were conducted independently of each other and varied greatly in design. Each was conducted to address real-time learning needs attempting to understand how best to use these experimental baits within the spatial, temporal, and logistic limitations of the eradication programs, not because they were intended to be an integrated research program.

Materials

Commonalities among the studies

We acknowledge that the three designs have varying levels of inherent pseudoreplication (Hurlbert 1984) because the treatment (infestation) is not always replicated, but this was unavoidable especially in Studies 1 and 3 which could only treat a single infestation. Likewise, in Study 1 it was not possible to fully resolve spatial autocorrelation issues (i.e. one treatment was spatially separated from the others) which constrains the ability to discern between treatment and spatial effects. These issues are common within invasive species research. In line with recommendations of how to minimise these pseudoreplication issues (Hurlbert 1984) we conducted sampling across a broad area within the infested sites, and throughout comparable nearby surrounding uninfested areas subject to site access.

The delineation of the ant populations used, as well as of other populations in the broader areas in some instances to adequately select control sites, was conducted as part of the associated eradication programs, and those methods and data are not presented here.

Pitfall traps used in Studies 2 and 3 were 4.2 cm diameter specimen containers, partly filled with ethylene glycol as a preservative. Ants were sampled at each plot using an array of 9 pitfall traps set in a 3 × 3 grid with 10 m spacing between traps, operated for 48 hours.

Baits

Three types of hydrogel were used among the studies. First was Magic water beads, being clear 2.5 mm round polyacrylamide beads from Magic

Water Beads One, Inc. in Florida, USA. Second was Magic water crystals, being clear 2.5–4 mm irregularly shaped polyacrylamide crystals, also from Magic Water Beads One, Inc. in Florida, USA. Third was Water$ave Floragel, being clear 2–8 mm irregularly shaped polyacrylamide hydrogels, from Polymer Innovations in Australia. All hydrogel types were prepared for use as bait by being placed in 30% sugar-water solutions containing 0.006 mL/L fipronil (sourced from Termidor®) and allowed to absorb the solution for 24 hours. Sugar used in Study 1 on Norfolk Island was standard 1A sugar from the New Zealand Sugar Company, and in Studies 2 and 3 on the Australian mainland was standard white sugar from CSL Limited. In our conditions, the hydrogels were typically effective for a maximum of 24 hours before they dehydrated too much for ants to be able to imbibe the liquid.

Antoff (used only in Study 2) is an unregistered granular ant bait in Australia sourced from Animal Control Technologies in Brisbane, Australia. It consists of a dry (< 10% moisture) fishmeal matrix containing 0.001g/kg fipronil. The form used here was approximately 3–5 × 2 mm cylindrical granules.

Study 1 Norfolk Island

This study was conducted on Norfolk Island (29°02′S; 167°57′E) in the Pacific Ocean. The island has a subtropical climate with an average temperature range of 12–20 °C in winter,

19–25 °C in summer, and an average rainfall of around 1300 mm per year falling throughout the year but mostly in June and July (BOM 2022b). The natural vegetation is subtropical rainforest dominated by Norfolk Island Pine, *Araucaria heterophylla.*

A single infestation of *L. humile* covering 13 ha was chosen for toxic treatment using Magic water beads, with the infestation on one side of a road designated for high-rate treatments (77 kg/ha) (code H) and the other side for low-rate treatments (38 kg/ha) (code L) (Supplementary material Figure S1). The area surrounding the infestation was used as an uninfested and untreated control (code UN), and another spatially discrete infestation 3 km away that remained untreated was used as an untreated and infested control (code UI). Treatments were conducted by hand, commencing in late January 2016 and conducted approximately weekly until six treatments had been applied (Table 1).

Twenty locations in each of H, L and UI where *L. humile* was present were permanently marked for efficacy assessments. Efficacy assessments were conducted by placing a tea-spoon sized amount of catfood (lure) containing fish at the base of each marker. Lures were left for 30 minutes, then the number of Argentine ants at each lure were visually estimated. Assessments were conducted one day prior to each treatment, and after one and two weeks following the final treatment (Table 1).

Table 1. Sampling and treatment regimens for the three studies. Colours represent treatment codes for Study 2: blue is treatment code BB, red is code AF and green is code BACF.

Study	Sample dates	Treatment dates			Product used	Rate	Fipronil used	Polyacrylamid used
1	pre-treatment January 2016 3 days after first treatment April 2016 September 2017	Approximately weekly, January to March 2016			Magic water beads	4 or 8 beads/m² equating to 38 or 77 kg/ha respectively	1.368 or 2.772 g/ha	1.824 or 3.696 g/ha
2		Site 1 (10ha)	Site 2 (20ha)	Site 3 (66ha)				
	25 May 2016							
		29 May 2016	29 May 2016		Magic water beads	70 kg/ha	0.42 g/ha	0.56 g/ha
		2 June 2016	2 June 2016		Magic water beads	70 kg/ha	0.42 g/ha	0.56 g/ha
	22 June 2016							
				2 April 2017	Magic water beads	70 kg/ha	0.42 g/ha	0.56 g/ha
	1 August 2017							
			16 August 2017	16 August 2017	Antoff	10 kg/ha	0.01 g/ha	
				12 December 2017	Magic water crystals	70 kg/ha	0.42 g/ha	0.56 g/ha
			15 May 2018	15 May 2018	Floragel	70 kg/ha	0.42 g/ha	0.56 g/ha
	4 November 2019							
3	12 December 2017							
			16 December 2017		Floragel	70 kg/ha	0.42 g/ha	0.56 g/ha
			17 March 2018		Floragel	70 kg/ha	0.42 g/ha	0.56 g/ha
			23 June 2018		Floragel	70 kg/ha	0.42 g/ha	0.56 g/ha
	27 June 2019							

Non-target impact samples were collected from ten permanent sampling plots (4 m × 4 m) within each of the four treatment categories (H, L, UI, UN; Figure S1). To keep the environmental conditions as homogeneous as possible, all plots were positioned within native vegetation, not open paddocks or gardens. Samples were haphazard collection of leaf litter and soil to a depth of 1 cm from three 30 cm × 30 cm patches of ground within each sampling plot. The three samples from each plot were combined in a Winkler sac and hung for three days to extract invertebrates. Sampling was performed four times: in January 2016 pre-treatment, February 2016 three days after the first treatment, April 2016 one month after the final treatment, and September 2017 1.5 years after the final treatment. Pitfall traps were not used in this study because there were so few epigeic ants on the island.

