

Winter Abundances of Naked Amoebae in the Soil System of the Invasive Species Japanese Knotweed (*Fallopia japonica*) with Comparative Data from Adjacent Sites

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Abstract. Among the most prolific invasive plant species posing threats to the ecological balance of ecosystems in North America and Europe is Japanese knotweed (*Fallopia japonica*). In order to identify the potential role of protists in the invasive capacity of Japanese knotweed the winter abundances of naked amoebae in soils from three roadside thickets of Japanese knotweed were compared to nearby non-invaded sites. Japanese knotweed soils had higher abundances ($t = 5.43$, $df = 16$, $p < 0.001$) of amoebae than compared sites. This is one of the first studies to document higher abundances of soil naked amoebae associated with an invasive plant, and may indicate more generally that below-ground abundances of amoebae can promote soil fertility and support the successful adaptation and expansion of some invasive plant species. Moreover, analysis of encysted vs. trophic forms of naked amoebae in the winter soil, provided evidence of freeze-resistant, amoeba resting cells in soil samples from the natural environment in support of prior findings that were based solely on laboratory experimental evidence. Overall, high densities of naked amoebae in the winter soils of Japanese knotweed, some as resting cells capable of forming rapidly activated trophic stages, likely increase soil fertility and strengthen the species' invasive capacity.

Key words: Encysted amoebae, freeze-resistant resting amoebae, invasive species, microbial ecology, protozoan abundances, soil communities, soil fertility

INTRODUCTION

We have increasing evidence of the spread of invasive plant species and, our understanding of the environmental and biological bases for the invasive capacity of some of these introduced species is expanding. Studies addressing the enemy release hypothesis (e.g.,

Dukes and Mooney 1999, Keane and Crawley 2002) report that plants, removed from their native environs and introduced to an exotic region, experience a decrease in regulatory activity by herbivore and other biological enemies and as a result, increase in distribution and abundance. In support for the enemy-release hypothesis, Wolfe (2002) surveyed stands of the perennial *Silene latifolia* as an exotic species in North America and in its native Europe environments in order to compare evidence of predation and biological regulatory activity. In support of the enemy-release hypothesis, Wolfe (2002) reports significantly greater predation and biological

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regulatory activity in Europe than North America. Another biological approach used to study and explain the rapid spread of invasive plants is the ‘novel-weapons-hypothesis’ which asserts that some exotic species have much stronger negative effects on competitive species in the introduced habitat than in the native region. For example, Callaway and Aschehoug (2000) found that *Centaurea diffusa*, an invasive weed in North America has much stronger negative effects on neighboring grass species in its new environs than it does on grass species in its native environment. Furthermore, studies reporting positive feedback mechanisms between soil biota communities and exotic plants (e.g., Bever *et al.* 1997, Reinhart *et al.* 2003, Klironomos 2002, Wolfe and Klironomos 2005) add to our increased understand of the biological basis for the rapid expansion of invasive plant species.

One, if not the most prolific and widespread invasive plant in North America and Europe is Japanese knotweed (*Fallopia japonica*). First introduced to the United States and Europe from Asia in the late 1800’s as an erosion control and ornamental plant, Japanese knotweed has uncontrollably spread along riparian sites and roadsides. The rapid expansion is due to several strategies including the ability of the plant to grow in soils with a wide range of pH and salinities (USDA 2016), effective dispersal by seeds and fragmentation of roots and stems (Groeneveld *et al.* 2014), and the exudation of allelopathic phenols known to decrease germination rates of competitive species (Dommanget *et al.* 2014).

Ecological concerns are many, including reduced summer stream flow attributed to high levels of transpiration from the broad leaves (Vanderklein *et al.* 2014). Moreover, leaf-bag studies (Shannon *et al.* 2014) attribute low colonization rates of macroinvertebrates on shed Japanese knotweed leaves to the overall low levels of nitrates and phosphates and high levels of low nutrient quality cellulose and lignin fibers. Likewise, Tamura and Nishanth (2014) report that the litter of Japanese knotweed is recalcitrant to microbial decomposition, thus feeding back to climate change by destabilizing the soil carbon cycle.

Although our knowledge about the biology and adaptive capacity of invasive plants is advancing, our understandings of the roles protists, including naked amoebae, may play in the proliferation of invasive plants species is less well understood. Naked amoebae are well documented as important members of soil nutrient cycling systems (e.g. Adl and Gupta 2006; Clarholm 1985, 1989; Fiore-Donno *et al.* 2016; Jahnke *et al.*

2007), and several studies have linked their abundances and community composition to soil moisture and organic matter concentrations (e.g. Bischoff 2002; Geisen *et al.* 2014). Recently, Anderson (2016) reported evidence for trophic (resting cell) stages of non-encysted naked amoebae in frozen winter soils that are capable of rapidly resuming growth in favorable (thawed and moist) soil conditions. That evidence was based exclusively on laboratory experimental studies, and additional evidence based on samples from the natural environment is needed to fully document the occurrence of amoeba resting cells in winter soil from the natural environment.

