Pedobiologia - Journal of Soil Ecology 83 (2020) 150669

Contents lists available at ScienceDirect

Pedobiologia - Journal of Soil Ecology

journal homepage: www.elsevier.com/locate/pedobi

Tools for monitoring and study of peregrine pheretimoid earthworms (Megascolecidae)

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ARTICLE INFO

Keywords:
Invasive earthworms
Management and control
Megascolecidae
Methods
Pheretimoids
Research
Sampling
Techniques

ABSTRACT

Peregrine pheretimoid earthworms, commonly known as jumping worms, are members of the family Megascolecidae that have become widely established outside of their native ranges. In many parts of the world this represents a second wave of earthworm invasions, following the introduction of peregrine European earthworms in the family Lumbricidae during the colonial era. Forest ecologists, turf managers, gardeners, and other land managers are concerned about the observed or presumed negative effects of jumping worms on invaded habitats. Although research on jumping worms has accelerated in recent decades, our understanding of their ecology remains limited. We compiled techniques useful to researchers working to fill voids in our understanding. Similar past efforts have focused on tools used to study common European species. Differences in life cycle, behavior, morphology, and physiology make it difficult to transfer experiences with European earthworms to pheretimoids. For example, the loss of reproductive features in many pheretimoid populations poses a challenge for identification, and techniques for individually tagging lumbricid earthworms have been less successful for megascolecids. The active and ongoing expansion of pheretimoid populations in many areas requires increased attention on distributed methods, such as citizen-science protocols, for detecting and tracking their expansion. Finally, the desire to limit populations of pheretimoids, including those invading gardens and other environments that might be successfully restored, has exposed the lack of options for targeted, effective control of unwanted earthworms. We identify opportunities to address these voids in our methodological tool kit and encourage the adaptation of techniques previously used in the study and management of other invasive animals.

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https://doi.org/10.1016/j.pedobi.2020.150669
Received 1 July 2020; Accepted 6 August 2020
Available online 16 August 2020

1. Pheretimoid biology and invasions

Pheretimoid earthworms of the family Megascolecidae refer to species in the *Pheretima* complex, including members of *Amynthas*, *Metaphire*, *Pheretima*, and related genera (Sims and Easton, 1972; Blakemore, 2009). Of particular interest is the set of approximately 20 pheretimoid species that have become widely established outside their native ranges. These pheretimoids, along with other earthworms that have become cosmopolitan, are commonly called peregrine species to reflect their apparent ease of introduction and the resulting pattern of global distribution (Lee, 1985). Pheretimoids are native to Australia and eastern Asia, but peregrine species have become invasive and abundant in portions of North America (Chang et al., 2016a), South Africa (Ljungström, 1972), South America (Mischis, 2004; Brown et al., 2006), and Oceania (Gates, 1959; Lee, 1981). In a sense, the diaspora of pheretimoid earthworms in many places represents a second wave of earthworm invasion (Chang et al., 2013; Szlavecz et al., 2018), following the first wave of European species in the family Lumbricidae (lumbricids) that began with colonization by European settlers (Hendrix and Bohlen, 2002; Hendrix et al., 2008).

Among the peregrine pheretimoids, *Amynthas agrestis*, *Amynthas tokioensis*, and *Metaphire hilgendorfi* have become widely distributed in the United States and are often found together (Chang et al., 2018). Certain characteristics of these co-invaders, including their annual life cycle (Kobayashi, 1937; Tandy, 1969, Fig. 1), large body sizes (Chang et al., 2016a), low predation or parasite pressure (Gorsuch and Owen, 2014), and broad diets (Bellitürk et al., 2015; Zhang et al., 2010), apparently allow them to quickly colonize and exploit many environments of temperate North America (Callaham et al., 2003; Görres et al., 2014). Cocoons of these species seem particularly resilient and long-lived (Nouri-Aiin and Görres, 2019), forming a bank of propagules that can persist for years during harsh weather or efforts to control populations. Pheretimoids are colonizing earthworm-free forests along with areas supporting existing populations of lumbricids (Snyder et al., 2011; Laushman et al., 2018; Moore et al., 2018; Szlavecz et al., 2018). In this report, we focus on techniques most relevant to the three co-invading pheretimoids of northern North America because of their potential ecological importance, broad and expanding distribution (Fig. 2), and uncertainties regarding their future invasion potential (Moore et al., 2018).

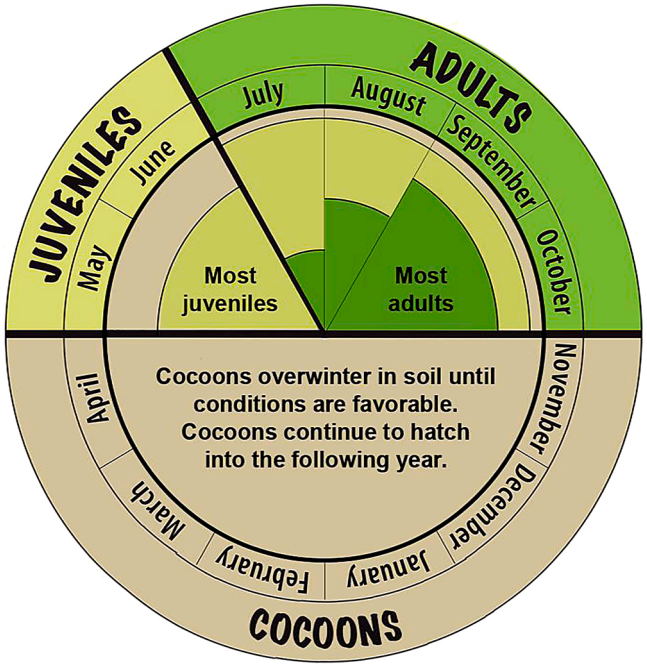

Fig. 1. The life cycle of *Amynthas agrestis*, *A. tokioensis* and *Metaphire hilgendorfi*. These three species share an annual life cycle in which they overwinter in the soil as cocoons, hatch in the spring (or when conditions are favorable), and mature from juveniles to clitellate adults over the summer months. Months of diagram are based on populations of jumping worms in the US Midwest (Wisconsin, Illinois); pie proportions are not drawn to scale.

The snake-like locomotion, surface-dwelling habit, and copious casts of pheretimoids cause dissatisfaction with infestations among landowners. In our experience in North America, European lumbricids are largely viewed positively by landowners whereas pheretimoids are viewed as harmful. Limited data suggests that invasive pheretimoids may cause less change in litter decomposition rate but greater change in aggregate structure than similarly sized litter-dwelling lumbricids (Greiner et al., 2012; Laushman et al., 2018). Consequences of pheretimoid invasion on soil function and biodiversity of the forest floor are poorly understood at present, but there is evidence that these earthworms are associated with diminished diversity and abundance of other litter-dwelling detritivores (Snyder et al., 2011). Public dissatisfaction, damage to turf (Potter et al., 2011), and ecological concern for the health of temperate forests (Greiner et al., 2012) have prompted research into methods for control of pheretimoids (Ikeda et al., 2015; Johnston and Herrick, 2019).

There is a critical need for basic information pertaining to peregrine pheretimoid earthworms. For example, our poor understanding of the thermal tolerances of these species has made predicting their potential for invasion of high latitudes difficult (Moore et al., 2018). The urgent need for more information on these invasive earthworms motivated our attempt here to compile relevant information on methods that can be used to study pheretimoids and to indicate gaps in our methodological tool kit. Recent reviews of methods in earthworm ecology have emphasized the value of simple research tools (Butt and Grigoropoulou, 2010) and integrating combinations of technologically advanced techniques (Bartlett et al., 2010). There is ample opportunity, we believe, to apply the full spectrum of available research tools in earthworm ecology to the pressing issue of pheretimoid invasion.

