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What role does human activity play in microbial biogeography?: The revealing case of testate amoebae in the soils of Pyramiden, Svalbard.

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ABSTRACT

The role of human activity as a vector in the movement of soil microorganisms is uncertain and disputed. It is increasingly clear that some larger microorganisms have restricted distributions and plausible to imagine that many human activities could lead to exotic species introductions, but concrete examples are lacking. We investigated an unusual case study: the former mining settlement of Pyramiden on the island of Spitsbergen in the Svalbard archipelago. In around 1983 large quantities of chernozem soils were imported from the southern USSR as part of an urban greening initiative, bringing large numbers of soil organisms to a very different physical environment. Focusing on a readily-identifiable group of protists with documented regional endemism (testate amoebae), we assessed morphospecies assemblages after thirty years. We analysed communities from Pyramiden imported soils and conducted comparisons to: i) nearby locations with non-imported near-natural soils; ii) previously-established datasets from near-natural Svalbard soils and chernozem soils in southern Russia, and iii) regional species inventories from both regions. Our aims were to assess how the community has adapted to the change in physical conditions and identify any evidence for the import of exotic taxa. Our results show significant differences between the assemblages of imported soils and those of nearby reference sites but strict comparisons are complicated by the different treatment of soils in different locations. No taxa were identified which can be unambiguously identified as atypical for the region. Assemblages of imported soils were more similar to those of other sites on Svalbard than to assemblages from southern Russia. Our data are equivocal but suggest that the testate amoeba assemblages of soils transported more than 3000km northwards have substantially acclimated to the new conditions, contrasting with micro-invertebrates in the same site. 'Accidental experiments' like the soils of Pyramiden provide unique opportunities for long-term, large-scale, soil biological research.

KEYWORDS: Testate amoeba; Protist; Biogeography; Svalbard; Ecology

INTRODUCTION

The study of biogeographic patterns in microscopic organisms has lagged that of macroscopic organisms (Kleinteich et al., 2017; Martiny et al., 2006). While many key factors structuring large-scale distribution of larger organisms are well-understood there remains uncertainty as to whether and how these rules relate to microbes. This situation is arguably particularly acute in soils due to limited understanding of microbial diversity and the likelihood for complex spatial patterning at scales from individual soil pores (Ruamps et al., 2011) through to regions (Drenovsky et al., 2010) and biomes (Fierer et al., 2012).

Much research in microbial biogeography has focused on distinctive flagship species and groups. Testate amoebae have proven a useful group for such studies due to their highly variable shells ('tests'), and a substantial established data resource (Payne, 2013). Research on testate amoebae has made a number of interesting and occasionally surprising contributions to understanding of global soil microbial biogeography (Heger et al., 2011; Lara et al., 2015; Smith et al., 2008). A key debate has concerned the Baas-Becking hypothesis that 'everything is everywhere but the environment selects' (De Wit and Bouvier, 2006; O'Malley, 2007; O'Malley, 2008). While there is little doubt that 'the environment selects' there has been considerable debate whether 'everything is everywhere' for microbial eukaryotes (Azovsky and Mazei, 2013; Azovsky et al., 2016; Finlay, 2002; Finlay and Fenchel, 2004). Testate amoebae provide convincing examples of morphospecies with restricted geographic distributions (Heger et al., 2011; Smith and Wilkinson, 2007) and molecular methods are demonstrating complex biogeographic patterning which shows similarity to macroscopic organisms (Lara et al., 2015). It is increasingly accepted that, at least for larger protists, restricted distributions are relatively frequent and although 'most things may be in most places', everything is not truly everywhere (Wilkinson, 2001; Yang et al., 2010).

Once it is accepted that at least some microorganisms have restricted distributions an important secondary question is whether the distributions of microorganisms, like macroscopic organisms, may have been affected by human activity. Recent studies have suggested that human activity is a plausible mechanism for inter-hemispheric similarity in microbial assemblages (Kleinteich et al., 2017) and Wilkinson (2009) argues that this may be a significant gap in current knowledge. It is clear that there is considerable global movement of soil both for its own sake and as incidental material associated with the global plant trade (Wilkinson and Smith, 2006). It is probable that long-distance movement of small quantities of soil on the footwear and clothing of air travellers happens accidentally all the time (Wilkinson, 2009). The global aggregates trade transports more material than all the worlds rivers and much of this material is from near-surface deposits (Peduzzi, 2014). Soil and sediment moved by human activity will mean that soil microorganisms are also moved by human activity. If these soils and sediments contain different microbial communities what role does this have in soil microbial biogeography?

This study addresses the question of human translocation of microorganisms by focusing on an unusual case-study. Pyramiden is a now-abandoned settlement on the island of Spitsbergen in the Svalbard archipelago. The site was originally founded as a coal-mining town by Sweden in 1910, before being sold to the USSR in 1927. The site was abandoned in 1998 and is now largely derelict apart from some tourist visitors in summer. From a soil biological viewpoint the key interest in