Parepa *et al.* (2013) examined the role of soil biota in the invasion success of exotic knotweed (*Fallopia x bohemica*), by creating artificial native plant communities that they experimentally planted with invasive knotweed, using a range of substrates where different fractions of soil biota were included. They found that invasive knotweed benefited more from the overall presence of soil biota than any of the six native species, but were not able to fully determine why the soil biota (including protozoa and fungi) were beneficial. In this study we provide the first evidence that soil naked amoebae associated with knotweed are more abundant than in nearby native plant sites, and this study may help to explain the results obtained by Parepa *et al.* Moreover, we have found evidence of proportionally high concentrations of winter resting cells of naked amoebae in the soils of Japanese knotweed plants.

Our data speak to the potential role amoebae, including those that are resting cell/non-encysted, play in increasing the fertility of the soil in knotweed stands thus enhancing productivity and, spread of this potentially harmful invasive plant.

MATERIALS AND METHODS

Soil samples within three rural roadside Japanese knotweed monoculture thickets and from adjacent mixed vegetation sites (deciduous trees, mixed perennials and grasses) were collected in winter (February and March 2016) from sites within three km of the State University of New York, College at Oneonta, New York, USA. Site 1 (42°30’9.13”N; 75°1’43.24”W) was snow covered on collection day. Following recent night-day, freeze-thaw cycles, sites 2 (42°28’20.38”N; 75°3’12.10”W) and 3 (42°29’13.95”N; 75°4’4.50”W) were not snow covered on collection day. For all sites, the immediate surface layer of organic detritus deposited the previous fall was removed exposing the surface soil. Soil at site 1 was frozen on collection day and a chisel and hammer were used to

loosen and extract frozen soil samples to a depth of ~ 2 cm. At sites 2 and 3 the surface soil to a depth of ~ 3 cm had thawed atop the frozen subsoil. Three independent soil samples from each site were placed into labeled Whirlpac™ bags for transport to the nearby lab for immediate processing. Soil temperature was measured in-situ. On return to the lab the percent soil moisture was measured gravimetrically as percent (w/w) per dry weight (dw) based on water loss upon drying (90°C) overnight, using a Metler Toledo Excellence XS Analytical Balance (Greifensee, Switzerland). The percent organic matter of the dried soils was obtained by combustion after 2 h at 650°C.

The number of naked amoebae per g dry matter from Japanese knotweed and adjacent comparison soils was determined using a standard culture-enrichment method (e.g. Anderson and Rogerson 1995, Brown and Smirnov 2004). In this method a small piece (ca. 1 mm³) of malt-yeast agar (Page 1983) is used to support bacterial growth as a food source for the amoebae. Densities of amoebae are then determined through proportional analysis. The samples were prepared for analysis immediately on return to the laboratory before substantial thawing or warming occurred. Four quality control measures were used to ensure accuracy in the recording of the naked amoebae in each well of the Falcon™ culture dish. First, to prevent bias in the observation process, we labeled and recorded in a notebook page, each Falcon™ tissue culture plate with a random number identifying it as a Japanese knotweed (J) soil, or a comparison (C) soil. Thus, the observer did not know what type of soil was analyzed until all plates for a particular site was fully recorded. Second, the undergraduate researcher was new to this work and every observation made was double-checked by the more experienced observer. Third, almost every culture dish well was checked after two weeks of incubation and then again after three weeks in an effort to accurately detect amoebae that may have excysted after a more extended time in incubation. Fourth, our Zeiss Axiovert 40C inverted microscope was equipped with phase contrast optics, camera and software, and an ocular micrometer, thus allowing capture of amoeba images and their measurement. Except in a few cases where a good photo-image could not be obtained, pictures were taken of amoebae representing the population(s) in each well. Often, several pictures were taken in an effort to more clearly document the changes in morphology as the amoebae moved. Length and width were recorded for the documented amoebae as we progressed through the observation process. The observed amoebae were grouped into one of four morphotypes (MTs) commonly used in ecological studies (e.g. Anderson 2016) designed to reveal general patterns in amoebae densities and not phylogenetic affiliations (e.g., Geisen *et al* 2015). Type 1 MTs (e.g. *Acanthamoeba* and *Korotnevella*) exhibit lobose or fine sub-pseudopodia usually emerging from the anterior hyaline edge during locomotion. Type 2 MTs (e.g. *Hartmannella* and *Saccamoeba*) are limax, sometimes with a distinct hyaline cap. Type 3 MTs (e.g. *Acrasis* and *Vahlkampfia*) are limax and distinctly eruptive during locomotion. Type 4 MTs (e.g. *Vannella*) are flattened, fan-shaped or discoidal. The number of encysted naked amoebae was determined by the air-drying modification of the enrichment culture technique (Anderson 2006) and expressed as the ratio of encysted to total enumerated amoebae for the first sample collected at each site. Statistical analyses were done with SPSS 17.0 for Windows (SPSS Inc. Chicago, IL).