2. Field sampling

2.1. Designing sampling protocols for density estimation

The specific objectives of a field study dictate the most appropriate sampling design, but it is always desirable that samples accurately represent the populations from which they are drawn. Sampling soil animals is often difficult due to spatial and temporal variability and the ability of animals to escape detection (Lee, 1985; André et al., 2002). Earthworms are typically sampled within a two-dimensional area at the soil surface or a three-dimensional area extending to a certain depth. When a two-dimensional area is sampled, a complete sampling of the earthworms beneath that area is often implied or explicitly assumed. Procedures for the estimation of density and associated confidence intervals using these sorts of quadrat samples are provided by Krebs (1999).

Quadrats can be arranged randomly or systematically, and with or without stratification among predefined habitats, but care must be taken to avoid the human tendency to locate quadrats within easily sampled areas. Small-scale variability in earthworm populations is common and can dramatically affect estimates. Global Positioning Systems (GPS) or locally referenced sampling grids may be used to objectively position quadrats. The number and size of quadrats (i.e., the fraction of the study site sampled) should be related to the spatial variability in the sampled populations, which is often unknown before sampling. However, the precision of the density estimate likely will be a function of the total number of earthworms sampled within the quadrats (Krebs, 1999). So, sites with sparse populations will require more intensive sampling than sites with dense populations. Density can change dramatically throughout the year for some species (Görres et al., 2016), and sampling

intensity may be adjusted accordingly. Investigators have calculated density estimates of pheretimoids and their propagules within an invaded forest using as few as five 0.25-m^2 (0.04-m^2 for cocoons) quadrats (Görres et al., 2014, 2016, 2019).

2.2. Cocoon sampling

Estimation of cocoon abundance begins by excavating a known volume of soil. Most cocoons are contained in the casting layer, which is often about 5 cm deep. Görres et al. (2018) extracted cocoons from cylinders of soil 23 cm in diameter and 7.5 cm deep; Johnston and Herrick (2019) extracted large numbers of cocoons from cylindrical cores with a diameter of 7.6 cm and depth of 7.6 cm in heavily infested habitats. The soil collected at these depths may include underlying mineral material. In some areas the casting layer may be deeper, and the depth of the sampling may be fruitfully extended. Because abundances are usually reported per unit area of soil surface, the depth of the sample is ideally designed to include the layer that includes all cocoons, and the diameter of the sample should be large enough to accommodate many cocoons given the density of cocoons at the site.

Diameters of cocoons of common invasive pheretimoids in North America range between 2.0 and 4.5 mm (Nouri-Aiin and Görres, 2019). Cocoons dehydrate and change shape when the soil freezes and during drought conditions. So, it is prudent to condition soils first to room temperature and to moisten them before extraction. Sieves with mesh sizes around 1 mm are used to separate cocoons and materials of similar size from the remainder of the soil using a wet-sieving procedure (Blackmon et al., 2019). After placing the soil on the sieve, wetting and mechanical disruption allow soil to pass the mesh. Sieving does not achieve complete separation; organic debris and pebbles are also retained. Because viable cocoons are not buoyant, organic material can be floated away by submerging the sieve slowly in water.

Detecting pheretimoid cocoons after the separation process can be done directly on the sieve or after turning the sieve contents onto a tray. White trays provide the best contrast. Visual and tactile cues can both be employed to detect the cocoons. Pheretimoid cocoons are nearly spherical (Fig. 3). Thus, when running fingertips over the retained fraction, pheretimoid cocoons will feel like tiny ball bearings. Cocoons may be visually identified based on size, shape, and color. A lighted magnifier can greatly assist in this process. Once detected, cocoons are best removed using forceps. A step-by-step description of the protocol to separate cocoons from soil is provided in Appendix A.

2.3. Sampling (hatched) earthworms

Density of juvenile or adult pheretimoids can be estimated by enumerating organisms within quadrats, as defined above. A variety of techniques for extracting earthworms from the soil have been devised, spanning the gamut from applying electrical fields to hand removal (Singh et al., 2015). Most invasive pheretimoids are epi-endogeic, living close to the soil surface. Thus, they are often easily brought to the surface with extraction methods and removed. Techniques that have been used to sample pheretimoids within a fixed area include electrical extraction (Snyder et al., 2011), chemical extraction (Bernard et al., 2009), and hand sorting (Görres et al., 2016).

The efficiencies of both electrical and chemical extraction depend on soil moisture, which may lead to seasonal biases (Eisenhauer et al., 2008). Chemical extraction additionally can cause changes in soil chemistry, which may affect other ecological work. Hand sorting is especially suitable for epi-endogeic pheretimoids because they occupy soils only to a shallow depth and deep excavation is not necessary as it is for other species. Görres et al. (2016) excavated quadrats to a depth of 10 cm to sample pheretimoids in Vermont. Animal depth will vary depending on soil moisture, temperature, and perhaps other factors.

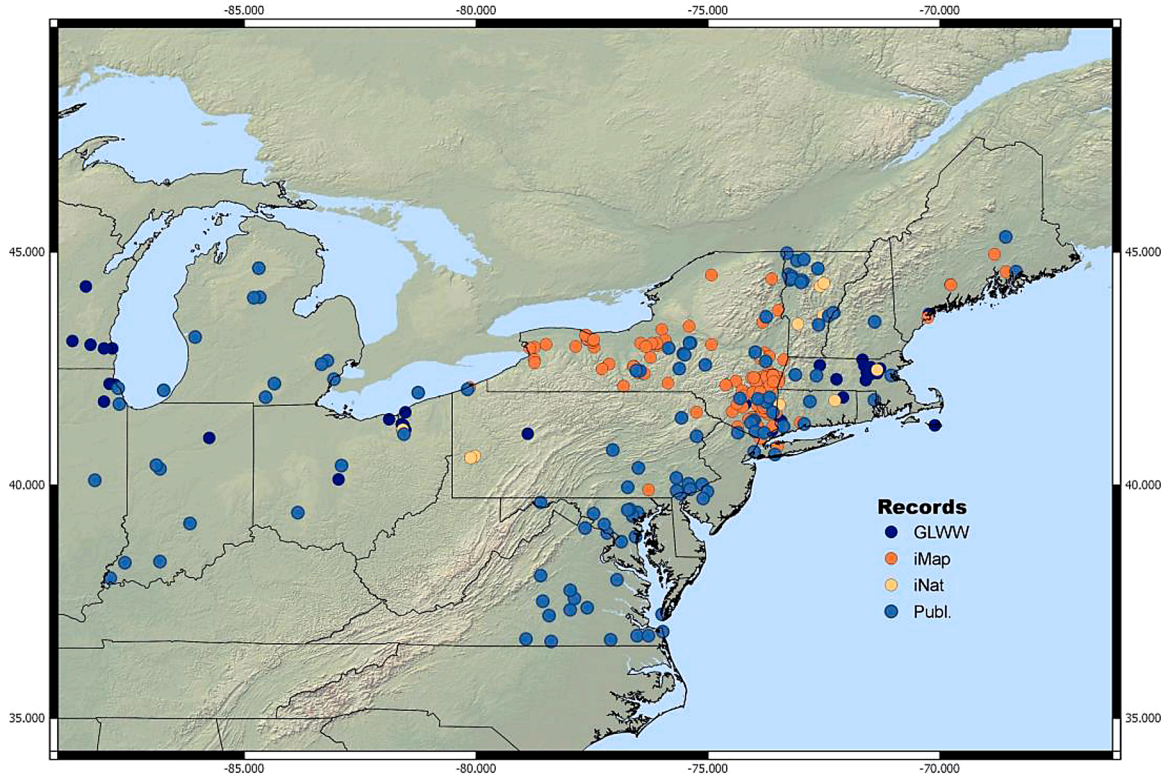

Fig. 2. Locations of records of pheretimoid earthworms in the Northern United States and Canada based on published records (Publ.) and reports from citizen-science efforts (iMapInvasives.org [iMap], iNaturalist.org [iNat], and Great Lakes Worm Watch [GLWW]). Citizen-science data were accessed 30 May 2020. Note that these species have a broader distribution than represented here and that the reported locations were strongly dependent on spatially patchy monitoring and research efforts.

