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Invasive earthworms *Amyntas tokioensis* and *Amyntas agrestis* alter macronutrients (Ca, Mg, K, P) in field and laboratory forest soils

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ABSTRACT

Pheretimid earthworms of the family Megascolecidae are invading deciduous forests across the northern and northeastern United States with unknown consequences on soil and nutrient dynamics. This study investigated plant macronutrients (Ca, Mg, K, and P) at partially invaded forest sites in Madison, WI, USA, and in forest soil incubated with *Amyntas agrestis* or *Amyntas tokioensis*. Exchangeable, organic-bound, and/or inorganic Ca, Mg, K, and P concentrations and pools were typically greater inside than outside the invasion front. Exchangeable K and organic K were significantly increased by *Amyntas* earthworms. These changes in nutrient status were associated with an increased pH and greater soil organic matter (SOM), suggesting litter decomposition by *Amyntas* was likely responsible for nutrient additions. Under laboratory soil incubations, increases in Ca, Mg, K, and P occurred for *A. tokioensis* but this was less consistent for *A. agrestis*. Soil incubations also demonstrated that *A. tokioensis* stabilized more nutrients and SOM than *A. agrestis* despite being fed one-third less oak litter. Thus, while *Amyntas* earthworms alter forest soils due to differences in feeding, digestion, and mineralization among species, whether these differences facilitate co-invasion or bring about long-term changes in ecosystem soil pools remains unclear.

1. Introduction

Invasive pheretimid earthworms have been introduced across forests of the northern and northeastern United States by human activities (Chang et al., 2018; Moore et al., 2018). The introduction of pheretimid earthworms of the family Megascolecidae, particularly the *Metaphire* and *Amyntas* genera, represents a second wave of earthworm invasions (Görres and Melnichuk, 2012; Chang et al., 2018, 2021; Laushman et al., 2018). Three primary species from Asia have been identified for their propensity for invasion: *Metaphire hilgendorfi* (Michaelson 1892), *Amyntas agrestis* (Goto and Hatai 1899), and *Amyntas tokioensis* (Beddard 1892) (Chang et al., 2018; Chang et al., 2021). This group of Megascolecids share important distinctions that set them apart from the widely introduced European earthworms (e.g. *Lumbricus* spp., *Apporctodea* spp.). First, they are epi-endogeic and can thus drastically transform the soil surface into granular castings of several millimeters diameter (Görres et al., 2019; Greiner et al., 2012). Also, these three species of Megascolecidae reproduce parthenogenetically and exhibit an annual life cycle; their cocoons over-winter in the soil, juveniles emerge in the spring, and adults die with freezing temperatures (Gates, 1956,

1958). They are prolific breeders with faster growth rates and can occur in higher densities than Lumbricids (Greiner et al., 2012; Moore et al., 2018). Finally, they negatively impact native fauna of soil and litter such as millipedes (Snyder et al., 2011) and salamanders (Ziemba et al., 2016). Where they have been introduced, Megascolecid earthworms can replace all other functional groups of European earthworm species in deciduous forests (Laushman et al., 2018; Szlavecz et al., 2018). Little is known about the consequences of these recent invaders on forest soil biodiversity and nutrient dynamics.

Invasive *Amyntas* (*agrestis* and *tokioensis*) earthworms can substantially change physical and chemical properties of surface soils, generally to a greater extent than Lumbricids (Görres et al., 2019; Greiner et al., 2012). *Amyntas* earthworms consume leaf litter and decomposing organic matter, causing the loss of the organic horizon (Richardson et al., 2016; Dobson et al., 2017; Frelich et al., 2019). While this is similar to the initial impacts of European earthworm introduction, *Amyntas* also thoroughly mix the mineral soil of the A horizon, which is a different effect on soil than that caused by litter-dwelling epigeic earthworms (e.g. *Dendrobaena octaedra*) or vertical-burrowing earthworms (e.g. *Lumbricus terrestris*) (e.g. Richardson et al., 2015). The

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Fig. 1.—Example of casting activity of *Amynthus* species. Two *A. tokioensis* individuals are shown among their castings (A), and a close-up photograph of the castings' granular structure (B). Casts are approximately 2 mm diameter. Photo credit: Susan Day, UW-Madison Arboretum.

transformation of the forest floor and upper mineral soil cascades into changes in soil physical and chemical properties. From previous work on Lumbricid European earthworms, soil porosity and bulk density are altered by burrowing and ingestion (Satchell, 1983; Alban and Berry, 1994; Hale et al., 2008; Pagenkemper et al., 2015). As a result, earthworm invasions can cause subsequent shifts in water infiltration and storage, root growth, erosivity, and gas exchange (Hale et al., 2005; Bohlen et al., 2004; Frelich et al., 2019).

Quantifying the impacts of invasive *Amynthus* on forest soils is key to characterizing their broader changes to forest ecosystems. On one hand, earthworms can cause increased litter decomposition rates (Satchell, 1983; Alban and Berry, 1994; Qiu and Turner, 2017), the disruption of mineral soil aggregates (Jouquet et al., 2011) and subsequent increases in the availability and mobility of essential plant nutrients. *Amynthus* can cause higher soil pH (Laushman et al., 2018), the conversion of litter to organic matter to mineral soil (Laushman et al., 2018; Richardson, 2019), and altered root abundance (Hale et al., 2005; Dobson et al., 2017) generating nutrient-rich A horizons. Resner et al. (2015) demonstrated that soil nutrient concentrations can increase following earthworm invasion through the addition of new nutrients from litter decomposition. Alteration of macronutrient (Ca, Mg, P, K) availability and distribution in soils can affect growth and sustainability of understory and canopy trees in forest ecosystems (Bohlen et al., 2004; Dobson et al., 2017; Frelich et al., 2019). Operationally-defined fractions of elements in soils, such as exchangeable, organic-bound, and inorganic forms (oxides, insoluble salts) can indicate their solubility, chemical speciation, and potential availability or uptake by plants (see Rao et al., 2008). In general, exchangeable elements are most readily accessible to plants, while organic-bound and inorganic forms can be taken up or lost by leaching over the growing season or several years. Moreover, the loss of base saturation (specifically exchangeable Ca and Mg) can decrease pH buffering capacity and increase uptake of potentially toxic elements such as Al and Mn (Kobe et al., 2002; Clair and Lynch, 2005). Such changes in nutrient availability and storage have been understudied, especially for forest soils invaded by *Amynthus* earthworms and conversion of organic-bound and inorganic forms to exchangeable nutrients can lead to greater plant uptake or leaching out of the rooting zone.

Both field and laboratory approaches are needed to link mechanisms to specific changes and allow assessing species-specific attributions. Richardson et al. (2016) investigated changes by *A. agrestis* and *Lumbricus rubellus* in organic horizons under laboratory conditions but did not find a shift in nutrient availability, largely due to the high organic matter content of the forest floor. Qiu and Turner (2017) and Bethke and Midgley (2020) used collected field soil in mesocosm and indoor Quonset hut experiments, and Dobson et al. (2017) studied changes along an earthworm invasion front but could not establish changes in nutrient availability. One of the challenges to study these earthworms is that they can travel quickly across the landscape, exist in patches, have varying impacts across forest soils, and often coincide with European species.

Here, we quantify soil macronutrient concentrations (mass of nutrients per mass of soil) and pools (mass of nutrients per unit volume or area) on both sides of an *Amynthus* invasion front in forested sites and in soils incubated two with *Amynthus* species under laboratory conditions. Concentrations can determine differences in changes with regard to nutrients per mass of soil, but since earthworms can change soil density, it is important to also determine if nutrient pools per unit area are affected. We tested several related hypotheses regarding the effects of *Amynthus* on soil physicochemical properties. First, we expected forest soils where *Amynthus* earthworms are present (i.e. inside the invasion front) to have decreased nutrient concentrations and pools than soils where the earthworms have not invaded by converting nutrients to more exchangeable, soluble phases, which can be leached from the surface soil out of the rooting zone. Second, we expected a shift in nutrient fractions from organic and inorganic phases to exchangeable phases in field soils and especially laboratory studies. This is because *A. agrestis* and *A. tokioensis* consume organic matter and disrupt soil aggregates. Lastly, we hypothesized that soil macronutrients are positively associated with increases in soil organic matter content and higher soil pH due to the incorporation of litter by *Amynthus*. This combined approach of both laboratory and field work is needed to help assess the external validity of laboratory studies on earthworm invasions.