Study 2 Nhulunbuy

This study was conducted around Nhulunbuy within northeast Arnhem Land (12°11′S; 136°46′E) in the Northern Territory. The regional climate is tropical monsoonal with high temperatures (17–33 °C) throughout the year and an annual rainfall of approximately 1200 mm falling predominantly during the summer wet season (BOM 2022a). The landscape is open (20% cover) savanna woodland dominated by eucalypts to heights of approximately 15 m, with an understorey to three meters predominantly of acacias and grasses (Williams et al. 1996).

Three spatially discrete *A. gracilipes* populations (sites 1, 2 and 3) sized 1, 7 and 32 ha respectively were selected for toxic treatment (Figure S2). Ants were sampled using pitfall traps at two plots within each site, spaced 40, 120 and 220 m apart respectively. These infested plots were all paired with closely located uninfested control plots that were not subject to toxic treatment. But because the treatments included a 100 m buffer zone around the infestation giving treatment areas of 10, 20 and 60 ha respectively, these paired plots were always more distant to each other (range of 250–1000 m) than treatment plots within a site were to each other. One additional plot was also established in each of two other *A. gracilipes* populations (sites 4, 5) within the surrounding landscape that were not treated with toxic bait to serve as infested controls of *A. gracilipes* abundance. These two infested control plots were also paired with an additional uninfested control plot each. Ants were sampled using pitfall traps four times, on 25 May 2016, 22 June 2016, 1 August 2017 and 4 November 2019.

The study was designed to have all three sites treated identically and simultaneously. However logistic constraints, product supply issues, weather issues, and adaptive management arising from real-time results all resulted in the three sites receiving vastly different treatments using three types of hydrogels and Antoff (Table 1). All treatments were conducted aerially with the hydrogel treatments being applied at 70 kg/ha, and Antoff treatments at 10 kg/ha.

Two sites (sites 1 and 2) received two treatments spaced four days apart with Magic water beads (treatment code BB). Commencing fourteen months later, site 2 which still contained *A. gracilipes* received an additional two treatments spaced nine months apart, the first treatment with Antoff and the second treatment with Floragel (treatment code AF). The third site received its first treatment with Magic water beads after the second sample time, but this treatment received rain interference. A triple treatment regime over nine months was re-attempted four months later immediately after the third sample time, first with Antoff, second with Magic water crystals and third with Floragel (treatment code BACF).

Study 3 Nome

This study was conducted in Nome, just south of Townsville in Queensland (19°22′S; 146°53′E). The region has a seasonal monsoonal climate, with high temperatures (17–33 °C) throughout the year and an annual rainfall of approximately 1140 mm falling predominantly during the summer wet season (BOM 2022c). The vegetation sampled was very open woodland dominated by *Eucalyptus platyphylla* often with a dense grassy understorey and with varying densities of the invasive shrub *Ziziphus mauritiana*.

This study had a single yellow crazy ant *A. gracilipes* population covering approximately 120 ha, with much of the area within a construction zone

for new residential development. We also sampled within the treatment buffer zone (the uninfested area extending out 100 m from the infestation that was also treated as a precautionary measure). Ants were sampled using pitfall traps from five infested-treated (IT) plots, three uninfested-treated (UT) plots (treatment buffer zone described above) and eight uninfested and untreated control (C) plots surrounding and 240–1100 m from the treated area (Figure S3), all within undisturbed natural vegetation, albeit with some cattle grazing in plots C1–3. Note that the design was originally 4 IT and 4 UT plots, but the pitfall trapping revealed the presence of *A. gracilipes* within one of the UT plots.

The pre-treatment sample was conducted on 12 December 2017, treatments of Floragel applied at 70 kg/ha were conducted aerially on 16 December 2017, 17 March 2018 and 23 June 2018, and the post-treatment sample was conducted on 27 June 2019 (Table 1).

Analysis

All samples from all studies were taken to the CSIRO Darwin laboratory for processing. Ants were sorted to species level, and for Study 1 all other invertebrates were sorted to ordinal level. Lepidoptera and Coleoptera larvae were also counted separately from adults. Adult flying invertebrates, such as Diptera, Lepidoptera and Coleoptera were removed from the data as they could have easily flown from outside the treatment area.

For all studies, the abundance of the target species, total ant abundance (excluding the target species) and species richness (excluding the target species) were compared among the treatment categories and sample times. Whenever statistical analyses were deemed pertinent to eliminate human bias for interpreting the data (Benjamini et al. 2021), we used One-way ANOVAs if the data satisfied assumptions of normality within Cochran's test, otherwise we used Kruskal-Wallis ANOVAs. For Study 1 Kruskal-Wallis ANOVA were applied to each non-ant invertebrate category and for total non-ant invertebrate abundance, and due to the large number of tests performed we reduced P to 0.01 to reduce the likelihood of type I errors. All univariate analyses were conducted using Statistica 11.

Ant community composition (presence/absence) and structure (abundance), both excluding the target species, were also compared in multivariate analyses using non-metric multi-dimensional scaling (nMDS). The association matrix was constructed using the Jaccard method for presence/absence data and the Bray-Curtis method for abundance data. Statistical separation of data by treatment category and sample time was tested using Analysis of Similarity (ANOSIM). ANOSIM returns an R-statistic which gives a measure of how spatially distinct groups of data are, with values ranging from −1 to 1, most commonly 0 to 1. The closer the R-value is to 1 the more separated the groups are in ordination space, whereas a value close to zero indicates no spatial separation of groups (Clarke and Warwick 2001).