RESULTS

The National Oceanographic and Atmosphere Administration report that the El Niño winter of 2016 in Northeastern USA was the warmest in 121 years. The impact at the sampling sites was a significant fluctuation in temperature and the intermittent freezing and thawing of the soils in the days preceding sampling. Given the likely impacts of the freeze-thaw soil conditions (obtained from the campus meteorological database) on amoebae abundances, summary temperature data from five days preceding sampling and in-situ soil temperature readings are provided for each site. On sampling day 2/9/16 the in-situ temperature at site 1 (both J and C) was -0.5°C. Weather data from the five days preceding sampling shows a mean high of 1.7°C and a mean low of -0.5°C. The temperature was unusually high (8°C) on 2/7/16. On sampling day 2/19/19 the in-situ temperature at site 2 (J and C) was 1°C. The collected soil was thawed above the frozen subsoil to a depth of 3 cm. The five-day mean high was 6°C and the mean low was -4°C. These soil likely experienced freeze-thaw events in the days preceding sampling. The in-situ temperature at site 3 on 3/1/16 (J and C) was 1°C. The five-day mean high was 5°C, and the mean low was -5°C. Similar to sample 2, the soil at site 3 was collected above the frozen subsoil and likely experienced freeze-thaw condition in the days preceding sampling.

Amoebae abundances by morphotype, percent moisture (w/w), and percent organic matter (w/w) for each Japanese knotweed and comparison site are shown in Table 1. At each site the mean total abundances of naked amoebae in the soils of the Japanese knotweed was at least three times that of the adjacent comparison sites. An independent samples t-test ($t = 4.9$; $df = 16$; $p < 0.001$) supports the conclusion that the winter soils within Japanese knotweed thickets are highly favorable habitats for amoebae. At sites 1 and 3, the Japanese knotweed soils had much higher mean organic matter concentrations than the adjacent mixed vegetation sites. Total amoeba abundance data was positively correlated with organic matter ($r = 0.44$; $p < 0.03$, $N = 18$). Likewise, soil moisture in the Japanese knotweed sites ($X = 58\%$ moisture, w/w) was higher than control sites ($X = 32\%$ moisture, w/w). Overall there was a strong correlation ($r = 0.63$; $p < 0.02$, $N = 18$) between amoebae abundances and moisture. The data demonstrate a strong trend of higher populations of naked amoebae in the organically rich and more moist Japanese knotweed soils.

The highest ratios of encysted vs. total amoebae were in the more moist soils and organically rich Japanese knotweed soils (Table 1). The data for site 1 serves as an example. This sample from Japanese knotweed site 1 (1JA) was very moist (78%) and organically rich (45%). It had a ratio of 116/253 revealing 45% encysted and, 55% trophic amoebae. This soil was also hard frozen on collection day demonstrating the proportionally high presence of freeze-resistant amoebae in this Japanese knotweed soil. The total abundance ratio at 2JA was 51/132 showing that 81 or 61% of the observed

amoebae were trophic forms when the sample was taken. Likewise at site 3, the moisture content of the soil sample used to compare encysted/total amoebae in the Japanese knotweed soil was 71% and the organic concentration was 32%. This soil had the second highest total abundance (249/g dry soil). The fraction shows that 179 amoebae were encysted and 70 were in the trophic form at the time of sampling.

In addition to careful observations of naked amoebae in our tissue culture plates we also noted the presence of flagellates in many of our wells demonstrating

Table 1. Amoebae abundances/g dw by morphotype (Ty) in Japanese knotweed (J) and Comparison (C) sites with percent (w/w) organic matter (O. M.) and percent (w/w) moisture (H₂O) in soil samples.^a