2. Materials and methods

2.1. Field study locations and sampling

Seven paired forest sites in Madison, WI, USA (43° 4' 22" N, 89° 24' 2" W) were sampled for the field portion of this study (University of Wisconsin-Madison Arboretum and Madison's Wally Bauman Woods within the UW-Madison Lakeshore Nature Preserve). These urban forests were selected because they are currently undergoing invasion by *Amynthus tokioensis* and *Amynthus agrestis*, and, specifically, they offer similar soil, plant, and earthworm communities both before and since invasion by *Amynthus* spp. The soils consist primarily of Military loam and Dodge silt loams (USDA-NRCS, 2020). The forests are hardwoods typical of the US Midwest: *Acer saccharum* dominates the canopy and sapling layers, and *Quercus* spp. (primarily *Q. alba* and *Q. velutina*) are common canopy trees. The ground vegetative layer is dominated by species found in US Midwest deciduous forests where European earthworms are present (see Laushman et al., 2018 for a more detailed description of site vegetation). Each of the forested sites are managed for invasive plants, primarily *Rhamnus cathartica*, *Lonicera* spp. and *Alliaria petiolata*, and the sites were all previously invaded by European earthworms (e.g. *Lumbricus* spp.; *Aporrectodea* spp.). Each of these urban forests are bordered on one side by a paved road and are accessible by the public for hiking via narrow dirt trails. Human settlements in the forest occur but are limited. Although *A. agrestis* and *A. tokioensis* have become established in these managed forests, a clear invasion boundary was present in each area as of 2018 (Laushman et al., 2018; Herrick,

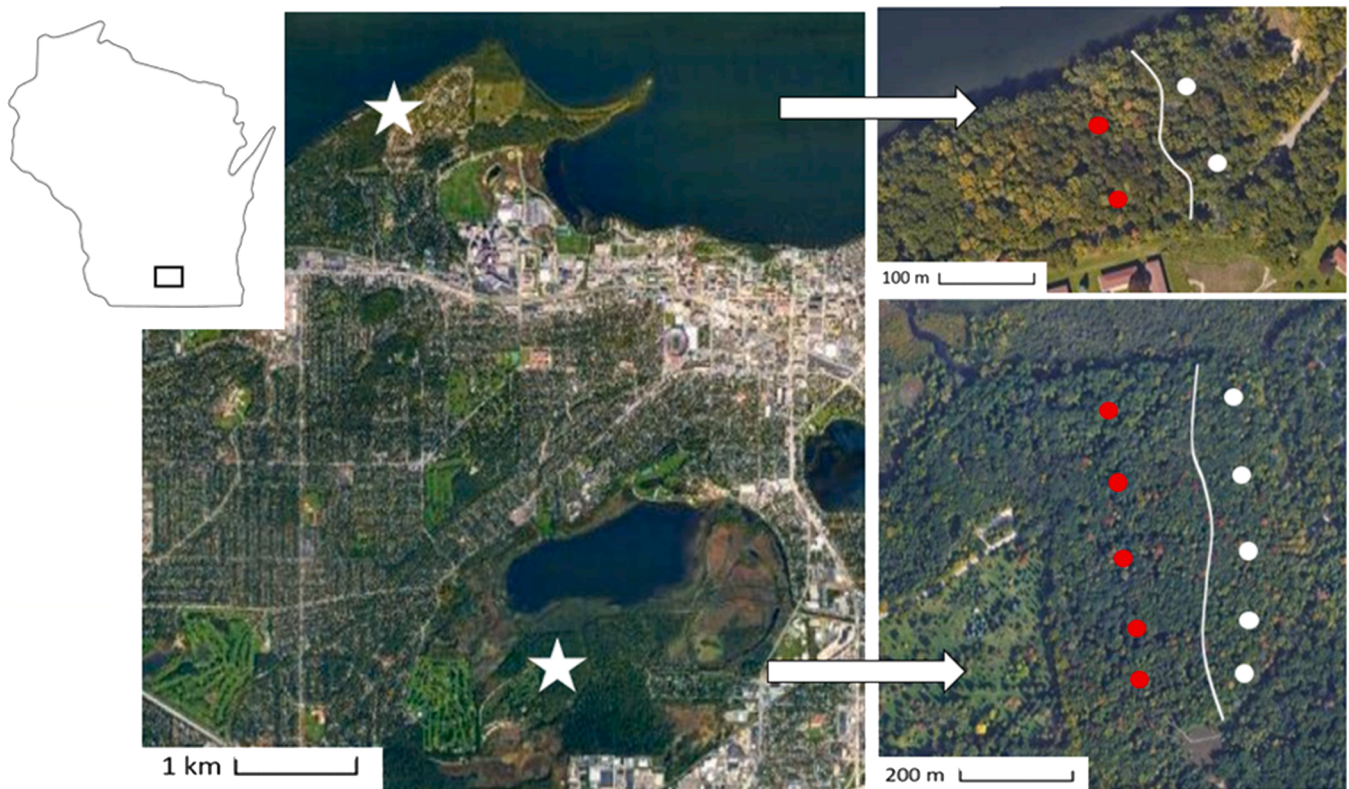


Fig. 2. – Field study area of managed forests in the city of Madison, in south-central Wisconsin, USA. Within each forested site, soil samples were collected at paired locations representing the inside (red dots) and outside (white dots) of the invasion front of invasive earthworms of genus *Amyntas*.

unpublished data, 2018). The identifiable difference in earthworm abundance across the invasion front and visual inspection of the soil for the unique *Amyntas* casting activity (Fig. 1), were the primary justifications for site selection in this study. Earthworm sampling was conducted using liquid mustard extraction (Gunn, 1992; Hale et al., 2005). Out of 121, 0.39 m² plots sampled in 2018, five contained a total of 14 lumbricids with no *Amyntas*. Thirty-three *A. agrestis* individuals were found in 20 plots, and 906 *A. tokioensis* individuals were found in 106 plots. Sixteen plots had both *Amyntas* species present. Ten plots did not contain any earthworms. The lumbricid plots were located on the further east end of each site that had not been invaded by *Amyntas*. The plots without earthworms were often close to the lumbricid only plots, possibly representing a transition zone along the invasion front. The boundary line representing outside and inside the invasion front, was selected as close to the lumbricid only areas as possible. Yet, visual evidence of *Amyntas* disturbance was present to ensure that the soil samples accurately represented *Amyntas* and non-*Amyntas* areas.

In the fall of 2018, seven paired forest sampling locations straddling the *Amyntas* invasion boundary were established within the forested areas (Fig. 2). Due to differences in parcel size, two pairs of locations were sampled in the northern site, and five pairs of locations were sampled in the southern site. At each paired sampling location, the forest floor (Oi, Oe, and separable part of the Oa) was removed and six replicate soil cores (347 cm³, 0–10 cm depth) were collected randomly from an area of approximately 100 m² on both sides of the invasion front. Soil samples (700–800 cm³, 0–10 cm depth) were also collected with a hand trowel from within ~20 cm from where each soil core was extracted. In total, 84 soil cores were collected and stored at 4 °C until analysis of soil bulk density, which was used to convert nutrient concentration data to areal pools. Soil samples were placed in plastic bins, air-dried, and then divided with a stainless-steel riffle splitter with 12-mm slots to acquire representative subsamples; a representative subsample was sieved to < 2 mm and later used for analyses.

Earthworm plots were re-sampled in summer of 2021. Only one plot contained a single lumbricid individual. Seventy-four *A. agrestis* individuals were found in 41 plots and 658 *A. tokioensis* individuals were found in 113 plots. Forty-two plots had both *Amyntas* species present. Five plots did not contain any earthworms. This suggests that *A. tokioensis* and *A. agrestis* are spreading into previously uninvaded areas of these forests.