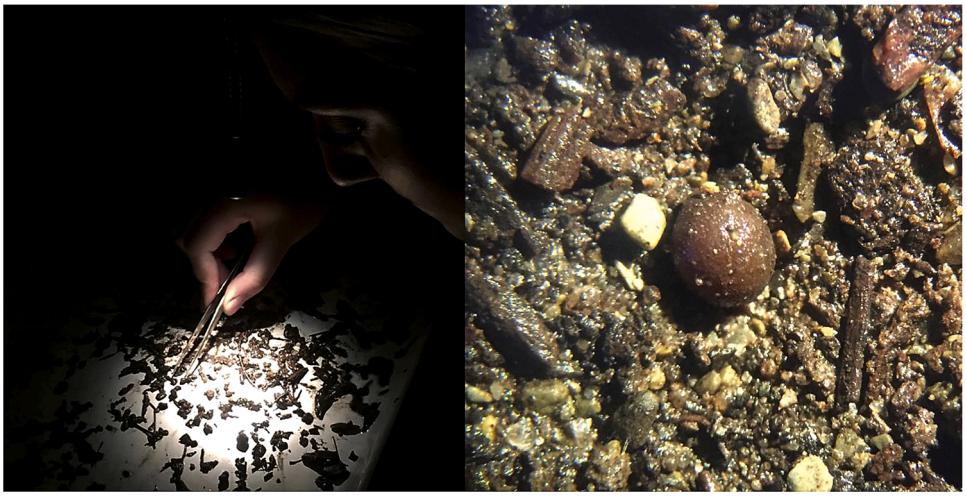

Fig. 3. Investigator sorting sifted material for pheretimoid cocoons (left) and single cocoon of Metaphire hilgendorphi under 10X magnification (right). Photo credit Sierra DeAngelo.

When hand sorting, it is essential to examine both leaf litter and the underlying mineral soil. This is especially important for juvenile earthworms and when litter is moist. We recommend the use of hand sorting for pheretimoids when possible.

When sampling pheretimoids to detect presence, but not to estimate density, hand collection and pitfall sampling (Callaham et al., 2003) may be used. We use the term "hand collection," rather than "hand sorting," to indicate the manual collection of animals from the soil surface and under physical structures such as rocks and logs. Hand sorting is always conducted within defined sampling areas and is intended to be exhaustive; whereas, hand collection is usually conducted opportunistically. Although the haphazard nature of hand collection typically precludes density estimation, it can be used to establish presence and estimate species richness of earthworms (Pinder and Robinson, 2019). Pheretimoids sometimes will emerge from the soil for hand collection if a tool (e.g., pitchfork) is inserted into the soil and agitated rhythmically. Hand collection protocols can be standardized to allow site comparisons (Pinder, 2013).

Pitfall traps, which may consist of small (e.g., 500 mL) containers partially filled with preservative, collect worms that are travelling along the soil surface from within an unknown sampling area. Pheretimoids often travel on the soil surface, especially at night during or after rains, and are readily collected in pitfall traps. These traps also can lead to the by-catch of vertebrate animals, including salamanders and shrews, which may be a conservation concern. Callaham et al. (2003) identified *Amynthas agrestis* across a large area of the Southern Appalachians using the by-catch from pitfall traps that were used for sampling other animals.

3. Processing living animals

3.1. Identification of cocoons

There are no keys for the identification of pheretimoid cocoons. Rearing cocoons to maturity may be an option, but this may bias estimates of species composition if survival and hatching rates differ among species. At a Vermont, USA site inhabited by *Amynthas tokioensis* and *A. agrestis*, *A. tokioensis* cocoons were much smaller than *A. agrestis*, and cocoon size distribution was bimodal (Nouri-Aiin and Görres, 2019). In cases like this, fitting probability (e.g., Normal) distributions to the empirical size distributions might allow for a cocoon to be attributed to a species with estimated probability. Invasive jumping worm populations at different locations differ significantly in body size, which often restricts the application of cocoon size in species determination. Characterization of pheretimoid cocoons based on other characteristics (e.g., color, shape, surface texture) has not been sufficiently developed to allow species discrimination, but we see potential for this. Cocoons contain sufficient DNA for identification by genetic barcoding (see the sections below on molecular analyses).

3.2. Identification of living earthworms

Identifying live specimens is possible for people who have learned the basics of pheretimoid earthworm identification. Although the total number of pheretimoid species is large, historical records and published regional keys and checklists can greatly narrow the range of possibilities. For instance, in the USA and Canada, 16 pheretimoid species have been reported (Chang et al., 2016a). However, only about half of the 16 species can survive in the temperate part of the two countries, and so in northeastern North America there are relatively few species in the regional species pool, making it possible to identify many live specimens in the field.

Live specimen identification relies heavily on genital markings (or papilla), the number of spermathecal pores, and the morphology of male pores. This requires that the specimens are adults. Color and size are useful as well, and are usually the first thing people look at, but these two characters vary intraspecifically and there are considerable interspecific overlaps. Some species are thinner than others (e.g. *Amynthas corticis* versus *A. agrestis*), but recognizing these differences usually requires experience. While many pheretimoids "jump" when disturbed, some do not. For instance, *Amynthas hupeiensis* typically coils, rather than thrashes or jumps.

In the field, after the first impressions of body size, shape, and color, species identification of live specimens, when possible, is usually achieved by examining the spermathecal pore area and the male pore area. The male pore area, which is on the ventral side of segment 18, hosts a pair of male pores and associated genital markings. The shape, size, location and arrangement of these structures with respect to the male pores usually differ among species. Many species are parthenogenetic, however, and their male pores and the associated genital markings may be completely absent. Spermathecal pores are located anterior to the clitellum, usually between segments. Most species have a fixed number of spermathecal pores at specific locations. For instance, *Amynthas corticis* has four pairs of spermathecal pores in intersegmental furrows 5/6/7/8/9; whereas, *Metaphire hilgendorfi* has only two pairs in 6/7/8 (Chang et al., 2016a). In both species, unique genital markings can also be found in the same area. In some species, such as *M. hilgendorfi*, these genital markings provide the most efficient and reliable way for species

identification.

It is generally not possible to identify live juvenile pheretimoids. However, genital markings may start to take shape before the male pores and clitellum appear. In the case of *M. hilgendorfi*, the unique preclitellar genital markings on one or some of the 7th-11th segments quite often allow unambiguous species identification for juvenile specimens. Additional details regarding species identification are available in the section below titled *Identification of Sacrificed Earthworms*.

3.3. Anesthesia

Anesthesia may be necessary for handling live worms with the objective of marking or otherwise manipulating them. A solution of 60 % (by volume) carbonated water (sold as "club soda" or "sparkling water") is an effective anesthetic, as is 10 % ethanol. Earthworms are placed in the anesthetic solution for 5 min and then rinsed in water before beginning the next protocol. The effects generally last 5−10 min. Anesthetized worms do not respond with the typical contraction to disturbances, such as tapping their tails or heads with forceps. Further details are given by Shannon et al. (2014).