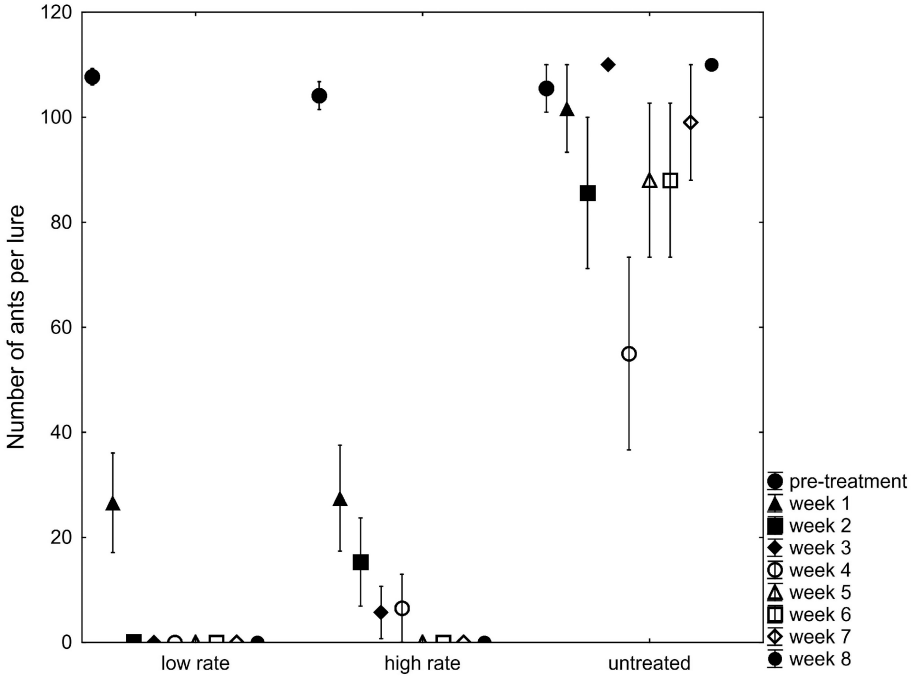

Figure 1. Mean (± SE) abundance of *Linepithema humile* in Study 1 at cat food lures at assessment locations in areas treated at a low rate (38 kg/ha), a high rate (77 kg/ha), and in an infested and untreated control area.

A multivariate analysis was also applied to the non-ant invertebrate abundance data in Study 1. All multivariate analyses were conducted using the analysis program Primer 6 (Clarke and Gorley 2003).

Specific to Study 2, the two infested control sites were only utilised for the efficacy analysis, not the non-target impact analyses because these forms of controls were not necessary. Also, for the efficacy analysis, we could not meaningfully statistically analyse the data from the AF and BACF treatments due to too few plots (2), but the results are clear with no possibility of biased interpretation (see Benjamini et al. 2021). Specific to Study 3, one of the post-treatment uninfested control plots was excluded from analysis because more than half of the traps received interference from small mammals resulting in only one ant species being caught.

Results

Treatment efficacy

Study 1 Norfolk Island

Treatments using both application rates had clear negative influences on *L. humile* populations such that at lures no *L. humile* were detectible at the monitoring locations in the two treatment areas after four treatments over five weeks (Figure 1) while activity at the infested and untreated control assessment locations remained high. Few *L. humile* were collected in the soil samples, but they were present in all three infested plot categories pre-treatment. In the third and fourth samples (1 month after the 6th treatment and 1.5 years, respectively) no *L. humile* were collected in the two treatment

categories but they remained present in the infested and untreated control plots (Figure S4), despite none being collected in the third sample time due to some rain interference.

Study 2 Nhulunbuy

Pre-treatment, *A. gracilipes* abundance in pitfall traps from the BB plots (sites 1 and 2) ranged from 201–846 (Figure S5). No *A. gracilipes* were collected in the first post-treatment sample (20 days), six individuals were collected from site 2 in the next sample (425 days), and one individual was collected from site 1 in the final sample (1250 days). These post-treatment abundances in the treated plots differed significantly from the two control infested plots (Kruskal-Wallis ANOVA: $H = 19.39$, $P = 0.007$).

The AF treatment at site 2, which followed the BB treatment 14 months later only had *A. gracilipes* present in very low abundance at the commencement of this treatment regime (6 and 0 individuals in the two plots) and no *A. gracilipes* were collected in the final sample. The rain-spoiled treatment in site 3 which commenced the BACF treatment regime greatly reduced the *A. gracilipes* population from 600 and 2140 individuals in the two plots to just 1 and 14, respectively. No individuals were collected in the fourth sample following the subsequent three treatments.

Study 3 Nome

Pre-treatment *A. gracilipes* abundance in pitfall traps within the infested plots averaged 8480 per plot (range of 2001 to 18150). One year after treatment, no *A. gracilipes* were collected.

Non-target impacts

Study 1 Norfolk Island

Ant abundance varied significantly among some treatment categories and sample times (Kruskal-Wallis ANOVA: $H = 81.68$, $P < 0.0001$). Ant abundance declined across the four sample times within all treatment and control categories, reflecting the cooling of environmental temperatures from January to April 2016 (Samples 1–3), and relatively cool temperatures dominating the September 2017 sample (Sample 4) (Figure 2A), but these differences were not statistically significant among the two control categories. Likewise, there were no differences among any categories pre-treatment (Sample 1) and after a single treatment (Sample 2). But after six treatments (Sample 3) there were considerably fewer ants collected in the areas given toxic treatments relative to the uninfested and untreated control (UN), but only the High treatment category was statistically lower. Notably the data of the UI control category for this timeframe are unreliable due to rain interference. Ants were only collected in 4/10 and 3/10 plots within H and L plots respectively compared to all UI plots (10/10). After 1.5 years (Sample 4), the same pattern persists with ant abundance remaining