2.2. Laboratory earthworm soil incubations

A laboratory study was designed to incubate forest soil with either *A. tokioensis* or *A. agrestis* for sequentially longer periods from 3 to 15 weeks. Soil for laboratory incubations was obtained from the West Madison Agricultural Research Station, Madison, WI; the soil was a silt loam of local origin with no known previous contact with *Amyntas* earthworms. Due to their agricultural background, they have higher nutrient concentrations than forest soils in the area. Plastic bins (5.7-L with tight-fitting lids) were established with 300 g of air-dried soil that was previously ground with a mortar and pestle to pass a 1-mm sieve. The soil was moistened to 0.30 g g⁻¹ water content and allowed to rest for 24 h before earthworms were added. Clitellate *A. agrestis* and *A. tokioensis* earthworms were collected from UW-Madison Arboretum forests and manicured gardens in late summer of 2018. Earthworms were examined to confirm species identity by the presence, number, and location of male pores on *A. tokioensis* and spermathecal pores on *A. agrestis* (Chang et al., 2016). Twelve earthworms (of one species or the other) were placed into each bin, with three replicates per species combination and length of incubation (3, 6, 9, 12, and 15 weeks); three additional bins received no earthworms (control). The numbers were chosen to correspond with survey data at the site and partly from prior observations of how many earthworms persist in these bins (Johnston and Herrick, 2019). Dried oak leaf litter was crumbled to pass a 5-mm sieve (Ikeda et al., 2015) and added to all bins. Bins containing

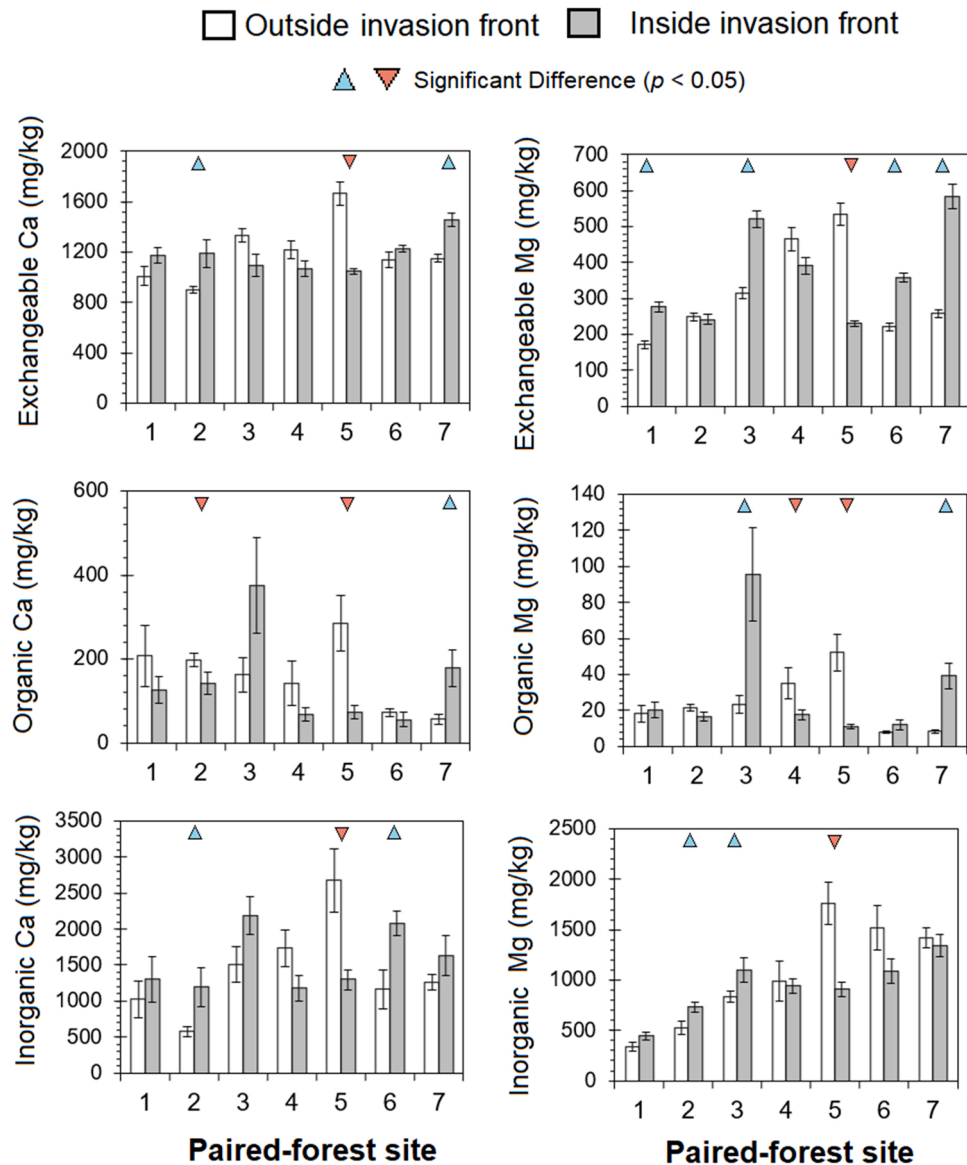


Fig. 3. – Differences in forest soil Ca and Mg concentrations (0–10 cm depth); white bars are outside (*Amyntas* absent) and gray bars are inside (*Amyntas* present) the invasion front. Blue upward triangles indicate significant increases while red downward triangles indicate significant decreases across the invasion front among paired-forest sites using the nonparametric Wilcoxon Rank Sign test. Error bars are standard error of six replicates.

A. tokioensis received ~1 g of litter every 2–3 days, and bins containing *A. agrestis* received ~3 g of litter every 2–3 days; these amounts of litter per feeding met consumption rates for each species (Johnston and Herrick, 2019). All bins were held at 20 °C as per Reinecke and Kriel (1981). The intention of the incubation design was to assess differences in soil nutrient pools as *Amyntas* earthworms consumed litter, burrowed through the soil, and created new aggregates larger than 1 mm. The study design also assumed that three control bins per species group were sufficient as these would have low data variability.

Earthworms were removed and discarded after the designated length of incubation (e.g., after 3 weeks in the 3-week treatment). Particulate organic matter > 2 mm was removed, while particulate organic matter < 2 mm was well-mixed into the soil. The soil was then allowed to air-dry in the bins until all incubations were complete. Mortality rate for both earthworms were ~50% by week 12 and > 90% by week 15. Bins lacking earthworms (the controls) were opened and allowed to dry at the same time as the 15-week treatment. Air-dry soil samples were divided with a stainless-steel riffle splitter with 12-mm slots to acquire representative subsamples; these were later used for analyses.

2.3. Soil physicochemical analyses

Field and incubated soil samples received the same set of analyses. Soil organic matter was determined by loss-on ignition in which soil was combusted for 4 h at 500 °C. Soil pH was measured using a 2:5 soil to 0.1 mol L⁻¹ CaCl₂ slurry. The soil-CaCl₂ mixtures were shaken for 1 h using an Eberbach shake table and filtered gravimetrically with a Whatman 40 filter. The pH of the supernatant extract was measured with a pH meter in triplicate (8015 VWR). Soil textural class was determined by hydrometer. Briefly, 40 g of air-dried soil was dispersed with 100 mL 5% (g g⁻¹) sodium hexametaphosphate for 24 h, and remaining aggregates were destroyed using a motorized mixer for 2 min. Hydrometer data were collected at 40 s, 4 h, and 7.5 h for 1-L columns held in a water bath at 22 °C.

2.4. Three step-sequential extractions, and instrumental analyses

To characterize the different phases of nutrient sorption (i.e., exchangeable, organic-bound, and inorganic-bound nutrients), we

performed a three-step sequential extraction on all (forest and incubation) soil samples. For the first step, we characterized the exchangeable (weakly-bound) nutrients. One gram of soil was extracted with 20 mL of 0.1 mol L⁻¹ ammonium acetate. Samples were shaken for 24 h and then soil slurries were centrifuged at 3000 rpm for 60 min and the supernatant was decanted. For the second step, organic complexed nutrients were extracted. The residual solid phase was extracted with 5 mL of 30% H₂O₂ to oxidize organic phases. After 24 h of degassing, the samples were diluted to 40 mL with deionized water and heated to 50 °C to remove excess H₂O₂. The resulting mixture was centrifuged at 3000 rpm for 1 h and the supernatant was collected. For the final step, a pseudo-total digestion using a modified EPA method 3050B in 5 mL of 8 mol L⁻¹ reverse aqua regia (9:1, HNO₃ to HCl) and digested at 90 °C for 45 min on an insulated hot plate. This method is often referred to as a pseudo-total digestion due to the inability to dissolve silicates and other refractory compounds. Since our study focuses on plant available nutrients, we henceforth refer to the pseudo-total concentrations as inorganic concentrations.