3.4. Marking and tracking

The marking of individual earthworms, usually for purposes of studying their movement, is difficult due to their small size, cryptic lifestyle, and tendency to eject foreign objects from their bodies (Mathieu et al., 2018). Three techniques show promise in allowing the persistent tagging of pheretimoids: visual implant elastomers (VIE; González et al., 2006; Butt and Lowe, 2007), radio frequency identification (RFID) tags (Mathieu et al., 2018), and coded wire tags (CWT; Jefferts et al., 1963).

VIE tagging is most well-developed in earthworms and is very reliable in lumbricids (Butt et al., 2009) and rhinodrilids (González et al., 2006). Our experience with VIE, however, has suggested much lower reliability in megascolecids. In particular, we have observed a nearly universal tendency for VIE material to migrate and be ejected, something that happens only occasionally in lumbricids (Butt et al., 2009). We and others (Mathieu et al., 2018) have successfully implanted earthworms with RFID tags and used this tagging to identify individuals in the lab. As in VIE tagging, however, we noticed a much higher rate of tag ejection in megascolecids than in lumbricids. Currently, the smallest RFID tags we have found (Beijing Raybaca Technology Co., Ltd., Beijing, China) are 1.25 × 7 mm, which restricts their use to only the largest pheretimoid individuals. CWTs are small (0.25X × 1.1 mm) injectable magnetized pieces of stainless-steel wire that are etched with a unique number. Our experience indicates that these are much less likely to be ejected than VIE or RFID tags. Animals can be identified as marked without sacrificing them; however, the tag must be removed to read the unique number. More work is necessary to evaluate the efficacy of these methods for marking pheretimoids.

3.5. Depuration

We use the term "depuration" to mean the removal of materials from within the gut of earthworms. Depuration is necessary for many analyses, such as mass-dependent or chemically sensitive measurements, which may be confounded by the soil or other materials in the alimentary canal. Typical methods for live depuration begin with thoroughly rinsing earthworms in deionized water and then placing them individually in Petri dishes with filter papers or other porous media. Media should be moistened, but not saturated, with deionized water. Earthworms are kept in the Petri dishes in constant darkness while the soil is being evacuated from their alimentary canal. To prevent the ingestion of their own casts, media should be changed every 24 h. The length of time for complete depuration ranges from 12 h to 7 d, depending on gut transit time and earthworm size (Arnold and Hodson, 2007; Ernst and Frey, 2007; Nahmani et al., 2007; Bade et al., 2012).

For pheretimoid species, we recommend 48 h for small species, such as *Amynthas tokioensis*, and up to 72 h for large species, such as *M. hilgendorfi*. Richardson (2019) observed that *M. hilgendorfi* were not fully depurated after 48 h. For larger earthworms, depuration may need to be complemented or replaced by physical removal of soil particles through dissection, in which a longitudinal cut is made along the alimentary canal and soil particles are detached with a small brush or washed with deionized water (Arnold and Hodson, 2007). Although physical removal can be very effective, passive depuration can be more time-efficient for large numbers of earthworms and can prevent loss of sensitive tissues (Arnold and Hodson, 2007).

For molecular analysis of whole-body tissue, soil particles can be removed by centrifugation of the previously frozen and powdered tissue of the earthworm, avoiding the use of depuration. Moreover, in the case of transcriptomic analyses, gene expression may change while earthworms are in the dishes, and contaminated RNA-seq reads can be removed *in silico* afterwards. Depuration can also be helpful for study of the animal microbiome, where removal of the soil can be useful to better assess and understand bacterial communities within earthworm tissues and not adhered to ingested soil particles (Pass, 2015). However, analysis of soil samples and the active transient microbiome is also important for understanding the comprehensive earthworm phenotype (Pass et al., 2015).

4. Processing specimens

4.1. Preservation and fixation

Long-term storage of earthworm specimens requires preservation with alcohol and sometimes fixation with formalin. Preservation with ethanol or isopropanol do not render tissues firm and resistant to softening over long periods of time, but formalin does. Fixation with formalin ensures that specimens taken as vouchers will be morphologically identifiable for a long time, assuming correct long-term care. On the other hand, formalin is toxic and irritating, must be handled with care, and it depurinates DNA. Fixed specimens will retain small amounts of formalin through multiple changes of alcoholic preservatives. Specimens preserved in ethanol at high concentrations (90–95 %) without fixation are stable in the fluid but fragile during dissection, making identification a little more difficult. Specimens can degrade badly if the alcohol concentration gets too low through evaporation or errors in mixing. The details of preservation and fixation for pheretimoids do not differ from preservation and fixation of other earthworms. See Appendix B for details of preservation and fixation protocols and Appendix C for instructions on preparation of the fixative.

4.2. Identification of preserved earthworms using morphology

Detailed examinations of the external and internal anatomy are useful for learning about variation in populations, presence or absence of reproductive organs and external genital features, as well as being necessary for positive identification. Our preferred method of examination is to use fixed specimens (see above) after they have been cycled through two or more changes of alcohol preservative. Specimens should be placed in a shallow dish (Appendix D) and covered with tap water or a dilute (≤ 50 %) alcohol solution, to prevent desiccation of the specimen and improve visual clarity. Good ventilation is advised during microscopy (Appendix E).

Characters of the external anatomy to note in pheretimoids include the following: pigmentation, number of segments, body dimensions, genital markings, clitellum location and shape, and locations of dorsal, spermathecal, female, and male pores. If male pores are within an invagination of the body wall, that is an important distinction. One can count numbers of setae per segment in several segments, typically the 7th or 8th, 20th or 30th, and the number of setae between the male pores

on segment 18. Some asexual morphs will lack some or all of the pores mentioned, other than the dorsal pores. Certain characters, such as the shape of the spermathecae, require internal examination. Dissection procedures for pheretimoids do not markedly differ from dissection procedures for other earthworms (Appendix F).

A key to North American pheretimoids is provided by Chang et al. (2016a). Species found in South Africa are listed by Plisko (2010). A key to the genera of South American megascolecids is given by De Assis et al. (2017). Blakemore (2010) includes a key to peregrine earthworms of the world, including peregrine megascolecids. Additional regionally specific aids to identification would help scientists and land managers in early detection and monitoring of pheretimoids.

4.3. Cocoon dissection and embryonic development

Cocoons can be dissected under a dissecting microscope, using two fine forceps. One set of forceps is used to pierce cocoon membranes. Then together with another set of forceps the cocoon is gently pulled apart. Care has to be taken not to disrupt the embryo. Study of early life stages of pheretimoids as components of the life cycle can be done under a dissecting microscope. For description of the embryonic development of *Amynthas agrestis*, a classification system (Table 1) has been described by Nouri-Aiin and Görres (2019), which was adapted from Boros et al. (2008). The stage with no visible embryo (stage one) may reflect a non-viable condition (Johnston and Herrick, 2019) or an early stage of cell division (Nouri-Aiin and Görres, 2019). A lengthy incubation period may be necessary to confirm viability given that cocoons are apparently viable for more than one year.

4.4. Processing earthworms for elemental analyses

For elemental analysis or stable isotopic composition (such as ^{15}N or ^{13}C) of earthworm tissues, individuals are first washed to remove soil particles. Animals may be depurated alive or using dissection (see *Depuration* section above). Animals should be euthanized by dipping in boiling water for 1−2 s or freezing to avoid contamination with chemical killing agents. Specimens may be stored frozen until time allows for dissection and removal of gut contents if the dissection method of depuration is to be used. Tissues are then frozen to −20 °C, −40 °C (Richardson, 2019), −70 °C (Dang et al., 2015), or in liquid nitrogen (Ireland, 1975) and lyophilized (freeze-dried) to a constant mass. Drying an earthworm in a convection oven can cause the formation of recalcitrant organic compounds that are difficult to digest with strong acids and may produce pungent odors. After freeze-drying to a constant mass, earthworm dry weight should be recorded and earthworm tissues may be ashed at 550 °C or can be digested directly.