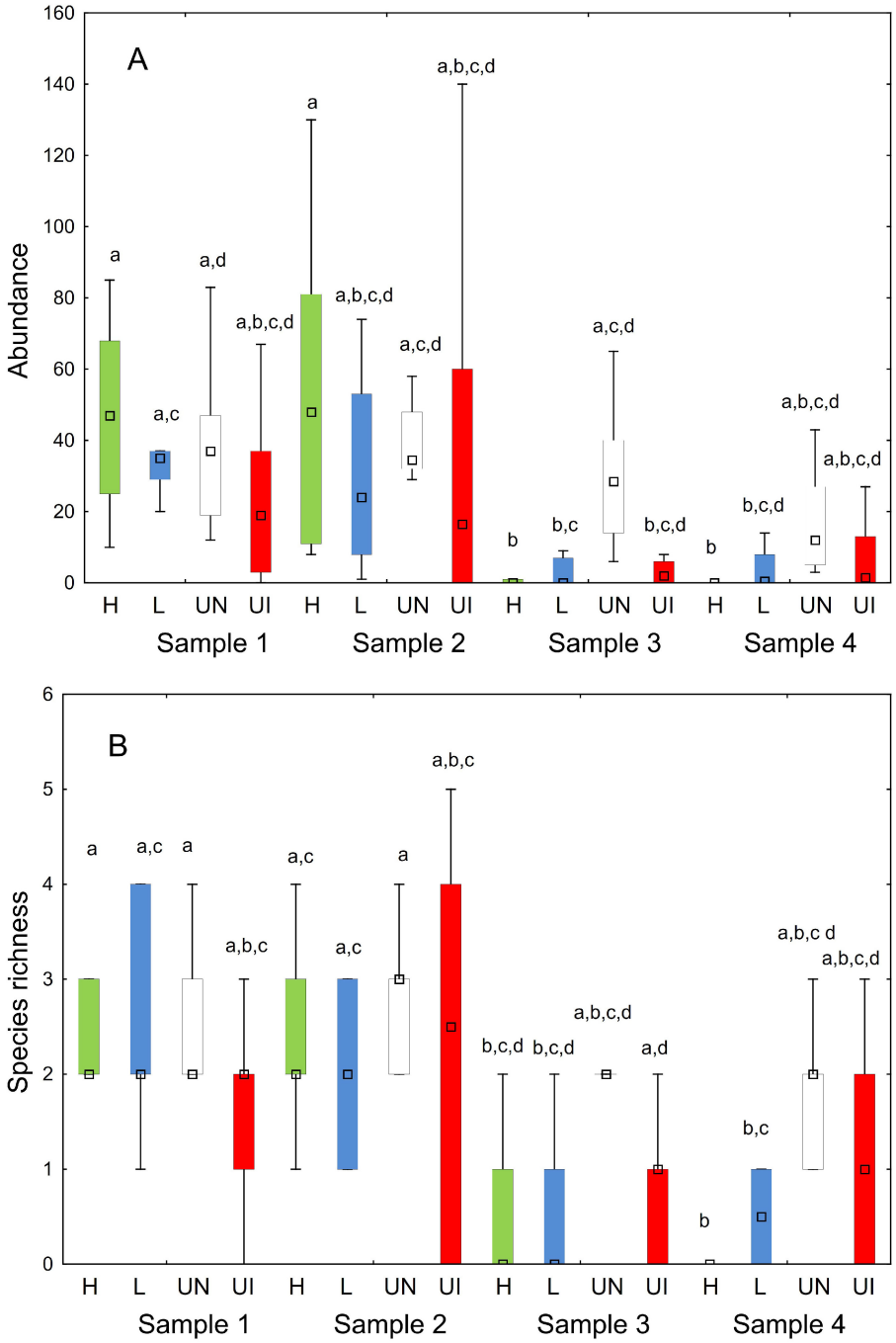

Figure 2. Box plots of ant abundance (A) and species richness (B) (excluding *Linepithema humile*) in Study 1 in the four treatment categories (H = high, L = low, UI = untreated and infested, UN = untreated and uninfested) at four sampling times (Sample 1 = pre-treatment, Sample 2 = three days after the first treatment, Sample 3 = one month after the sixth weekly treatment, and Sample 4 = 1.5 years after the final treatment. Letters indicate statistical separation within each treatment time.

lowest in the two areas that had undergone toxic treatments, but not statistically significantly from the two controls.

Almost identical patterns were observed for sample-level species richness (Kruskal-Wallis ANOVA: $H = 80.35$, $P < 0.0001$) (Figure 2B). The greatest difference being that the total number of ant species (excluding *L. humile*) collected in each category in each sample time remained consistent in the

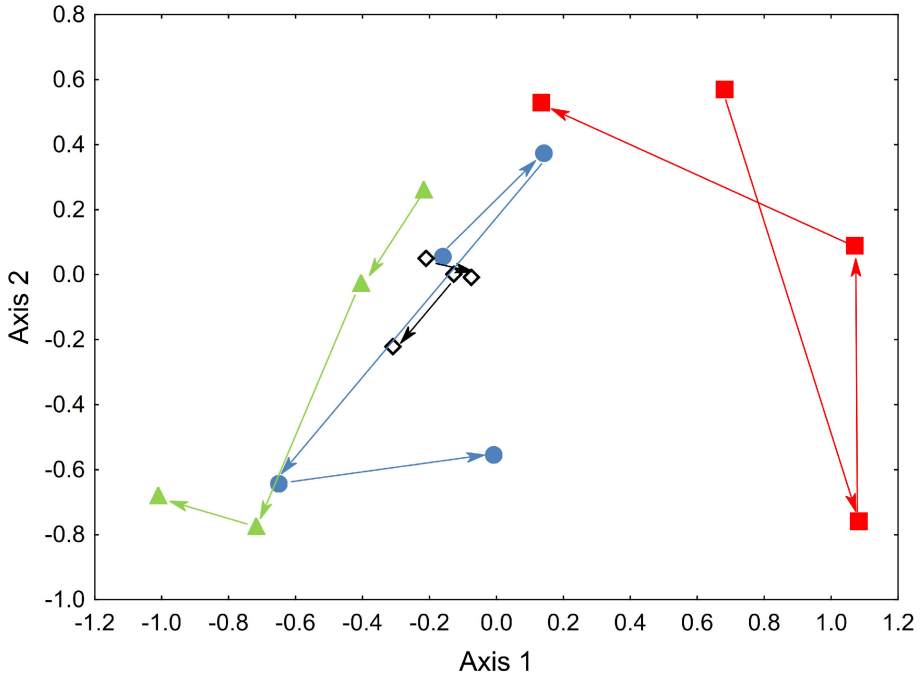

Figure 3. NMDS ordination of ant presence/absence data (excluding *Linepithema humile*) in Study 1 for the four treatment categories of high rate (green triangles), low rate (blue circles), untreated and uninfested (white diamonds), and untreated and infested (red squares), in the four sampling times with the order of samples indicated by the arrows. For ease of visualisation each point represents the midpoint of the plots for each treatment/sample time combination, not individual plots. 2D Stress = 0.07.

two untreated controls (UI and UN) but was clearly reduced within the two areas that underwent toxic treatments, especially the High rate (Figure S6).

Notably, after six toxic treatments *L. humile* appears to have been extirpated from the monitoring areas, yet other ants persisted.

Ordination of presence and absence data (Figure 3) showed that species composition in the categories was more related to spatial location than treatment. When all sample times were considered simultaneously, the three categories that were in the same general area (High, Low and UN) clustered together away from UI (ANOSIM: Global R = 0.25, P = 0.001), which was in another location on the island. When only the first and final sample times were considered, the final midpoint for the two controls (UI and UN) were within the vicinity of their first midpoints and with no statistical separation, indicating little change in ant community composition within the control categories, whereas ant community composition changed markedly in the two areas that underwent toxic treatments (High and Low) (ANOSIM: Global R = 0.23, P = 0.001). Ordination of ant abundance gave identical patterns and statistical outcomes (Figure S7).

The abundance of macro-invertebrates varied greatly among plots within a sample time and statistically significantly among treatments and sample times (Table 2), but there were no discernible patterns relating to treatment, seasonality or spatial location. Notably no invertebrate Order was found to be affected by the toxic treatments, even in the first two post-treatment samples. An anomaly is the almost complete lack of Amphipoda in the final sample, and because the samples were not retained it remains

Table 2. Median (± SD) abundance of invertebrate Orders collected in plots of four treatment categories (High, Low, UI = untreated infested, UN = untreated uninfested,) at four sampling times (Sample 1 = pre-treatment, Sample 2 = three days after the first treatment, Sample 3 = one month after the sixth weekly treatment, and Sample 4 = 1.5 years after the final treatment). Letters indicate statistical separation ($P \leq 0.01$) within columns.