Extracts and digests were further diluted with deionized water and analyzed for Al, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn with an Agilent 5110 Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Agilent, Santa Clara, CA, USA). Every 25 samples included a digestion blank, a duplicate and NIST San Joaquin 2710a standard reference material (SRM) (National Institute of Standards and Technology, Gaithersburg, MD). Extracts and digests had relative standard deviations (RSDs) among SRMs and duplicate samples were less than < 4% for exchangeable, organic-bound and inorganic soil concentrations. Preparation blanks had elemental concentrations below the detection limit for Mg, Mn, and P. Aluminum, Ca, and K concentrations within blanks were < 2 µg L⁻¹. As expected, summed concentration recoveries for San Joaquin 2710a SRM were between 60% and 90% for all nutrients particularly due to the indigestion of silicate and other residual compounds. Inorganic concentration recoveries for San Joaquin 2710a SRM were Ca (82%), Mg (86%), K (97%), Mn (81%), and P (79%). Concentrations of Al, Cu, Fe, Mn, S, and Zn are reported in the [supplemental material](#) and are not discussed in the present paper.

2.5. Data analyses

We tested for changes in soil A horizon (0–10 cm) macronutrient Ca, Mg, K, and P concentrations (mg kg⁻¹) and pools (g m⁻²) and with variations with soil chemical properties pH and SOM (%) using methods appropriate to the two approaches of study (field sampling vs. laboratory manipulations). For the field study forested sites were compared between invasion status (inside the invasion front where *Amyntas* spp. are present, and outside the invasion front where there is no evidence of *Amyntas* spp.) using the nonparametric Wilcoxon Rank Sign test due to nonnormality and/or unequal distributions. To determine if a significant positive or negative effect on soil macronutrients occurred among all of the sites, a One-Way ANOVA was calculated using Matlab (Mathworks, Natick, MA, USA). Variables were tested for normality using the Lilliefors test and Levene's Test to determine equal variance. Macronutrient pools (g m⁻²) were calculated by multiplying the nutrient concentration by the measured bulk density at that respective sample location. This calculation converts concentration data (mg kg⁻¹) to mass per unit area (g m⁻²) for the 0–10 cm depth sampled. The mean bulk density was 1.06 g cm⁻³ and the range 0.67–1.29 g cm⁻³ for all locations in this study. Bulk density data were obtained to answer other research questions and are not discussed further in the present paper. Linear regressions were used to explore relationships between soil pH and SOM and exchangeable, organic-bound, and inorganic Ca, Mg, K, and P.

For the laboratory study, we examined soil chemical and nutrient properties through time (i.e., length of incubation) for each *A. tokioensis* and *A. agrestis* versus a control which lacked earthworms. Due to the limited number of replications per treatment combination, non-parametric statistics (Kruskal-Wallis, Wilcoxon Rank Sign Test) were

Table 1 –

Average values with one standard error and One-Way ANOVA results for soil pH, soil organic matter (SOM), and plant macronutrients (Ca, Mg, K, and P) as concentrations and pools in forest soils of Madison, WI. The ANOVA tested whether there was an increase or decrease across the *Amyntas* invasion front (n = 7 paired forest sites).

	Concentrations		Pools	
	Mean ± SE		Mean ± SE	
pH	5.42 ± 0.14	(+)**		NA
	%		kg m ⁻²	
SOM	7.9 ± 0.7	ns	8.0 ± 0.5	ns
	mg kg ⁻¹		g m ⁻²	
Calcium				
Exchangeable	1191 ± 64	ns	122 ± 6	ns
Organic	153 ± 37	ns	15 ± 4	ns
Inorganic	1489 ± 205	ns	150 ± 19	ns
Magnesium				
Exchangeable	344 ± 37	(+)*	35 ± 3	ns
Organic	27 ± 8	ns	2.7 ± 0.8	ns
Inorganic	998 ± 130	ns	101 ± 12	ns
Potassium				
Exchangeable	89 ± 7	(+)**	9.1 ± 0.7	(+)**
Organic	11 ± 1	(+)**	1.1 ± 0.1	(+)*
Inorganic	201 ± 27	ns	21 ± 3	ns
Phosphorus				
Exchangeable	4.4 ± 0.7	ns	0.4 ± 0.1	ns
Organic	26 ± 6	ns	2.7 ± 0.6	ns
Inorganic	363 ± 39	ns	37 ± 3	ns

* p < 0.05,

** p < 0.01; (+) and (–) indicate a significant increase or decrease where *Amyntas* species are present (inside the invasion front) compared with where they are yet absent (outside the invasion front); ns indicates no significance; NA indicates not applicable.

applied to test for differences between the two species and for earthworms compared with control samples.

3. Results

3.1. Field study of forest sites with an invasion front

3.1.1. Soil macronutrients concentrations

Exchangeable nutrient concentrations were a substantial portion of the total concentrations for Ca (37%), K (29%), and Mg (22%) but not P (1%). Organic-bound nutrients were a small portion of their total respective concentrations of Ca (5%), K (4%), Mg (2%), and P (7%). Inorganic nutrient concentrations were the largest portion of their respective concentrations for Ca (59%), K (67%), Mg (77%), and P (92%).

Forest soil Ca and Mg exhibited mixed differences across the *Amyntas* invasion front. Site 5 had significantly lower Ca across all three fractions (exchangeable, organic, inorganic) inside the invasion front while Sites 2, 6, and 7 had some significant increases in Ca fractions (p < 0.05; Fig. 3). Based upon the ANOVA results, Ca was not consistently significantly different between inside and outside the invasion front (Table 1). Forest soil Mg exhibited mixed differences across the *Amyntas* invasion front for all three fractions. Sites 1, 2, 3, 6, and 7 had some significant increases in Mg fractions but Site 5 had significantly lower Mg across all three fractions inside the invasion front (p < 0.05; Fig. 3). Few differences between inside and outside the invasion front were consistent across Ca and Mg fractions. Based upon the ANOVA results, the exchangeable Mg fraction was significantly different between inside and outside the invasion front but not organic-bound or inorganic Mg (p < 0.05; Table 1).

Forest soil exchangeable and organic-bound K fractions were significantly greater inside the *Amyntas* invasion front for Sites 1, 2 and 6 than outside of the invasion front (p < 0.05; Fig. 4). Forest soil P exhibited mixed differences across the *Amyntas* invasion front for all three fractions. Sites 1, 2, 5, 6, and 7 were significantly higher inside the