Specimens (whole earthworm, sections, or individual organs) used in isotopic analyses should be ground to a fine powder. Dried, homogenized powders in sealed vessels are recalcitrant and can be submitted for analysis by continuous-flow isotope-ratio mass spectrometry (Snyder et al., 2009; Melody and Schmidt, 2012; Chang et al., 2016b). For inorganic elemental analyses, digestion methods typically entail strong acids (e.g. 15.8 M nitric acid, 18.4 M sulfuric acid or 15.2 M *aqua regia*) with or without oxidizers (e.g. H_2O_2), and under pressure (e.g. sealed chambers in a microwave digestion system such as in USEPA Method 3051; Richardson et al., 2015; Wang et al., 2018). Earthworm tissue digests should be diluted and may be analyzed by Inductively Coupled Plasma-Optical Emission or Mass Spectrometry or Atomic Absorption Spectroscopy.

Table 1
Embryo characteristics for classifying stage of embryo development according to Nouri-Aiin and Görres (2019). Stages 4 and 5 are regarded as ready to hatch.

Stage	Elongation	Segmentation	Pigmentation	Other
1				No embryo visible, albumin white to clear; if yellow likely not viable
2	< 2.5 mm	Near head	none	Aspect ratio ~ 2
3	>2.5, <6 mm	Throughout, but faint	none	
4	>7 mm, < 12 mm	Clear segmentation	none	
5	> 12 mm	Clear segmentation	pigmented	Looks like hatchling

4.5. Processing earthworms for molecular analyses

Molecular analyses may be based on whole animal samples, which obviously require that the animal is sacrificed. However, analyses also may be based on a small sample of the tail (a "tail snip" ≥ 3 mm long or ~ 20 mg), secreted coelomic fluid, or secreted mucus. In these cases, samples may be taken without sacrificing the animal. Secreted coelomic fluid can be collected by placing earthworms in sterile autoclavable polypropylene bags. Using ethanol precipitation, DNA can be extracted from 50 µl of secreted coelomic fluid in the bag; desalted and concentrated DNA is then dissolved in Tris–EDTA buffer and stored at −20 °C (Minamiya et al., 2011). DNA also may be isolated from small pieces of Whatman FTA cards that have collected mucus from traversing pheretimoid earthworms (Minteer and McHugh, Unpubl. data). Before extracting DNA or RNA from embryos, it is advisable to wash cocoons in 10 % bleach for 30 s, rinse in distilled water and then dry.

For isolation of DNA or RNA, worm tissue should be fluid preserved in 100 % ethanol or RNALater (SigmaAldrich), or be flash frozen with dry ice or liquid nitrogen. For DNA analyses, ethanol preservation usually is sufficient; animals are sacrificed in ethanol and then individually placed in tubes with fresh ethanol at -20 °C. It is advisable to change ethanol again after about one week to avoid dilution. For RNA isolation, two options are available. Firstly, tissue can be stored in RNALater. For storing of entire animals, especially large individuals, it is recommended to make a dorsal cut in the worm to allow the RNALater solution to perfuse the animal. Samples may be stored at ambient temperature or ~ 4 °C (basic refrigeration) for up to several days but should be transferred to -20 °C for longer term storage. Fluid preservation permits the dissection of different tissues from individual earthworms to be analyzed separately, which is very difficult to do with frozen samples. As a second option for RNA isolation, animals can be flash frozen in dry ice or liquid nitrogen and then stored at -80 °C. Depending on the purpose of the study, earthworms may be depurated beforehand, taking into account that gene expression may change during this time. Alternatively, frozen tissues can be homogenized with a pestle and mortar so that 25−50 mg can be suspended in 1.5 mL of TRIzol (Invitrogen) and centrifuged (12,000*g* at RT for 5 min) to eliminate most of the soil within the animal, with the supernatant then used for isolation.

Using readily available commercial kits, tissue or cocoon digestion and DNA isolation is routine and deviations from manufacturers' protocols are not usually necessary. Standard kits with spin columns are recommended (e.g., DNeasy Blood & Tissue Kit [Qiagen]), because they generally avoid inhibitors of PCR. Total RNA can be isolated using commercially available kits (e.g., RNAwizTM [Ambion] or RNeasy Mini Kit [Qiagen], see Novo et al., 2015a) and reverse transcribed to cDNA (e.g., SuperScriptTM II [Invitrogen]) to eliminate highly variable introns.

4.6. Analyses of mitochondrial and nuclear genes

Analyses of mitochondrial or nuclear genes can reveal the origins and phylogeography of invasive species, and studies of variation at the genomic level are key in understanding population genetics, mating systems, and adaptations of invading populations (Pearse and Crandall, 2004). The study of invasive pheretimoids has benefitted from the application of molecular analyses (e.g., Novo et al., 2015b; Schult et al., 2016), but much remains to be done, and the use of molecular

techniques for understanding invasions of pheretimoid species is in the early stages.

Fragments of mitochondrial genes or nuclear ribosomal genes can be amplified using standard PCR protocols (e.g., Novo et al., 2015b; Schult et al., 2016) with universal primers (e.g., Folmer et al., 1994 for cytochrome oxidase I [COI]; Perez-Losada et al., 2009 for 16S rRNA; Colgan et al., 2000 for nuclear Histone 3). For the commonly used COI barcode fragment, degenerate primers have been designed to be used for megascolecids in general, and pheretimoids in particular (Schult et al., 2016):

MEGA-Forward: 5′-TAYTCWACWAAYCAYAAAGAYATTGG- 3′

MEGA-Reverse: 5′-TAKACTTCTGGRTGMCCAAARAATCA- 3′

For nuclear coding genes, amplification from cDNA is necessary if the inclusion of highly variable introns is to be avoided; the PCR methodology is generally the same in this case, although touchdown PCR and a second round of amplification may be required depending of the specificity of primers available for the targeted gene (e.g., Struck et al., 2011). This could be helpful to study variability of genes with key functions in relation with environmental variables. Given the use of universal or degenerate primers for either mitochondrial or nuclear markers, it is highly recommended that forward and reverse primers have specific 18-nucleotide-long M13REV or M13(-21) sequences added to aid in direct sequencing of PCR products from genomic or cDNA (Regier and Shi, 2005).

PCR amplification with a mix of species-specific primer pairs can allow species identification without sequencing of amplified fragments (e.g., Koester et al., 2013; Zhang et al., 2016). With this technique, each primer pair in a mix of primers generates a characteristically sized fragment of amplified DNA for a given species; thus, it is sufficient to examine amplicon sizes using agarose gel electrophoresis, because of the species-specific variation in amplicon length. This technique can be used in species-level identification of pheretimoid DNA extracted from tissue, cocoons, coelomic fluid or mucus, with the possibility of false negative or false positive results being excluded by including appropriate controls. Keller et al. (2017) developed a PCR-based method to distinguish between *Amynthas agrestis* and *A. tokioensis* based on PCR fragment sizes amplified by a mix of species-specific primers. This method has been extended to include *M. hilgendorfi* (Nouri-Aiin et al., in review) and can be adapted to include more species.