Site	% O. M.	% H ₂ O	Ty 1	Ty 2	Ty 3	Ty 4	Total
1Ja	45	78	81/185	0/0	35/56	0/12	116/253
1Jb	59	83	79	0	106	26	211
1Jc	54	86	107	0	40	13	160
\bar{X} (S.D.)	53 (7)	82 (4)	124 (55)	0 (0)	67 (34)	17 (8)	208 (47)
1Ca	12	28	55/142	0/0	11/11	0/22	66/175
1Cb	19	10	14	0	3	0	17
1Cc	32	45	24	0	19	0	43
\bar{X} (S.D.)	21 (10)	28 (18)	50 (71)	0 (0)	11 (8)	7 (13)	78 (85)
2Ja	14	35	51/76	0/6	0/44	0/6	51/132
2Jb	8	27	79	6	11	17	113
2Jc	14	37	118	0	33	7	158
\bar{X} (S.D.)	12 (3)	33 (5)	91 (23)	2 (3)	29 (17)	10 (6)	134 (22)
2Ca	10	35	13/13	0/0	13/13	6/13	33/39
2Cb	8	28	34	6	23	0	63
2Cc	24	34	26	0	46	20	92
\bar{X} (S.D.)	14 (9)	32 (4)	24 (11)	2 (4)	27 (17)	11 (10)	65 (27)
3Ja	32	71	159/159	0/20	20/40	0/30	179/249
3Jb	24	48	104	0	0	13	117
3Jc	37	61	105	0	0	7	112
\bar{X} (S.D.)	31(7)	60 (12)	123(31)	7 (12)	13 (23)	17 (12)	159 (78)
3Ca	13	36	12/4	0/0	12/0	6/22	30/26
3Cb	14	35	13	0	6	0	19
3Cc	13	37	13	0	0	0	13
\bar{X} (S.D.)	13(1)	36 (1)	10 (5)	0 (0)	2 (4)	7 (13)	19(7)

^aRatios are abundances/g dw for encysted naked amoebae/ total amoebae observed in the first sample of each sampling site as explained in the Materials and methods.

no observable allelopathic effects of the extracted soil suspension on flagellates, at least. Similarly *Diffugia* spp. were commonly observed in our wettest Japanese knotweed soil samples (mean *Diffugia* spp. density in J site 1 was 85/g dw compared to mean of 8/g dw at the comparison site) as further evidence of the rich microbial communities inhabiting the rhizosphere of Japanese knotweed soils during this el Niño winter.

DISCUSSION

In overview the data show that organically rich and moist soils in Japanese knotweed thickets are exceptionally productive habitats for naked amoebae. High overall densities (including encysted forms that excysted in the culture plates), and exceptionally high proportions of resting cell/non-encysted amoebae, even in these winter soils, are likely part of reason for the success of the invasive plant.

Until recently (Anderson 2016), it was widely assumed that soil amoebae would revert to an encysted form as soil temperature approached the freezing point and that they would remain essentially inactive under such adverse condition until a sustained spring thaw. The soils (J and C) at site 1 were hard frozen when sampled and had high numbers of recoverable, apparently resting, trophic amoebae, when prepared immediately for analysis after return to the laboratory. The soils at sites 2 and 3 (J and C) were undergoing intermittent freeze-thaw cycles and also had measurable populations of trophic, apparently resting forms, as well as encysted forms. These observations further confirm evidence that naked amoebae can form non-encysted, resting stages as reported by Anderson (2016), and support the conclusion that trophic amoebae are able to quickly respond to favorable (e.g. thawed) soil conditions and resume active growth rapidly as a trophic stage. Some of the invasive properties of Japanese knotweed, including its documented dispersal mechanisms, may be attributed to increased soil fertility associated with abundant organics and greater densities of protists, especially naked amoebae as reported here. Thus, the improved fertility could lead to enhanced emergence of the knotweed during spring and more robust growth during the growing season.

Wolfe and Klironomos (2005) describe ways in which varied exotic plants can affect soil microbial community structure. For example, the timing, quality,

quantity and spatial distribution of organic matter inputs can result in changes to the microbial community structure. The results of this study, showing particularly high densities of naked amoebae in the moist and organically rich soils of Japanese knotweed plants supports their findings. In the case of some exotic species (including Japanese knotweed), the release of allelochemicals from plant roots (e.g., Callaway and Aschehoug 2000) has been used as an explanation for success of exotic species in plant to plant competitive interactions. Vivanco *et al.* (2004) reported that the root exudate 8-hydroxyquinoline from Eurasian knapweed (*Centaurea diffusa*) is an effective antimicrobial agent. Although the results reported here for Japanese knotweed show no evidence of allelopathy on naked amoebae, follow up studies on are needed to more fully understand the potential impacts of allelopathy on soil microbial community structure.

This research showing particularly high densities of naked amoebae in the winter soils of Japanese knotweed and high ratios of freeze-resistant, amoeba resting cells may explain in part the ability of the exotic plant to rapidly invade new ecosystems. Further studies are needed to determine the generality of our findings with other invasive species and at different geographic locales.

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