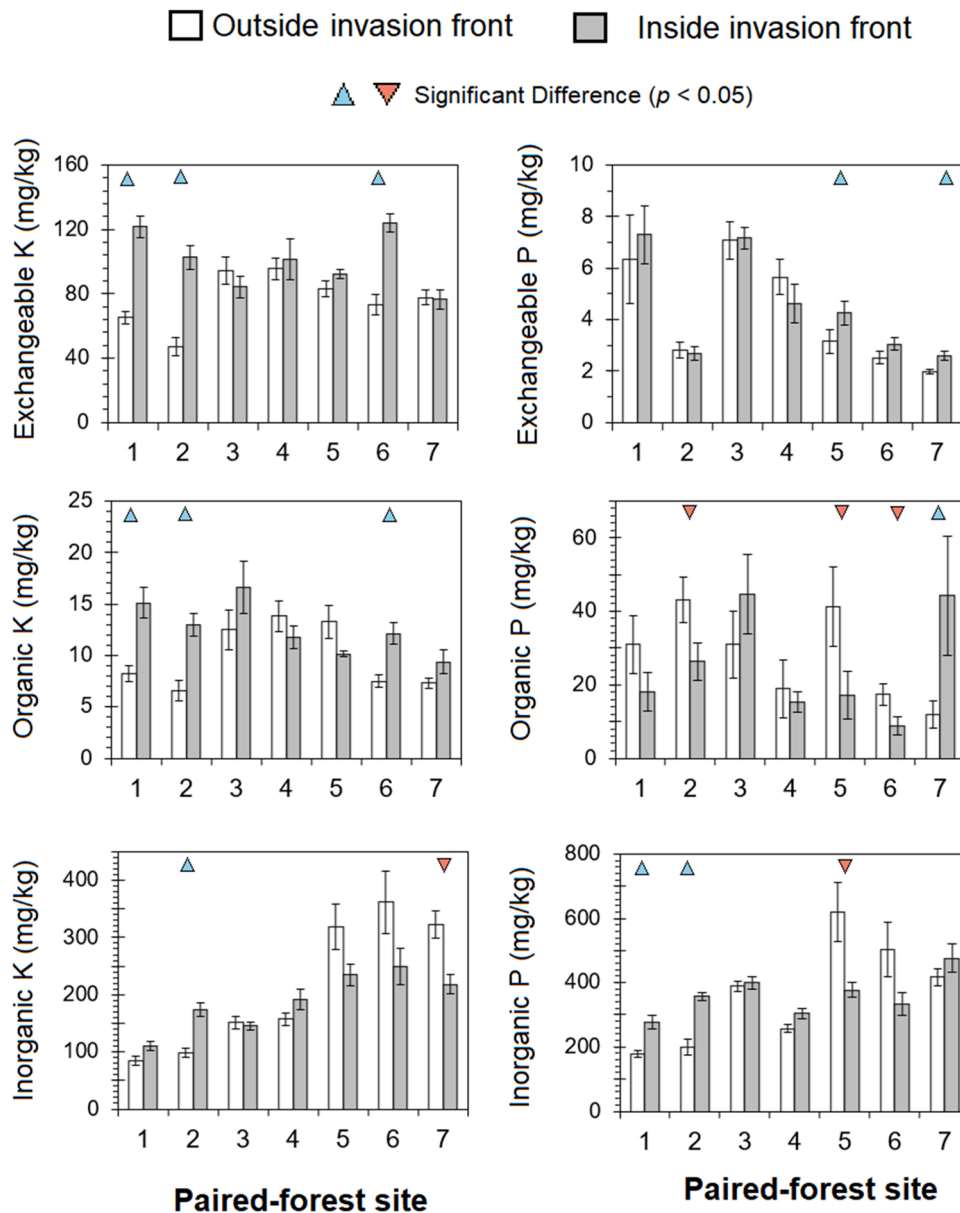


Fig. 4. – Differences in forest soil K and P concentrations (0–10 cm depth); white bars are outside (*Amyntas* absent) and gray bars are inside (*Amyntas* present) the invasion front. Blue upward triangles indicate significant increases while red downward triangles indicate significant decreases across the invasion front among paired-forest sites using the nonparametric Wilcoxon Rank Sign test. Error bars are standard error of six replicates.

invasion front for one P fraction, but Sites 2, 5, and 6 had significantly lower P inside the invasion front ($p < 0.05$; Fig. 4). The one-way ANOVA results revealed that exchangeable and organic-bound K were significantly greater inside than outside of the invasion front ($p < 0.01$; Table 1). Based upon the ANOVA results, no P fraction was significantly different between inside and outside the invasion front (Table 1). Nutrient pool distributions across the exchangeable, organic, and inorganic fractions were the same as their concentrations. Similar to K concentrations, only the exchangeable and organic-bound K pools were consistently, significantly different between inside and outside of the *Amyntas* invasion front (Table 1; Supplemental Figs. S1 and S2).

3.1.2. Soil pH and soil organic matter

Soil organic matter averaged $7.8 \pm 0.7\%$ across the seven paired forest sites. There was no significant change in SOM across the invasion front when evaluated across all sampling locations (Table 1), as the percentage of SOM showed mixed effects across the forest pairs. Sites 2, 3, 6, and 7 had significantly greater SOM inside the invasion front,

whereas Sites 4 and 5 had significantly less SOM inside the invasion front ($p < 0.05$; Fig. 5).

Soil pH averaged 5.42 ± 0.14 across the seven paired forest sites. Soil pH was significantly higher inside the invasion front ($\text{pH } 5.23 \pm 0.10$) than outside ($\text{pH } 5.61 \pm 0.09$) across all sites ($p < 0.05$; Table 1). Soil pH was significantly higher inside the invasion front than outside for Sites 2, 5, and 6 ($p < 0.05$; Fig. 5). There was no statistical difference in soil pH at Sites 1, 3, 4, and 7, yet the trend was a higher pH inside the invasion front (Fig. 5).

3.1.3. Linear relationships among nutrient concentrations, pH, and SOM

Relationships among nutrient concentrations, pH, and SOM were assessed with individual linear least-square regressions. Exchangeable, organic-bound, and inorganic macronutrient concentrations were each strongly associated with soil pH and SOM. Exchangeable and organic-bound Ca, Mg, K, and P concentrations were all positively and significantly related to SOM ($R^2 > 0.25$, $p < 0.01$). Exchangeable K and P and organic-bound Ca, Mg, and K were each correlated with higher pH.

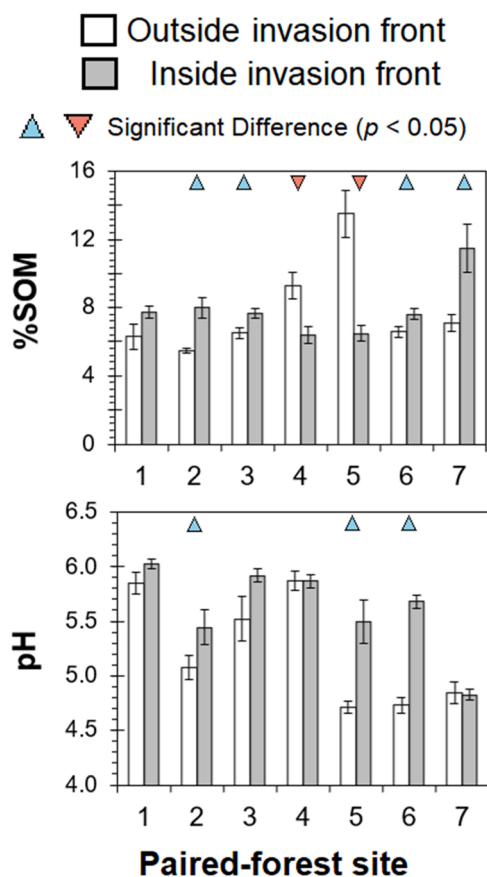


Fig. 5. – Forest soil (0–10 cm) soil organic matter (%SOM) and pH; white bars are outside (*Amyntas* absent) and gray bars are inside (*Amyntas* present) the invasion front. Blue upward triangles indicate significant increases while red downward triangles indicate significant decreases across the invasion front among paired-forest sites using the nonparametric Wilcoxon Rank Sign test. Error bars are standard error of six replicates.

Inorganic Ca and P were each correlated with higher SOM ($R^2 > 0.25$, $p < 0.01$), while higher inorganic Ca, K, and P were each correlated with higher pH ($R^2 > 0.25$, $p < 0.01$).

3.2. Laboratory incubations under *Amyntas* earthworms

3.2.1. Macronutrient concentrations

Overall, soil Ca and Mg concentrations increased with the presence of *A. agrestis* or *A. tokioensis* when forest soil was incubated in the 15-week laboratory experiment. Under incubation with *A. tokioensis*, exchangeable, organic-bound, and inorganic Ca were each greater than the control soil at all dates over the 15-week incubation ($p < 0.01$; Fig. 6). Under incubation with *A. agrestis*, organic-bound Ca was often greater and exchangeable Ca was always greater than the controls over the 15-week incubation ($p < 0.01$; Fig. 6). For both earthworm species, the exchangeable and organic-bound Mg fractions were greater than in the control over the 15-week incubation ($p < 0.01$; Fig. 6). The inorganic fraction of Mg was greater than in the controls after 6 weeks of incubation with *A. tokioensis*, but not with *A. agrestis* ($p < 0.01$; Fig. 6).

The presence of *A. agrestis* or *A. tokioensis* over the 15-week laboratory experiment similarly altered soil K and P fractions, yet some fractions did not change for one or both earthworm species. Under incubation with *A. tokioensis*, all three fractions of K were greater than in the controls over all dates of the 15-week incubation ($p < 0.01$; Fig. 7). *Amyntas agrestis* did not consistently increase exchangeable or inorganic K, but this species did significantly increase organic K for all dates of the experiment (Fig. 7). Incubation with *A. tokioensis* or *A. agrestis*

increased exchangeable and inorganic P relative to the controls over the 15-week incubation ($p < 0.01$; Fig. 7). However, neither earthworm species altered organic P concentrations compared with the control soils (Fig. 7). It must be noted that temporal differences in the earthworm populations due to mortality occurred, in which ~50% of both earthworms were deceased by week 12 and > 90% of both earthworms were deceased by week 15.