4.7. Analysis of variation across genomes

Microsatellites provide a reliable and consistent source of easily available genomic-level variation that can be used in analyses of dispersal patterns, population admixture and reproductive strategies, in addition to testing hypotheses about any adaptation mechanisms that might explain observed distribution patterns of invasive pheretimoid species. Cunha et al. (2017) presented a large set of primers for microsatellite amplification for *Amynthas corticis* and provided full details of the pipeline for primer design from low coverage genomes and validation of the markers. Additional microsatellite resources are being developed for *A. agrestis, A. tokioensis* and *M. hilgendorfi* by M. Nouri-Aiin. If microsatellite primers are available for the species of choice, amplification of microsatellite loci proceeds as per standard PCR methods (e.g., Novo et al., 2008). Forward primers may be tailed with an M13 sequence at their 5′end, and a universal fluorescent-labelled M13 primer is added to the reaction. This eliminates the need to label every primer with fluorescence, thus lowering the cost substantially (see Schuelke, 2000). Analysis of amplicons requires precise sizing using a size standard (eg., Genescan Rox 500) and automated fragment analysis (e.g., GeneScanner [Applied Biosystems]) and scoring (e.g., Peak ScannerTM Software 2 [Applied Biosystems]). Full analysis of genetic structure can then be undertaken using the microsatellite genotypes, with consideration given to effects of ploidy level on such analyses as corrected by, for example, GenoDive (Meirmans and Van Tienderen, 2004).

With recent advances in Next Gen Sequencing (NGS), the scoring of variation in hundreds of thousands of Single Nucleotide Polymorphisms (SNPs) for specimens across multiple populations is becoming routine, and such data would be invaluable in assessing fine-scale population structure, dispersal, and adaptation in invasive pheretimoids. Methods like Genotype by Sequencing (GBS) and Restriction Site Associated DNA (RAD) markers use restriction enzymes to reduce genome complexity and genotype multiple DNA samples, thus providing information on SNPs. These, and other similar methods are reviewed in Davey et al. (2011) and have been applied in some earthworm species (Giska et al., 2015; Anderson et al., 2017).

4.8. Further useful molecular analyses

Transcriptome analyses could help unravel the molecular mechanisms by which invasive earthworms adapt to many different habitats. Construction and sequencing of cDNA libraries can provide data to study gene expression patterns under different environmental conditions (e.g., in earthworms, Novo et al., 2015a) as well as to analyze selection signatures in protein coding genes (Hawkins et al., 2019). Molecular techniques have also been used to assess the microbiota associated with earthworms, with recent application of high throughput sequencing in high resolution analysis of bacteria associated with the extremophile pheretimoid *Amynthas gracilis* from volcanic soils of the Azores (Pass, 2015).

5. Experiments and manipulations

5.1. Laboratory microcosms

Studies of the survival and reproduction of pheretimoid earthworms often use laboratory microcosms in the form of plastic pails, pots, or shoebox size tubs (Table 2). Laboratory studies of competition between pheretimoids and other species have been facilitated with microcosms (Zhang et al., 2010; Snyder et al., 2013; Chang et al., 2016b). Containers need tight-fitting lids to reduce the chance of escape. Larger pheretimoids (e.g., *M. hilgendorfi*) can push a glass Petri dish cover out of the way during escape attempts. Ventilation apertures must be kept small; earthworms can squeeze through openings much smaller than their diameter at rest. Soil should be kept at or close to water-holding capacity and checked frequently throughout the rearing period to avoid desiccation (Blackmon et al., 2019). Soil moisture content (by mass) has been maintained at 38 % (Chang et al., 2016b), 30 % (Greiner et al., 2012) and 20–25 % (Bernard et al., 2009). Rearing earthworms at room temperature (22 °C) seems to be adequate for survival and reproduction of most pheretimoids (Ikeda et al., 2015; Blackmon et al., 2019).

Ikeda et al. (2015) found that pheretimoid earthworms, reared in soil with litter that had been passed through a 4.75 mm standard sieve,

Table 2
Specifications of laboratory microcosms used in studies of pheretimoid survival and reproduction.

Type of Container	Dimensions (cm)	Inds Unit^{-1}	Surface Density (inds m^{-2})	Species	Reference
Pipe, PVC	10.2 (d) x 8 (h)	1	122	*A. agrestis*	Bernard et al. (2009)
Terraria, Plastic	17.5 (l) x 14.5 (w) x 4 (h)	10	394	*M. hilgendorfi*	Greiner et al. (2012)
Pot, Plastic	8.8 (d) x 14 (h)	2	329	*A. agrestis*	Ikeda et al. (2015)
Pipe, PVC	10.2 (d) x 15 (h)	2	244	*A. agrestis*	Richardson et al. (2009)
Container, Plastic	20 (l) x 12 (w) x 6 (h)	4	167	*A. agrestis*	Ziemba et al. (2015)

produced many cocoons. Johnston and Herrick (2019) similarly used dried fragmented litter (< 5 mm) to produce cocoons from *Amynthas tokioensis* and *A. agrestis* with similar success. They found that adding approximately 1 g and 3 g of litter per individual, for each species respectively, every 2–3 days was needed to maintain feeding rates. Microcosms are often provisioned with litter collected from the site of earthworm collection, which in a way ensures suitability. Captive pheretimoids also have been successfully cultured with a mixture of oak and maple (*Fagus*, *Acer*; Johnston and Herrick, 2019), apple (*Malus* sp.; McCay, Unpubl. data), basswood (*Tilia americana*; Greiner et al., 2012), tulip poplar (*Liriodendron tulipifera*; Chang et al., 2016b), and white pine (*Pinus strobus*; Richardson et al., 2009). *Amynthas agrestis* and *Metaphire hilgendorfi* have demonstrated large variability in food habits, which may aid in colonization success and dispersal (Zhang et al., 2010; Bellitürk et al., 2015; Chang et al., 2016b).

5.2. Field mesocosms and manipulations

Given increasing concerns regarding the effects of pheretimoid invasion in forests of North America, there have been some experimental manipulations in these ecosystems to evaluate the effect of jumping worms on their habitats. Moore et al. (2013) studied controlled populations of *Amynthas gracilis* (reported as *A. hawayanus*) in limed and un-limed soils at the northern limit of northern hardwood forest in North America using plastic pails (29 × 50 cm) with holes drilled in the bottom to allow heat, water, and gas exchange with the surrounding soil. In Michigan, effects of *M. hilgendorfi* on soils were studied using 1.2- x 1.2-m field enclosures constructed of medium density fiberboard (Greiner et al., 2012). Enclosure walls were 60 cm high, of which 15 cm were buried beneath the soil. Bowe et al. (2020) successfully excluded and contained pheretimoids with extra fine-gauge (0.4 mm) noseeum netting in field mesocosms. Ziemba et al. (2016) studied pheretimoid use of artificial cover objects (ceramic tiles) and their co-occurrence with native salamanders under these objects.

6. Management and control

6.1. Early detection and monitoring

Early detection of a newly invading organism requires that there are sufficient observers trained to identify the non-native species. As there are only a limited number of researchers and extension workers who are dedicated to pheretimoid research, only a few areas have good pheretimoid-distribution data. Clusters of sightings apparent on distribution maps are often associated with active research and monitoring groups (e.g. Fig. 2). Citizen-science efforts (e.g., *i*MapInvasives.org) have the potential to reduce this bias, filling gaps in geographic coverage. However, these efforts can be complicated by problems with identification and classification. For example, categories used by citizen-science platforms are sometimes ambiguous (e.g., "jumping worms") or misleading ("*Amynthas*" as a synonym for jumping worms).