Sample	Treatment	Amphipoda	Collembola	Isopoda	Arachnida	Larvae	Other	Total
1	High	$42 \pm 32^{a,e}$	0 ± 3.6^{a}	25 ± 16^{a}	$37 \pm 123^{a,c}$	$2.5 \pm 1.6^{a,b}$	$84 \pm 123^{a,b}$	$229 \pm 276^{a,b,c,d}$
1	Low	$5 \pm 7^{a,b,d,e}$	$8 \pm 17^{a,b}$	$5 \pm 7^{a,c}$	$198 \pm 145^{a,c}$	$3.5 \pm 35^{a,b}$	$214 \pm 170^{a,b}$	$430 \pm 356^{a,b,c,d}$
1	UN	$0 \pm 1^{b,d}$	$3 \pm 39^{a,b}$	$0 \pm 0.5^{b,c}$	$121 \pm 94^{a,c}$	$2.5 \pm 3.5^{a,b}$	$132 \pm 95^{a,b}$	286 ± 195^{a}
1	UI	$13 \pm 61^{a,b,d,e}$	$19 \pm 42^{a,b}$	$3.5 \pm 25^{a,c}$	$141 \pm 91^{a,c}$	6 ± 6.4^{a}	$163 \pm 106^{a,b}$	$436 \pm 243^{a,b,c,d}$
2	High	$30 \pm 60^{a,c,d,e}$	$20 \pm 49^{a,b}$	$5.5 \pm 15^{a,c}$	$206 \pm 369^{a,c}$	$1 \pm 0.9^{a,b}$	$212 \pm 368^{a,b}$	$516 \pm 811^{a,b,c,d}$
2	Low	$1 \pm 12^{a,b,d,e}$	$6 \pm 188^{a,b}$	$0 \pm 47^{b,c}$	$164 \pm 355^{a,c}$	$0.5 \pm 3.5^{a,b}$	$242 \pm 348^{a,b}$	$487 \pm 862^{a,b,c,d}$
2	UN	$0.5 \pm 1^{a,b,d}$	$1 \pm 468^{a,b}$	$0 \pm 0.7^{b,c}$	$107 \pm 248^{a,c}$	$1 \pm 2.3^{a,b}$	$115 \pm 249^{a,b}$	$226 \pm 914^{a,b,c,d}$
2	UI	$74 \pm 161^{a,c,e}$	$80 \pm 184^{a,b}$	$19 \pm 81^{a,c}$	$433 \pm 1882^{a,c}$	$0.5 \pm 1.7^{a,b}$	$459 \pm 1992^{a,c}$	$1285 \pm 4148^{a,b}$
3	High	$53 \pm 151^{a,c}$	$3 \pm 4^{a,b}$	$1 \pm 1.4^{a,b}$	19 ± 96^{a}	0 ± 0.4^{b}	23 ± 96^{b}	$117 \pm 296^{a,c}$
3	Low	$11 \pm 115^{a,b,c,d}$	$48 \pm 111^{b,c}$	$0 \pm 0.8^{b,c}$	$86 \pm 68^{a,c}$	2.5 ± 8.6^{b}	$106 \pm 67^{a,b}$	$306 \pm 143^{a,b,c,d}$
3	UN	$1 \pm 1.5^{a,b,d}$	$18 \pm 84^{a,b,c}$	$0 \pm 0.7^{b,c}$	24 ± 55^{a}	0 ± 0.7^{b}	$33 \pm 56^{a,b}$	90 ± 173^{c}
3	UI	$186 \pm 113^{c,e}$	$2 \pm 2.5^{a,d}$	$4 \pm 4^{a,b}$	$82 \pm 93^{a,c}$	0 ± 1.3^{b}	$113 \pm 109^{a,b}$	$383 \pm 306^{a,b,c,d}$
4	High	0 ± 1.1^{d}	157 ± 212^{b}	$1.5 \pm 2.9^{a,b}$	$376 \pm 138^{b,c}$	0 ± 0^{b}	388 ± 141^{c}	974 ± 422^{d}
4	Low	$0 \pm 0.6^{b,d}$	$120 \pm 205^{b,c,d}$	0 ± 0^{b}	$458 \pm 206^{b,c}$	0 ± 0^{b}	467 ± 209^{c}	1114.5 ± 354^{b}
4	UN	$0 \pm 0^{b,d}$	$113 \pm 415^{b,c}$	$1.5 \pm 1.7^{a,b}$	$134 \pm 119^{a,c}$	0 ± 0.3^{b}	$135 \pm 122^{a,b}$	$313 \pm 615^{a,b,c,d}$
4	UI	$0 \pm 0^{b,d}$	67 ± 43^{a}	$0 \pm 0.7^{b,c}$	$224 \pm 115^{a,c}$	0 ± 0^{b}	$233 \pm 119^{a,b}$	$525 \pm 270^{a,b,c,d}$