3.2.2. Soil pH and soil organic matter

Soil pH and SOM showed different trends under incubation with the two species of *Amyntas* earthworm (Fig. 8). Soil organic matter was significantly higher in soils with *A. tokioensis* (10.5–11.2%) than the control soils (7.1–8.9%) after 6 weeks of incubation. Although *A. agrestis* increased SOM slightly (8.3–10.0%), this change was not statistically different from SOM in the control soils. Soil pH values were initially greater in soils incubated with *A. agrestis* (pH 5.26–5.39) and *A. tokioensis* (pH 5.23–5.25) than the control soils (pH 5.02–5.15) after 3, 6, and 9 weeks, but the soil pH was not significantly different than control soils after 12 or 15 weeks.

4. Discussion

4.1. Site-specific changes in nutrient concentrations and pools upon *Amyntas* invasion in field soils

In our initial hypothesis formed from previous work across the northern and northeastern United States, we expected broad nutrient depletions under *Amyntas* earthworm invasion. We observed consistent and significant increases in exchangeable and organic K concentrations and pools from outside to inside the invasion front across the paired forest sites (Fig. 3; Supplemental Fig. S2). This suggests *Amyntas* earthworms promote a shift in nutrient partitioning among soil phases, but not necessarily a loss, of K in forest soil. However, we found mixed results regarding Mg, Ca, and P (exchangeable, organic-bound, and inorganic fractions) concentrations and pools when evaluating inside versus outside the *Amyntas* invasion front. Increases in forest soil Ca, Mg, K, and P concentrations coincided with being inside the invasion front at some locations (Sites 2, 3, or 7; Figs. 3 and 4). Similar results are present for pools (Supplemental Figs. S1 and S2). Paired sites with significant increases in concentrations from outside to inside the invasion front demonstrated large swings in nutrient status, such as changes ranging from 32% to 367% difference. Conversely, one forest pair (Site 5) had significantly lower exchangeable, organic, and inorganic fractions of Ca, Mg, and P (except exchangeable P) inside compared to outside the invasion front (Figs. 3 and 4). The increases in Ca, Mg, K, and P concentrations and pools following earthworm invasion generally agree with other studies such as Hale et al. (2008), Resner et al. (2015), Dobson et al. (2017), and Ross et al. (2021) and contradict our hypothesis of decreased concentrations following *Amyntas* invasion. However, our results highlight the heterogeneity of the effect and demonstrate that the site-specific characteristics can obscure generalized trends for Ca, Mg, and P and that a temporal aspect of initial increases followed by net nutrient decreases is possible.

As common with survey-based studies, our field study has some confounding factors. One important factor is the previous long-term inhabitation by European earthworm species (*Lumbricus terrestris*, *Lumbricus rubellus*, *Apporectodea* spp; see Laushman et al., 2018). Lumbricids from multiple functional groups were present at all the forest study plots; their population size and density were not determined in this study. However, at our Arboretum study site, Laushman et al. (2018) documented the spread of *Amyntas* spp. into previously uninvaded areas with Lumbricids being displaced from the newly invaded areas. The displacement of predominately epi-endogeic and anecic earthworms has occurred following *Amyntas* invasion at these locations. A co-varying factor across sites is the length of time of this second wave of invasion. European species have been present in southern-Wisconsin

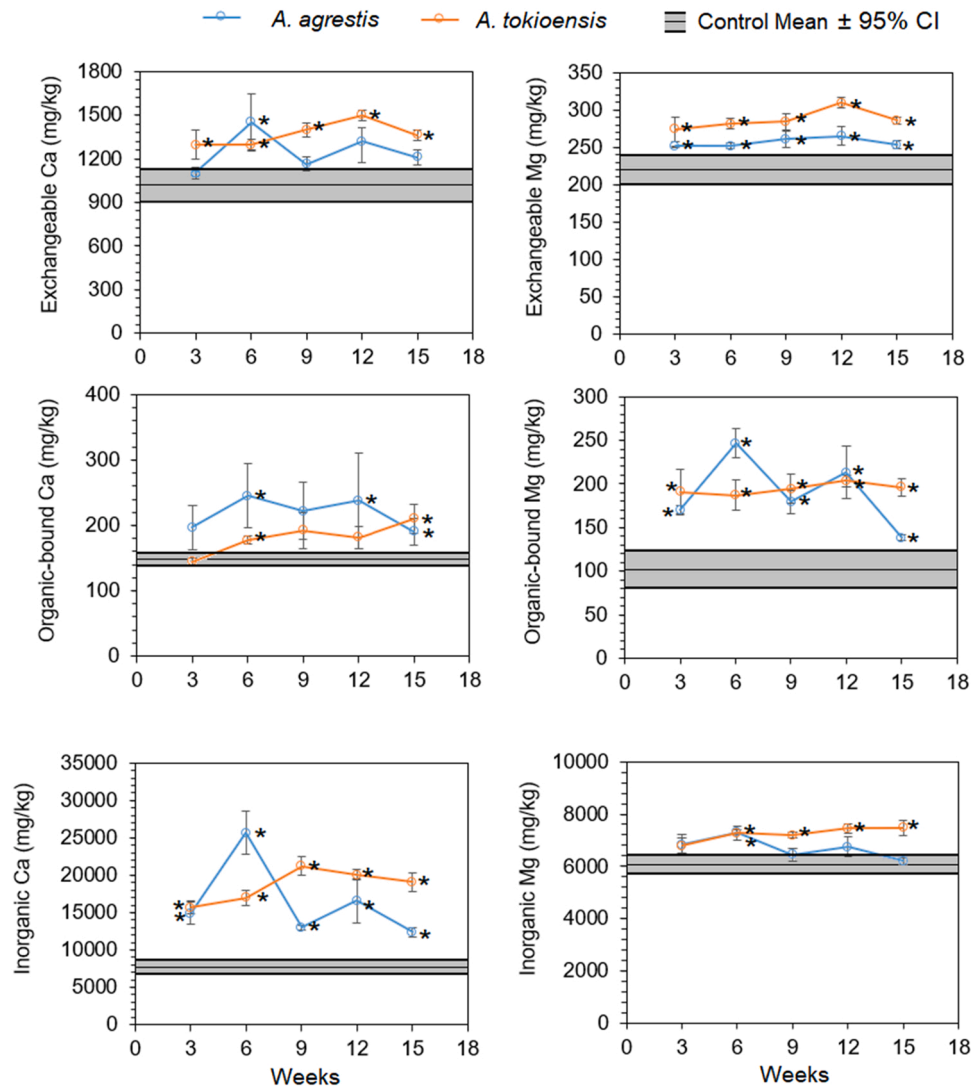


Fig. 6. – Exchangeable, organic, and inorganic Ca and Mg concentrations in soil over a 15-week incubation in the laboratory. Incubations either lacked earthworms (“control”), contained *A. agrestis*, or contained *A. tokiensis*. Values are means and standard error of three replicates, and asterisks (*) indicate a significant difference between an earthworm treatment and the control ($p < 0.05$).

forests for at least sixty years (Nielsen and Hole, 1964), whereas *Amyntas* invasions have occurred much more recently and not evenly among the paired plots. For example, *Amyntas* spp. were first confirmed at the UW-Madison Arboretum and Wally Bauman Woods sites in 2015 and 2017, respectively. However, the first *Amyntas* population in Wisconsin was discovered by chance in 2013 at a different location in the Arboretum. While it is likely that these earthworms arrived prior to 2013, we cannot confirm an exact date. Despite these limitations, our field study has captured the varying effects of *Amyntas* of early invasion increases on nutrients across paired plots.