In the USA, Extension Master Gardeners (EMGs; https://mastergardener.extension.org/) are excellent groups to train to identify pheretimoids. EMG hotlines now receive frequent calls that report pheretimoids and thus have an interest in this group of invasive species. Bellitürk et al. (2015) conducted a survey of EMGs and Extension Master Composters in Vermont, New Hampshire and Connecticut. Based on over 300 responses, almost all counties in these three states had pheretimoids. Sustained citizen science initiatives in North America are WormWatch Canada and Great Lakes Worm Watch whose volunteers might serve as early detectors. OPAL (Open Air Laboratories, www.opalexplorenature.org) is an effort that encourages citizen scientists in the United Kingdom to report information on earthworms and soils. These three initiatives are similar in that they have earthworm keys that are accessible to non-specialists.

6.2. Tracking expansion

At this point, many pheretimoids are well established outside of their native ranges, and eradication and containment are not possible. Successful management of these populations and mitigation of their negative effects will be based on knowledge of the factors that promote population growth and spread. Unfortunately, pheretimoid invasion rates and dynamics are still poorly understood. European lumbricid earthworms disperse relatively slowly (4–30 m year^{-1}) and are mainly spread through human transport (Marinissen and van den Bosch, 1992; Terhivuo and Saura, 2006) and during large storm events (Schwert and Dance, 1979; Moore et al., 2017). We believe that pheretimoids spread similarly, though intrinsic dispersal may be more important and human transport may be more dependent on gardening and land management practices and less dependent on fishing bait use.

Understanding the spatial distribution and spread of pheretimoids must be accomplished at multiple scales (local and regional), both because their distribution is inherently patchy (Burtelow et al., 1998), and because different tools can be used for management at different scales. Citizen science platforms such as *i*MapInvasives (iMapInvasives, 2020), EDDMapS (Bargeron and Moorhead, 2007), and Map of Life (Jetz et al., 2012) are tools available to collect and share these data. Concentrated data collection events such as the *i*MapInvasives Mapping Challenge link citizens, practitioners, and scientists in a webinar format to learn how to identify earthworm behavior and soil observations that make differentiation of lumbricid and pheretimoid earthworms easily accessible to citizen scientists. The connectivity between researchers and citizen scientists has been vital to maintain the quality and quantity of submissions. Some of the electronic tools that track invasive species require that the organisms are recognized as invasive species at the state level, which is not always the case. Presently, only a few US states (e.g., New York) regulate the use or release of jumping worms.

6.3. Physical control

Adult epi-endogeic pheretimoids, such as *Amynthas agrestis*, *A. tokioensis* and *M. hilgendorfi*, are usually conspicuous and spend time at the soil surface, making hand collection possible. However, it is exceedingly difficult to remove the entire population from even a small area by hand. Although many individuals may be conspicuous at the surface, a fraction of the population will burrow deeply, especially in dry conditions. An additional challenge is that a large proportion of pheretimoid populations is in the cocoon form at any one time. In recent studies it has been shown that there are some embryos ready to hatch year-round (Nouri-Aiin and Görres, 2019; Johnston and Herrick, Unpubl. data). Hand collection can be made more effective with application of a vermifuge. Hand collection, even with the use of vermifuge, is impractical over large areas. However, in nurseries and horticultural installations this may be an alternative to applying chemical controls.

Ikeda et al. (2015) found that adult *A. agrestis* showed some mortality when subjected to prescribed fire; however, there was no difference in the mean number of live adult earthworms in burn plots relative to unburned plots. In contrast, cocoon viability significantly declined in burn plots, suggesting that prescribed fire could offer a practical treatment to reduce the population of *A. agrestis* over time. In a laboratory experiment, Johnston and Herrick (2019) found that no cocoons of *A. agrestis* or *A. tokioensis* were viable after being subjected to 40 °C or above compared to ambient temperature for a minimum of three days. Identifying the thermal limits of cocoons could open up opportunities for treatments including composting parameters, solarization, and steam sterilization.

Diatomaceous earth is the skeletal remains of diatom algae that is marketed as a method of insect control. Although its abrasive properties make it feasible that it might cause earthworm mortality, there currently is no evidence of vermicidal action. Other abrasive mineral materials, however, have shown some effect on earthworms. These include angular

zeolites and slag particles, which have been tested on turf surfaces (Williamson and Hong, 2005). Herrick and Johnston (Unpubl. data) found that mortality rates of adult *Amynthas agrestis* and *A. tokioensis* were not significantly different between diatomaceous earth-amended soil and control after 7 or 14 d. No studies of the impact of diatomaceous earth or other angular materials on pheretimoid earthworms have been published.

6.4. Chemical control

There are no chemical pesticides currently certified for earthworm control, but several common pesticides are known to kill European lumbricid earthworms (Seamans et al., 2015; Boyle, 2018). Roberts and Dorough (1984) tested 90 chemicals against *Eisenia fetida* and rated them in categories from "supertoxic" to "relatively nontoxic." Carbofuran and eserine salicylate were supertoxic, whereas the remaining chemicals were distributed equally among the other categories. Dalby et al. (1995) found that 2,4-D as well as glyphosate and dimethoate had no effect on the growth or survival of *Aporrectodea trapezoides*, *Ap. rosea*, *Ap. caliginosa*, or *Ap. longa*. Potter et al. (1990) found that a single application of the fungicide benomyl or the insecticides ethoprop, carbaryl, or bendiocarb reduced populations of *Ap. caliginosa*, *Ap. trapezoides*, *Lumbricus terrestris*, and *Eisenia* sp. by 60–99 %. None of the herbicides tested (2,4-D, dicamba, triclopyr, and pendimethalin) significantly affected earthworms. Additionally, nitrogen fertilizers in turfgrass systems can cause a significant decrease in density and biomass of lumbricid earthworms (Potter et al., 1985).

Preliminary work indicates that vinegar and baking soda cause mortality of *Metaphire hilgendorfi* upon contact, when they are sufficiently concentrated. Cooney and McCay (Unpubl. data) applied a 30 % vinegar solution (Natural Armor™ 30 % Home and Garden) at a rate of 2.5 L m^{-2} and baking soda at a rate of 500 g m^{-2}. In both cases, adult animals suffered 100 % mortality within 10 d. However, it is unclear whether the mechanism of effect extends beyond the change in pH that occurs after vinegar or baking soda application. This vinegar treatment decreased the pH of microcosms by a full unit, and the baking soda treatment increased pH by over two units. Bernard et al. (2009) found that *Amynthas agrestis* was sensitive to soils with low buffer capacity taken from the Adirondack region of New York State.

Global interest in the use of biochar (partially combusted organic material produced using pyrolysis) as a soil amendment to increase soil fertility and sequester carbon has increased in recent years (Liesch et al., 2010). As a cause of earthworm mortality, biochar is considered a chemical method of control here because the method of action may be due to the change in pH that it causes (Weyers and Spokas, 2011). While there are documented cases of earthworm mortality in the presence of certain types of biochar, there appear to be no long-term impacts (Liesch et al., 2010). No published studies have looked at the effects of biochar on pheretimoid earthworms. In an unpublished mesocosm study, Herrick and Johnston found that mortality rates of adult *Amynthas agrestis* and *A. tokioensis* were not significantly different between wood biochar-amended soil and control after 7 or 14 d. In this study, biochar was only distributed on the soil surface, and earthworms were able to avoid direct contact by burrowing below the surface. Because the action of biochar may be dependent on particle size and shape, the wood source and processing may be important.