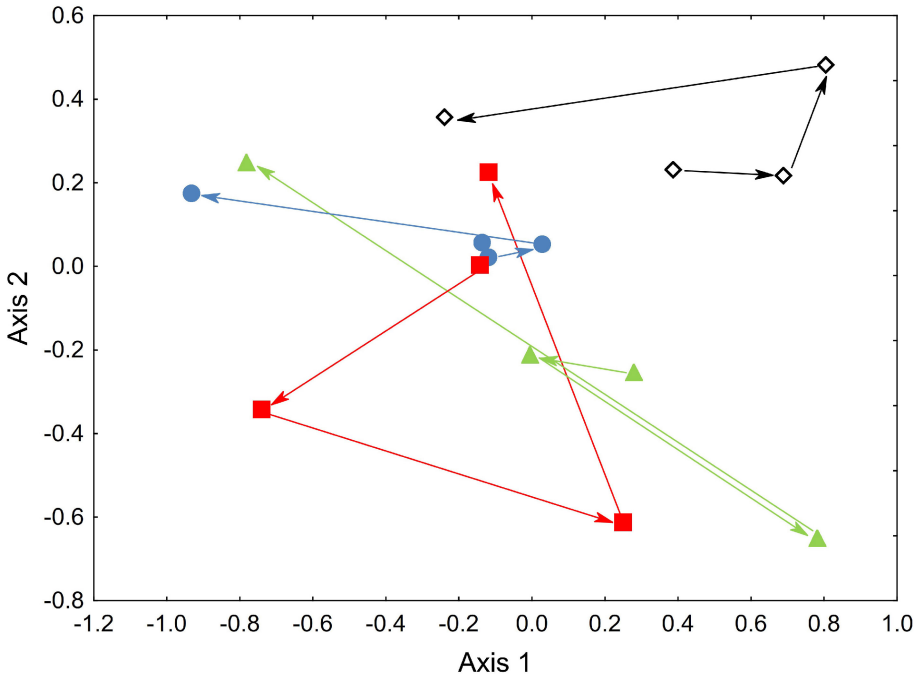

Figure 4. NMDS ordination of non-ant macro-invertebrate abundance data in Study 1 for the four treatment categories of high rate (green triangles), low rate (blue circles), untreated and uninfested (white diamonds), and untreated and infested (red squares), in the four sampling times with the order of samples indicated by the arrows. For ease of visualisation each point represents the midpoint of the treatment/sample time combinations, not individual plots. 2D Stress = 0.11.

unclear if this is a true result or sorting error. Ordination of non-ant macro-invertebrate abundance also statistically separated all sample times (ANOSIM: Global R = 0.17, P = 0.001), and there was much separation of categories within sample times (ANOSIM: Global R = 0.26, P = 0.001), but few consistent patterns relating to toxic treatment were evident (Figure 4). Pre-treatment H and L were not distinct from each other, H was distinct from

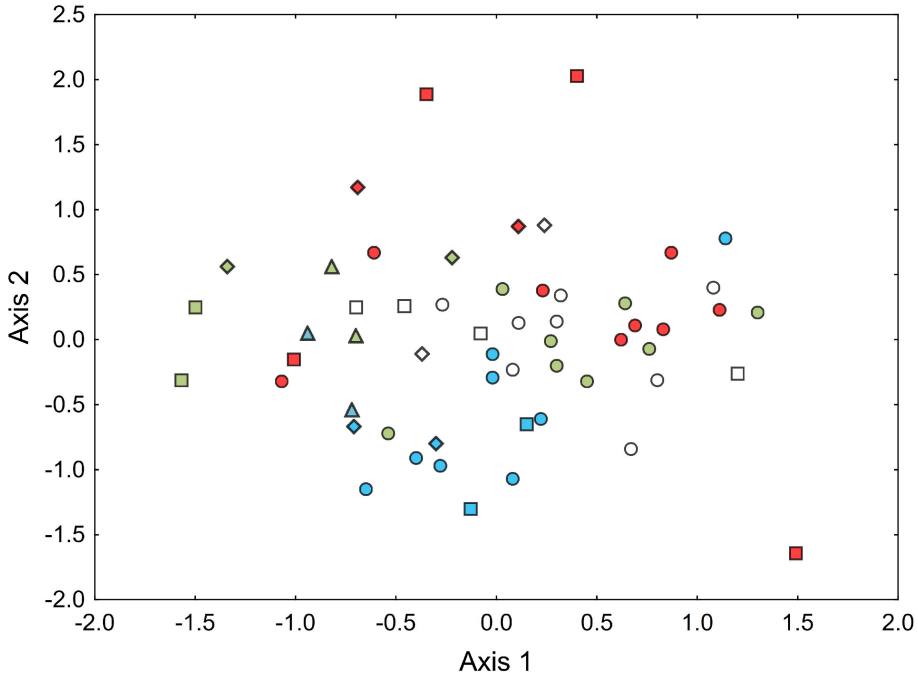

Figure 5. NMDS ordination of ant species composition (presence/absence data; excluding *Anoplolepis gracilipes*) in Study 2 within plots of BB treatments (squares), AF treatments (triangles), BACF treatments (diamonds) and controls (circles), in the first (white), second (red), third (green) and fourth (blue) sample times. 2D stress = 0.24.

the two controls, but L was not. In samples two and three H and L became statistically distinct from each other, with inconsistent relationships with the two control categories. In sample 4, 1.5 years after treatments ceased, H and L were no longer statistically distinct, nor were the two controls from each other, but both H and L were distinct from the two controls.

Study 2 Nhulunbuy

For the double treatment spaced only four days apart (BB treatment), species richness was significantly lower in the treated plots 20 days after treatment, (One-way ANOVA: $F = 2.68$, $P = 0.022$), but not different from controls after 425 and 1250 days. Similarly, abundance was lowest immediately after the treatments, but only significantly lower against the latter two sample times (Kruskal-Wallis ANOVA: $H = 21.94$, $P = 0.003$), not the pre-treatment sample, indicating that sample time and conditions were more influential than the treatments. For the double and triple treatments spaced months apart (AF and BACF treatments), species richness (One-way ANOVA, $F = 0.55$, $P = 0.74$) and abundance (Kruskal-Wallis ANOVA, $H = 10.88$, $P = 0.054$) did not differ at any time (pre-treatment and 538 days post treatment) among any categories.

Within ordination space, species composition of control sites alone differed significantly among sample times (ANOSIM: Global $R = 0.16$, $P = 0.02$) predominantly with the final sample being different to all other samples (Figure 5). When treatments were included there were also many statistical separations of categories (ANOSIM: Global $R = 0.36$, $P = 0.001$). In the first