Our laboratory study confirmed that *Amyntas* earthworms alter nutrient status, as there was increased exchangeable, organic, and inorganic Ca, Mg, K, and P (except organic-bound P) with the presence of *A. agrestis* and *A. tokiensis*. This finding similarly contradicted our hypothesis; we expected broad nutrient depletions or transitions from organic and inorganic forms to exchangeable forms of nutrients. The laboratory experiments show how quickly leaf litter added to the bins can be consumed by *A. agrestis* and *A. tokiensis* and then returned to soils as exchangeable, organic-bound and inorganic nutrient forms. These were enclosed containers, so the observed trends show that these earthworms increased the overall nutrient content of the soil. It must be noted that net leaching losses of nutrients could not occur. However,

under field conditions, the nutrients, particularly those present in the exchangeable phases, can be lost by leaching during large precipitation events or over several years. Further, nutrients in exchangeable, organic, and potentially inorganic forms can be taken up by understory and canopy plants.

There are several implications for changing forest soil nutrients under *Amyntas* earthworms. The abundance of nutrients can promote plant communities which are better-adapted to bioturbated soils and put understory plants that depend on organic horizons at a disadvantage for germination (see Dobson et al., 2020). In addition, the higher soil pH, SOM, and nutrients can promote tree growth (e.g. Suárez et al., 2004; Ross et al., 2021). Tecimen et al. (2020) observed a decrease in soil pH in *Amyntas* castings, which suggests that consumption of the forest floor and production of soil castings may neither increase soil pH nor base saturation (soluble Ca) of soils. However, soil nutrients could be more susceptible to loss by leaching and erosion (Bohlen et al., 2004), as earthworm castings promoted detachment and diminished aggregation (Wen et al., 2022). Thus, continued monitoring and studies on the long-term effects on forest soils under *Amyntas* invasion are warranted to ensure sustainability of forest health (e.g. Tecimen et al., 2020) and ecosystem services (Richardson, 2019).

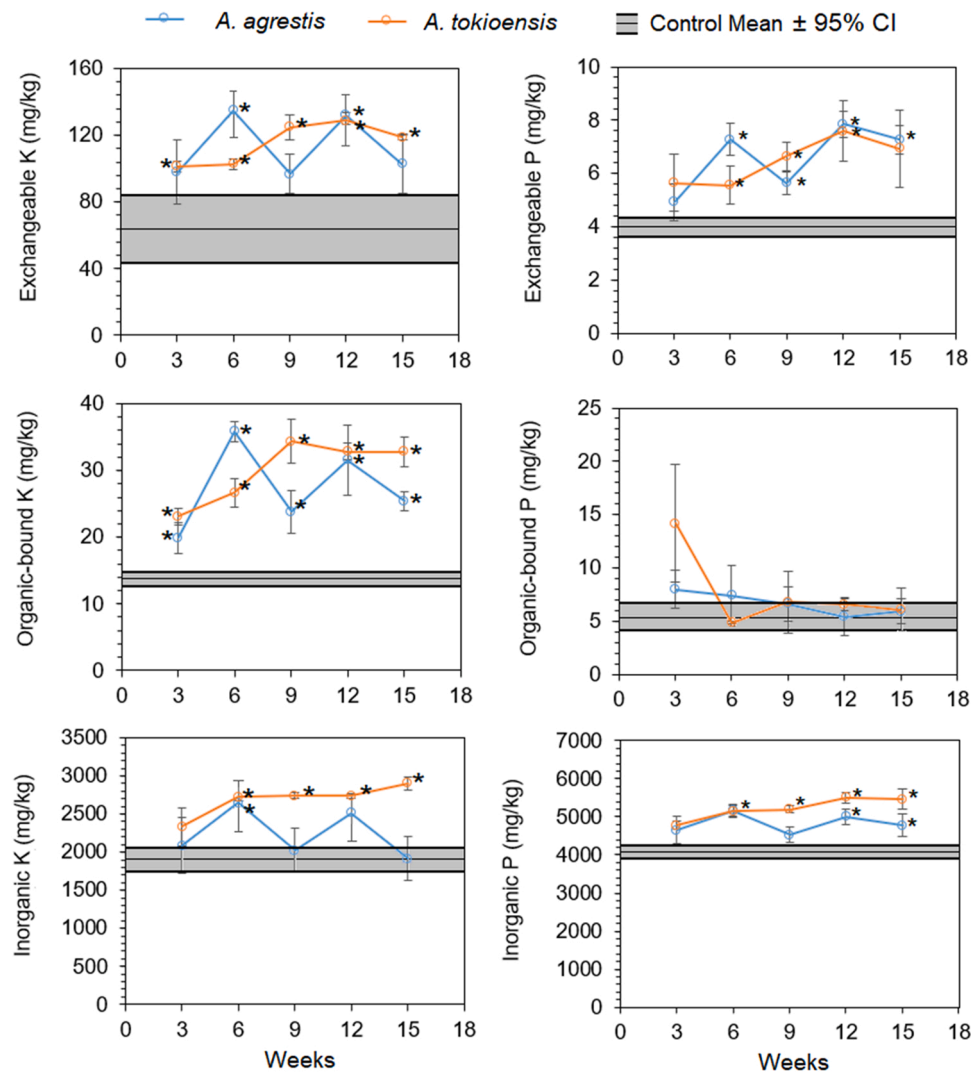


Fig. 7. – Exchangeable, organic, and inorganic K and P concentrations in soil over a 15-week incubation in the laboratory. Incubations either lacked earthworms (“control”), contained *A. agrestis*, or contained *A. tokioensis*. Values are means and standard error of three replicates, and asterisks (*) indicate a significant difference between an earthworm treatment and the control ($p < 0.05$).

4.2. Linkages among nutrients, SOM, and pH

We hypothesized that increases in SOM due to incorporation and mineralization of materials from organic horizons and leaf litter into the mineral soil would drive increased Ca, Mg, and K concentrations. As shown in Fig. 5, there were significant increases in SOM from outside to inside the invasion front for Sites 2, 3, 6, and 7. This increase in SOM co-occurred with increases in many Ca, Mg, K, and P fractions inside the invasion front at paired forest Sites 2, 3, or 7 (Figs. 3 and 4). Furthermore, exchangeable and organic-bound Ca, Mg, K, and P concentrations were all positively and significantly related to SOM. These observations support the hypothesis that *Amyntas* invasion has incorporated new organic matter from litter and the forest floor into the top 10 cm of the soil. However, we also observed that Site 5 had a significant decrease in SOM from outside to inside the earthworm invasion front (Fig. 5), which coincided with significant decreases in organic and inorganic fractions of Ca, Mg, and P concentrations (Figs. 3 and 4).

The increased incorporation of nutrients from organic horizons and litter into the mineral soil by earthworms has been observed by Hale et al. (2008) and Resner et al. (2015) in northern Minnesota USA and by Dobson et al. (2017) in central New York, USA. The invasive *Amyntas* can achieve this by either physically mixing litter into the mineral soil or stabilizing the organic matter in their castings (Angst et al., 2017;

Bottinelli et al., 2020; Ross et al., 2021). But as described by Hale et al. (2005), the increase or stability of organic matter content and nutrient concentrations in the forest A horizon are likely a short-term condition of an earthworm invasion. Bottinelli et al. (2020) found that *Amyntas khami* castings initially stabilize carbon, but the compounds shift as they age to less recalcitrant compounds (e.g. lignin reduction). Since Site 5 has exhibited a net loss in SOM and also decreased in nutrient concentrations and pools, it possible that this may be due to a longer term occupation by *Amyntas* spp. Conversely, Sites 2, 3, and 7 may be in an earlier stage of invasion where SOM is increasing, thereby experiencing increases in nutrient concentrations and pools.

Our laboratory study observations support increased organic matter and soil nutrients in the field study at Sites 2, 3, 6 and 7 and reinforce that early in the invasion of forests by *Amyntas* spp. can be a significant increase in nutrients due to litter consumption and incorporation into the mineral soil. This may be especially pronounced in soils with low SOM and low nutrient concentrations. However, our laboratory study did not evaluate the effect of soils without the addition of litter to capture thresholds of limited or abundant SOM and nutrient concentration. Thus, excellent future studies should consider the effect of SOM and nutrients present within the soil as a potential limiting or promoting factor.