Golf course managers have long used an organic fertilizer called Early Bird™ produced by Ocean Organics (https://oceanorganics.com/), to reduce the number of earthworm cast piles on fairways and greens (Boyle et al., 2019). Early Bird™ is a 3-0-1 fertilizer derived from *Camellia* spp. seed meal containing saponins. Recently, this product has increased in popularity among home gardeners as a way to reduce numbers of pheretimoid earthworms. Subsequently, Ocean Organics removed Early Bird from availability in 2019, probably due to extensive off-label use. Redmond et al. (2016) found that neonictinoid-pyrethroid combination insecticides decreased casts of *A. hupeiensis* by 95 % in a Kentucky golf course; whereas, tea-seed saponins did not significantly reduce casts over a single growing season. Preliminary work indicates that saponins derived from the soap bark tree (*Quillaja saponaria*) and the cocktail of natural saponins present in a natural soap (Dr. Bronner's Sal Suds Biodegradable Cleaner, https://www.drbronner.com/) cause mortality of adult *M. hilgendorfi* above background (control) levels in autumn (Feinbloom and McCay, Unpubl. data). If saponin-based treatments are to be advised, research into collateral effects on other organisms, soil quality, and water quality are necessary. Soapy drenches may be a useful tool in horticulture to expel and kill earthworms. Contact with earthworms must be greater than 5 min (Nouri-Aiin and Görres, Unpubl. data). However, there are no data on the vulnerability of cocoons to soapy water or any of the chemical controls mentioned above. Managing cocoons is likely to be the greatest challenge to any kind of control.

6.5. Biological control

Common, commercially available microbial insecticides such as *Bacillus thuringiensis* are not effective against earthworms (Saxena and Stotzky, 2001). However, several fungi and some bacteria have been shown to kill *Eisenia fetida* (Edwards and Fletcher, 1988), and some fungi infect earthworm cocoons (Nuutinen et al., 1991). Edward and Fletcher (1988) assessed the effect of sixteen species of fungi including *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Trichoderma* sp. on *Eisenia fetida*. Earthworms were dead after 10 days in six cultures and after 6 weeks in three cultures. Also, earthworms lost mass in five cultures, which may also reduce cocoon production. This research was undertaken in support of vermicomposting efforts, not as biocontrol measures. There are no studies that look at how specific these microbial agents are at controlling different earthworm species. Isolation of pathogenic microorganisms from field specimens of *Amynthas* would add to our knowledge on controlling these earthworms. This is common practice in biological control (Cook, 1993), and limited isolation was tried from cadavers of pheretimoids collected in Vermont forest. *Penicillium* sp., *Staphylococcus* sp., and *Bacillus* sp. were isolated and killed earthworms efficiently (Nouri-Aiin and Görres, Unpubl. data). In addition, *Beauvaria bassiana* was effective after reculturing it from a commercially available source (BotaniGard, BioWorks) but not in its commercial formulation (Nouri-Aiin and Görres, Unpubl. data).

7. Opportunities for methods development and conclusion

Pheretimoid earthworms can be challenging to identify, partly because individuals in invasive parthenogenetic populations often lack reproductive characteristics. As pheretimoids expand and become common in new areas, the availability of species lists and keys with illustrations will enable land managers to more quickly identify new invaders. Chang et al. (2016a) provide a model in this regard. Similar publications would be useful for portions of North America and other regions supporting invasive populations (e.g., South Africa; Brazil). Additionally, better tools for the determination of cocoons based on morphology would enable more detailed demographic studies of invasive populations.

The continued development of molecular techniques described above will increase our ability to positively identify individuals lacking diagnostic morphological features. Lumbricid earthworms have been identified using extracellular soil-based DNA (eDNA) samples in the French Alps (Bienert et al., 2012). Nested PCR improved detection of earthworms in soil samples taken from North American boreal forest, even samples archived for 30 years (Jackson et al., 2017). To date, an eDNA protocol has not been developed for pheretimoids. Such a protocol would be helpful in assessing invasion by peregrine pheretimoids, especially in the spring when populations may not include any adult individuals. Ficetola et al. (2014) argue for repeated sampling of sites to create an exhaustive species list. The use of eDNA to detect pheretimoids

would likely only be necessary when populations are small. When populations are large, the accumulation of castings usually makes the presence of pheretimoids obvious, even when populations may be at a low point in the annual cycle. The sampling of recently introduced, or otherwise small, populations of pheretimoids may require relatively intensive sampling for detection using eDNA.

Little is known about the local, regional, and continental distribution of pheretimoid earthworms beyond their native range. New methods of engaging interested citizens in documenting the presence of pheretimoids would expand our understanding of their current distribution and factors promoting spread. In particular, understanding the pattern of spread among gardens, sources of mulches and compost, and horticultural installations would help us to understand the role of gardens and supportive industries in spread of pheretimoids. At a smaller spatial scale, our understanding of intrinsic dispersal and spread would be enhanced by new and refined methods of marking and tracking individuals.

Museum specimens can provide important insights into the history of any invasion, not only as a record of the timing of an invasion but also as a source of molecular data (e.g., Hiller and Lessios, 2017). Even for soft-bodied invertebrate specimens like pheretimoids that are fixed in formalin before being preserved in ethanol, there is great potential for the analysis of DNA barcodes for museum specimens (e.g., Jaksch et al., 2016). Minteer and McHugh (Unpubl. data) amplified and sequenced COI barcodes from museum specimens of *Amynthas* spp. collected between 18 and 66 years ago from sites in the UK and the eastern US and deposited in the Museum of Comparative Zoology at Harvard University. The methods used followed standard protocols (see above), with the exception that tissue samples were washed twice in PBS buffer before DNA extraction; overnight incubation in TE buffer prior to PBS buffer washing was investigated, but it seemed to have no impact on the outcomes of PCR. Success rates in this preliminary study were variable. Interestingly, specimen age did not seem to be a factor in PCR or sequencing success. It will be worthwhile to compare haplotypes generated from historical specimens with haplotypes from earthworms in areas currently under invasion to assess alternative hypotheses regarding the patterns of pheretimoid invasions.

Although ecologists have long been concerned about earthworm invasion into natural habitats, management has largely been focused on preventing introduction rather than control and remediation (Hendrix and Bohlen, 2002). The spatial extent of invaded natural areas precludes most reclamation approaches, but control in anthropogenic habitats is achievable. In contrast to invasive European earthworms, which are often viewed positively in residential and gardening settings, pheretimoids are viewed negatively by gardeners, turf managers, and other land owners (Gale et al., Unpubl. data). The small spatial scale of these unwanted pheretimoid populations allows for the possibility of management. There currently exist few studies to inform approaches for management of earthworms in gardens, yards, and other cultivated areas. Many promising leads exist, and we encourage research toward finding targeted solutions that are compatible with cultivated plants and other conservation concerns.

Many techniques that were developed for the study of lumbricid earthworms (reviewed by Butt and Grigoropoulou, 2010 and Bartlett et al., 2010) have not been tested for study of peregrine pheretimoids. Limited initial evidence indicates that physiological, morphological, and behavioral differences between common lumbricid and pheretimoid species may complicate the simple transference of techniques and technologies originally developed for the Lumbricidae. We encourage the application of existing methodological techniques toward the study of invasive pheretimoids, with an eye toward refinement of techniques in ways that may more tightly match these species of concern.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

These ideas were discussed and developed at a symposium titled “Diversity and Ecology of Invasive Megascolecid Earthworms” supported by the Picker Interdisciplinary Science Institute (ISI) at Colgate University. We thank D. Schult and the Picker ISI for support of the symposium and follow-up interactions. We thank E. Alzaibak and M. Pugliese for help with the manuscript. D. Johnson compiled citizen science records of jumping worm occurrences. C.-H. Chang was supported by the Ministry of Science and Technology, Taiwan (MOST108-2621-B-002-001-MY3).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pedobi.2020.150669.

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