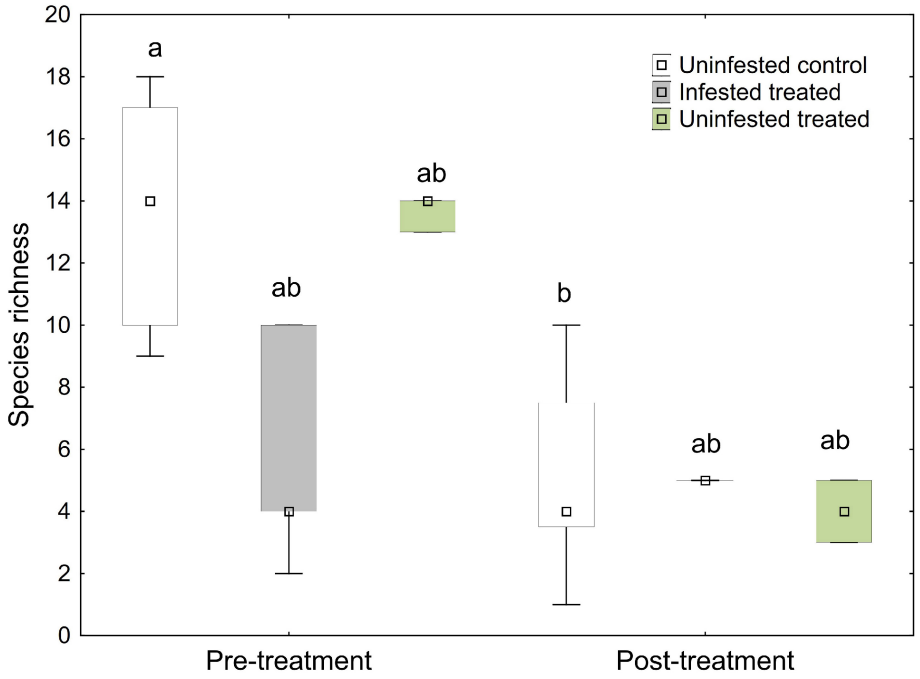

Figure 6. Box plots of ant species richness (excluding *Anoplolepis gracilipes*) in Study 3 in the two sample times, pre-treatment and 12 months post-treatment, at control plots (white) and plots of the two treatment categories of infested and treated (grey) and uninfested and treated (green). Letters indicate statistical separation.

sample the controls were statistically distinct from the infested sites most likely being the ecological effects of *A. gracilipes*. In the second and third samples, the infested plots that had been treated respectively were even more statistically separated from the controls than in the first sample, but there was no statistical separation of control and treated sites of any category in the final sample.

Study 3 Nome

Abundance of other ants did not differ significantly among any categories and between sample times (Kruskal-Wallis ANOVA: H = 10.99, P = 0.052) (Figure S8). Species richness varied significantly only between the controls of the two sample times (Kruskal-Wallis ANOVA: H = 17.2, P = 0.004) with there being many fewer species present in the post-treatment sample (Figure 6). The untreated controls didn't differ with the two treatment categories within each timeframe, indicating no treatment effects.

Species composition varied significantly in ordination space between the two sample times (ANOSIM: R = 0.315, P = 0.001; Figure 7), seemingly driven by fewer species being collected in the post-treatment sample in all categories. This sample time difference was prominent when categories and sample times were analysed simultaneously (ANOSIM: R = 0.43, P = 0.001), with the control plots of the two sample times differing significantly from each other in pairwise comparisons. Notably pre-treatment species composition within the two treatment categories differed significantly from each other and from controls, but post-treatment the two treated categories

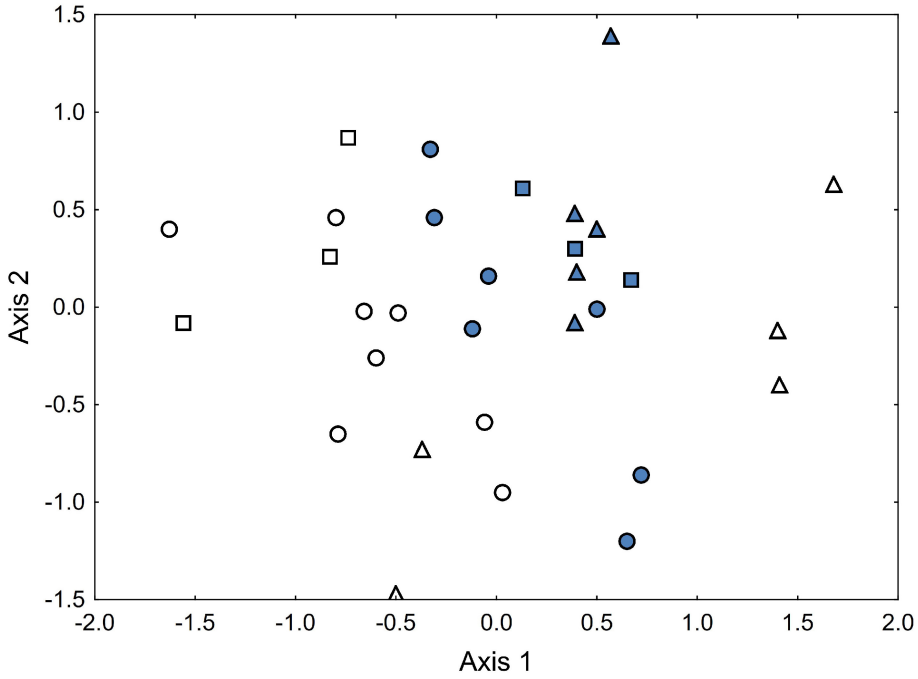

Figure 7. NMDS ordination of ant species composition (presence/absence data; excluding *Anoplolepis gracilipes*) in Study 3 within control plots (circles), uninfested treated plots (squares) and infested treated plots (triangles) pre-treatment (white) and 12 months post-treatment (blue). 2D stress = 0.21.

did not differ from each other and only the infested treated category differed significantly (P = 0.048) from control plots.

Discussion

Despite imperfect study designs and lower replication than preferred, all studies clearly demonstrated that the baits were highly efficacious against both invasive ant species. Results against *L. humile* were not as dramatic as for *A. gracilipes*, despite fipronil being known to be highly efficacious against *L. humile* (Soeprono and Rust 2004a, b; Choe and Rust 2008; Buczkowski and Wossler 2019), but numerous treatments did drive its abundance in all measures (lure counts and litter samples) to zero. The relatively slow decline is in line with the findings of Boser et al. (2014) who also treated *L. humile* with hydrogels, but with thiamethoxam as the active constituent. Such a slow decline has also been observed for *Trichomyrmex destructor* and *T. mayri* using Floragel baits (Hoffmann *unpublished data*) but those populations were not even close to being reduced to zero activity after six treatments.

There are many reasons why these efficacy differences can occur such as relative availability and seasonal preference of other surrounding food sources affecting forager food choice and hence bait uptake (Rudolph and Palmer 2013; Abbott et al. 2014; Buczkowski et al. 2014b), the concentration of the active constituent being too high for some species resulting in their workers dying before returning to the colony (Klotz et al. 1997), varying forms and rates of trophallaxis occurring back in the colony resulting in

different horizontal transfer of the active compound (Meurville and LeBoeuf 2021), and differing levels of necrophoresis, where by live ants carry the corpses of dead ants, affecting horizontal transfer of the active compound within the nest (Soeprono and Rust 2004a; Buczkowski 2019). We don't attempt to provide potential explanations here, but understanding and overcoming these limitations is clearly among the holy grails of invasive ant management (Silverman and Brightwell 2008; Hoffmann et al. 2016).