In addition to SOM, we also observed linkages between nutrients and

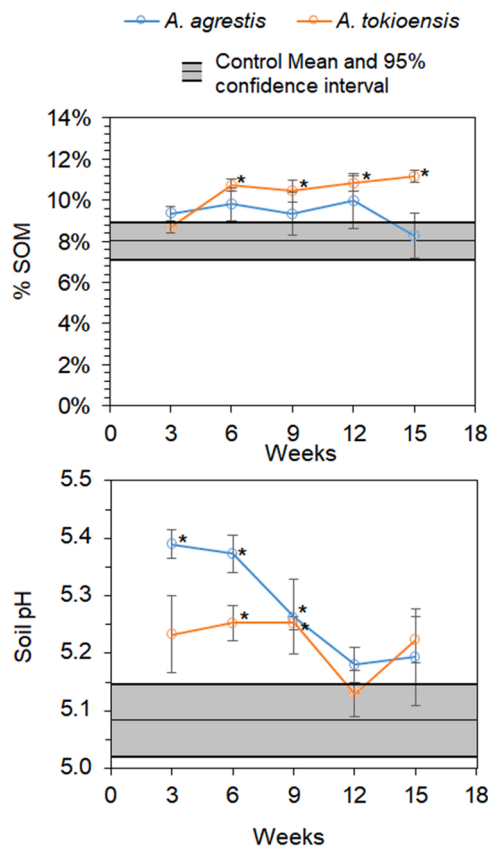


Fig. 8. – Soil organic matter (%SOM) and pH over a 15-week incubation in the laboratory. Incubations either lacked earthworms (“control”), contained *Amyntas agrestis*, or contained *Amyntas tokioensis*. Values are means and standard error of three replicates, and asterisks (*) indicate a significant difference between an earthworm treatment and the control ($p < 0.05$).

soil pH. Sites 2, 5, and 6 had higher soil pH inside than outside of the invasion front (Fig. 5). For Sites 2 and 6, this coincided with higher SOM inside than outside of the invasion front. Exchangeable fractions (exchangeable K and P), organic-bound fractions (organic-bound Ca, Mg, and K), and inorganic fractions (inorganic Ca, K, and P) were positively correlated with higher pH ($R^2 > 0.25$, $p < 0.01$). The correlation between higher nutrients and higher pH can result from a combination of losing the organic horizon, decreased acidity promoting sorption, and higher SOM can increase cation exchange capacity. Earthworms actively consume litter, which decreases organic horizon formation and results in an increase in soil pH (Alban and Berry, 1994; Dobson et al., 2017; Frelich et al., 2019). The higher soil pH from earthworm mixing and organic horizon consumption can promote sorption of macronutrients to soil (Resner et al., 2015; Dobson et al., 2017; Ross et al., 2021). The higher pH can deprotonate sorption sites as well as allows for pH-dependent, SOM-derived charge sites in the soil (e.g. Reich et al., 2005; Van Groenigen et al., 2019). However, as seen with the other elements, this is not consistent. The effect was primarily observed for Sites 2 and 6 and the opposite effect was observed for Site 5 (see Figs. 4 and 5). Thus, *Amyntas* earthworms can also change the ability to hold onto soil nutrients through increasing pH but this had mixed results in our study.

4.3. Differences among *Amyntas* species in a laboratory study

The two species *A. tokioensis* and *A. agrestis* both increased soil nutrients, with generally greater nutrient concentrations after inhabitation of *A. tokioensis* than *A. agrestis*. More specifically, exchangeable Ca and Mg and inorganic K, Mg, and P were greater for *A. tokioensis* than

A. agrestis. There was also less variability among replicates along the incubation period for *A. tokioensis* than *A. agrestis*. The greater soil nutrient concentrations of *A. tokioensis* than *A. agrestis* was unexpected as less litter and nutrients were added to *A. tokioensis* bins (1 g of oak litter per feeding) compared to *A. agrestis* bins (3 g of oak litter per feeding). One potential mechanism for this difference is the greater transformation of litter to organic matter via ingestion and digestion by *A. tokioensis* than *A. agrestis*. Here, our data support this potential mechanism as SOM increased in soils with *A. tokioensis* while soils with *A. agrestis* did not have an increase in SOM. Thus, the higher rates of transformation of litter to SOM by *A. tokioensis* could generate higher sorption capacity and nutrient retention than un-degraded litter in control treatments. A similar mechanism may be lower mineralization and greater stabilization of organic wastes by *A. tokioensis* than *A. agrestis*. Another potential mechanism for lower variability by *A. tokioensis* than *A. agrestis* may be due to feeding habit, as they may preferentially behave as an epigeic litter feeder causing greater consumption rates while *A. agrestis* may behave more as an endogeic mineral soil feeder and rely on mixing of litter into mineral soil prior to ingestion and digestion. However, this mechanism would lead to an increase in SOM by *A. agrestis*, which was not observed. The variation in feeding and incorporation of nutrients may be an important ecological feature to their combined invasion strategy (Chang et al., 2018). Laushman et al. (2018) observed differences in litter depth that was greatest when both *Amyntas* species were present and lower when only one was present. Richardson et al. (2019) found that *A. agrestis* can bioaccumulate higher concentrations of metals than *Metaphire hilgendorfi*, another Megascolecid, in their tissues, which can act as another pathway for nutrient losses from soil. Further work is needed to evaluate the long-term effect of their combined invasion of forest soils.

In our laboratory study, we observed significant increases in the concentration of Ca, Mg, K, and P in the inorganic phase (non-silicate) for *A. tokioensis* but only for inorganic Ca for *A. agrestis*. The cause of this difference is unclear. A potential cause is difference in the decomposition of SOM by *A. agrestis*, which could be limiting the formation of inorganic phases. For example, *A. tokioensis* castings may promote SOM stabilization and nutrient retention while *A. agrestis* may produce castings that do not allow physical protection (Angst et al., 2017) or formation of Al, Fe, and Mn complexes (e.g. Rietra and Hiemstra, 2001). *Amyntas* spp. do not have the pronounced calciferous glands of lumbricids which may explain the limited effects on soil pH and the drop in soil pH back to similar soil pH range as the control supports this idea. Instead, the liberation of exchangeable P and Ca from organic matter by *A. tokioensis* could promote the formation of hydroxyapatite in the soils (e.g. Li et al., 2012). However, without additional analyses, the inorganic forms being promoted by *Amyntas* invasion remain unclear and may explain the differences between the two species.

4.4. Conclusions and implications

In urban managed forests under invasion by *Amyntas* spp., we observed that exchangeable, organic bound, and inorganic forms of nutrients can be affected by invasion of *Amyntas* earthworms but this can be obscured as nutrients are often heterogeneous and site dependent. We conclude that soil conditions that correspond with initial stages of *Amyntas* invasion include higher soil pH, and SOM and can lead to an increase in nutrients. However, depletion of SOM is associated with lower Ca, Mg, and K was also observed at one paired-forest site. Thus, earthworm invasion can create site-specific nutrient depletion or enrichment. This may be further affected by other factors not studied here, including rates of litter addition, changes in nutrient solubility and leaching during the growing season, and earthworm population size.

Our laboratory study confirmed that the addition of litter plus earthworms added nutrients to the soil from all fractions, except for the organic-bound P fraction. We observed that *A. tokioensis* stabilized greater concentrations of nutrients than *A. agrestis* despite being fed one

third less litter. We observed differences between *A. tokioensis* and *A. agrestis* in litter consumption, soil mixing, mineralization, and formation of stable SOM. This result highlights that invasion effects can be species-dependent and can be difficult to observe at the field scale due to the common co-invasion of forest soils by several species of Megascolecidae.

Further studies are needed to quantify the long-term effects of earthworm invasion, particularly with impacts on understory and canopy trees acquisition of nutrients and leaching from the rooting zone. As nutrient accumulation plateaus following invasion, the long-term retention or losses will need to be quantified. This study captured changes in nutrient concentrations and forms (e.g., exchangeable vs. bound) following a 15-week incubation in the laboratory, but any long-term effects might be delayed until organic forest soil horizons are eliminated and litterfall no longer replenishes lost SOM. At that threshold, nutrient cycling may be vulnerable to the most substantial depletion with unknown effects on tree health.

Author Contributions

Mr. Bradley Herrick and Dr. Marie Johnston designed the experiment, collected, and processed samples, and contributed to writing of the manuscript. Dr. Justin Richardson analyzed the soil samples, led statistical analyses of the data, and contributed to the writing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pedobi.2022.150804.

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