Non-target impacts on invertebrates will always occur as long as ant baits contain general insecticides or growth regulators. But work to date of the impacts of broadscale applications of corn-grit baits broadcast within areas of conservation significance seems to indicate that such impacts are short term and for predictably susceptible species. For example, Plentovich et al. (2010, 2011) detailing treatments on small Hawaiian islets with few native invertebrates and no native ants found no non-target impacts of concern, just temporary reductions in non-native cockroaches, crickets and ants. Within pastures, Apperson et al. (1984) found no reductions of native ants. McNaught et al. (2014) found most local ant genera were not seriously impacted by treatments, with the greatest reductions occurring for *Pheidole* species which would predictably consume the corn grit because they are seed harvesters. Hoffmann (2010) found a similar temporary reduction, primarily of *Pheidole* species, but full ecological recovery was rapidly achieved within a couple years. Other eradication programs haven't had formal assessment of non-target impacts, but articulated that they saw no observable impacts of concern (Hoffmann and O'Connor 2004; Webb and Hoffmann 2013). A lack of impacts has also been found where bait was deployed within bait stations, rather than being broadcast, to limit non-target species from accessing the bait (Gaigher et al. 2012). Nothing can be stated about the non-target impacts of the single use of Antoff incorporated into Study 2, but what has been published already about this fishmeal bait from elsewhere (Marr et al. 2003; Stork et al. 2003, 2014) indicates that it has no significant non-target impacts other than on land crabs.

We found hydrogels did not have enhanced non-target impacts relative to what has been reported for the use of any modern granular bait. This is consistent with findings of the only other study quantifying the non-target impacts of hydrogel baits containing an insecticide to control invasive ant species. On the Channel Islands in California, Boser et al. (2014) found most non-target impacts were on other ant species, and these effects were small. Notably, here and for most other publications about non-target impacts due to ant management programs, treated areas were just small portions of bigger untreated landscapes, as opposed to entire areas (e.g. islands, isolated vegetation patches), which would help facilitate species recruitment and population recovery. Additionally, consistent among studies is that the short-term impact of baiting is typically smaller than the ongoing impact

of the target invasive ant species (Hoffmann 2010; Boser et al. 2014; McNaught et al. 2014).

Our work focused on ground-dwelling invertebrates, and didn't consider attractancy and potential impact on pollinators which potentially could be attracted to the sugar in hydrogels. But two comprehensive studies have also recently been conducted investigating pollinating-insect attractancy and feeding from various hydrogels containing sugar. On mainland USA, Buczkowski (2020) found honey bees and solitary bees rarely visited hydrogels and were never found on ground locations only on platforms. In one specific experiment at an apiary, bees were recorded less than 15 times, mostly in the first of four hours of observations. In Hawaii, Krushelnycky (2021) found honey bees fed from hydrogels containing sugar solution when hydrogels were placed adjacent to flowers, but not when the hydrogels were placed randomly on the ground, which suggests that the bees were not "attracted" to hydrogels, but will indeed feed from the hydrogels if they do discover them. Hydrogel baits, therefore, at least in the forms and ways currently used, appear to present minimal risk for pollinators.

This work also involved numerous other considerations and observations relating to understanding how best to use hydrogels as an ant bait. In the only study that has quantified ant liquid uptake from hydrogels, Cabrera et al. (2021) found that increasing the sugar content made it more difficult for the ants to imbibe the fluid resulting in less liquid being consumed by foragers. Studies 1 and 2 additionally manipulated sugar content in trials with less scientific rigour (data not presented), and it was determined that 30% sucrose was the best concentration to use for both ant uptake of the bait and for handling considerations, which was in line with the findings of Cabrera et al. (2021) as well as the decisions of other research and invasive ant management programs (Boser et al. 2014; Buczkowski et al. 2014a, b; Rust et al. 2015).

We also had informal observations of other work within Studies 1 and 2 that ants of both species were seemingly able to feed more from the irregularly shaped hydrogels than the round beads. Unfortunately, Cabrera et al. (2021) only used Magic water beads so we are not able to confirm this observation or not. But this, as well as other handling considerations (e.g. less residual liquid after bait preparations), resulted in the eradication programs associated with Studies 1 and 2 shift from using round hydrogels to irregularly-shaped hydrogels. Of the two irregularly-shaped hydrogels, magic water crystals produced a sloppy texture, and despite its apparent efficacy when used to produce bait, it was not desired for handling reasons.

Finally, none of the treatment regimens presented in Studies 1 and 2 achieved eradication of the target species at any site, but these were some of our earliest trials using hydrogels attempting to understand how best to use this bait form, as well as which hydrogels themselves were best to use for making ant baits. The work conducted in Study 3, to the best of our

knowledge, however, did achieved eradication demonstrated by the pitfall trapping as well as other visual assessments and subsequent use of a detector dog (data not presented). But the work detailed here for Study 3 covers only part of a larger infestation that involved ground-based treatments through a residential area and that work is still ongoing. Treatments conducted in Studies 1 and 2 in subsequent years, however, are currently being demonstrated to have achieved eradication, and these will be detailed in future publications.

In summary, invasive ant incursions and subsequent spread is increasing globally (Bertelsmeier et al. 2018), and consequently efforts to manage or eradicate these species from locations is increasing (Vanderwoude et al. 2015; Hoffmann et al. 2016; Wylie et al. 2020).

Hydrogels (Tay et al. 2020), as well as other novel baits and treatments (Buczkowski 2016; Allen 2021) are likely to have significant future roles to play for ant management and eradication, especially within areas of conservation significance.

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Authors' contribution

BH designed the experiments, BH, MP, AG, and SQ conducted the field work, MP, JA, JC, EF, AG, PH, TL, JM, SQ and TW conducted the laboratory identifications, BH conducted all of the data analysis, interpretations and led the writing, all authors provided comments and input into the draft manuscript and approved the final manuscript.

Ethics and permits

These studies were conducted under Australian Pesticides and Veterinary Medicines Authority permits 88159, 82931, 84820, 84817, 82749 and 85275.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Map of the non-target assessment plots for Study 1 on Norfolk Island.
Figure S2. Map of the non-target assessment plots for Study 2 in Nhulunbuy.
Figure S3. Map of the non-target assessment plots for Study 3 in Nome.
Figure S4. Box plots of *Linepithema humile* abundance in Study 1.
Figure S5. Box plots of *Anoplolepis gracilipes* abundance in Study 2.
Figure S6. Total number of ant species (excluding *Linepithema humile*) in Study 1.
Figure S7. NMDS ordination of ant abundance data (excluding Argentine ant) in Study 1.
Figure S8. Box plots of ant abundance (excluding *Anoplolepis gracilipes*) in Study 3.

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