Pyramiden comes from an experimental programme by the then authorities. Around 1983 as part of an 'urban greening' initiative large quantities of exotic soil were imported to Pyramiden by the state mining company. These imported soils were of chernozem type; typical of the steppes to the south of the former Soviet Union (Coulson et al., 2015). Exact details of the nature and origin of the soils imported are lost to history but it is a reasonable assumption that in terms of both their physico-chemical properties and soil biological communities the imported soils were very different to the indigenous soils. The steppe zone is characterized by a semi-arid continental climate, very different to the polar climate regime of Svalbard. Chernozem soils are characterised by a high carbon content and are usually of neutral pH, base-rich and fertile (Eckmeier et al., 2007). This contrasts with the soils of Svalbard which are heterogeneous but typically thin, infertile and often acidic (Kaštovská et al., 2005; Solheim et al., 1996). The total volume of soil transported is similarly lost to history but given that imported soils cover a substantial proportion of the non-built-up area of the settlement it is reasonable to suppose that this must amount to many tens of tonnes. In essence Pyramiden represents a large-scale and long-term 'accidental experiment' on the effects of a 3000km northwards soil translocation (HilleRisLambers et al., 2013). The situation of Pyramiden raises many intriguing questions. After more than thirty years do imported soils still harbour distinct species assemblages? Is there evidence of establishment by indigenous Svalbard taxa in the imported soil? Is there any evidence of exotic taxa spreading to indigenous soils? The answers to these questions can cast light on broader questions of the role of human activity in microbial biogeography. This study aims to address these issues using testate amoebae as a representative group of soil microeukaryotes.

MATERIAL AND METHODS

Field and laboratory work

We undertook sampling of the soils of Pyramiden (78°39.3N, 16°18.7E) in June 2013 (Supplementary Fig. 1, 2). We extracted 25 samples from five sites in the settlement where imported soils were located. The sampling locations were located throughout the settlement, typically had vegetation dominated by grasses and had previously been used for a range of land-uses including for recreation and agricultural activity. As a reference dataset of largely-natural soils in the region we extracted a further 20 samples from a site to the north-east at Petuniabukta (78°61.7N, 16°37.9E). The locations sampled at Petuniabukta are typical of Svalbard, with sparse cover of bryophytes and graminoids. At each sampling point we removed a sample of approximately 5g wet weight of surface soil (uppermost 50mm) and any plant litter. Samples were stored in cool conditions and prepared for analysis immediately on return to the laboratory (Mazei et al., 2015). Sampling was conducted in parallel with sampling for invertebrates (Coulson et al., 2015) but not in the same locations, precluding direct comparison of results. In the laboratory, samples were prepared for analysis of testate amoebae using a method based on suspension in water, physical agitation and settling (Mazei and Chernyshov, 2011). Samples were analysed using light microscopy at 160× magnification. All testate amoeba tests in the samples were identified following Mazei and Tsyganov (2006). We calculated both test concentrations (ind. g⁻¹) and relative abundance (%) from the raw data. Results are based on total tests, including empty tests not occupied by a living amoeba, and thereby reflect assemblages living over an extended period of time.

Data analysis

We analysed the data using univariate and multivariate methods to test for differences between imported and non-imported soils. We conducted three sets of data comparisons. We first compared data between our 'natural reference' site (Petuniabukta) and the Pyramiden imported soils. This test allowed us to compare the testate amoeba assemblages of the imported soils to a reference site which represents conditions typical of Svalbard at the same point in time. We tested for differences in test concentration, taxonomic richness and Shannon diversity using permutation t-tests (9999 permutations). We used non-metric multidimensional scaling (NMDS, function 'metaMDS') to inspect overall assemblage variability and tested for differences between imported and natural soils using permutational multivariate analysis of variance (PERMANOVA, function 'adonis'). We conducted separate analyses based on both relative abundance and test concentration.

Secondly, we compared the testate amoeba assemblages of Pyramiden imported soils to those of previously-established datasets for Svalbard and chernozem soils in southern Russia. The aim of these tests was to establish how the data from imported soils compare to other sites in Svalbard with different environmental conditions and to the soil source regions. For Svalbard we used data from three areas of the Barentsburg region (c. 70km SW of Pyramiden) previously presented by Mazei et al. (submitted). These sites encompassed natural soils with and without influence of seabirds. For chernozem soils we used the data of Mazei and Embulaeva (2008) and Mazei and Embulaeva (2009) from soils in the Middle Volga region of southern Russia. These data encompass a range of steppe and forested steppe vegetation and lie within the general source regions of the imported soils. All comparison datasets were analysed by the same research group using a consistent analytical and taxonomic approach allowing confidence in data comparability. We compared these reference datasets to our imported soil data in terms of test relative abundance using NMDS ordination and PERMANOVA tests. Finally, we compared our imported soils data to published species lists from other studies in the regions. All data analyses were conducted in R3.1.2 using packages including vegan and Deducer (Oksanen et al., 2007; R Development Core Team, 2014).

RESULTS

In total we identified 43 testate amoeba taxa in the 45 samples. The most abundant of these taxa were *Trinema lineare* (26.9% of all tests), *Euglypha laevis* (15.2%) and *Centropyxis orbicularis* (8.6%). Differences in the overall assemblage between imported and reference non-imported soils are clear in the NMDS plot (Fig. 1) and were highly significant (PERMANOVA relative abundance: $F_{(1,43)}=5.1$, $P<0.001$; concentration: $F_{(1,43)}=4.6$, $P<0.001$). The NMDS plot highlights greater variability in assemblage composition in the imported than non-imported soils. Six taxa were only found in the imported soil (*Centropyxis platystoma*, *Diffflugia bicornis*, *Euglypha cristata*, *Plagiopyxis declivis*, *Trinema enchelys*, *Trigonopyxis arcuata minor*) and 15 taxa only in the non-imported soil (Table 1). All of these taxa are minor occurrences which constitute a small proportion of the total assemblage. In independent permutation t-tests there were significant differences in relative abundance of 11 taxa between imported and non-imported soils (Table 1), but only two of these (*Heleopera petricola* and *Arcella arenaria compressa*) remained significantly different when using a Bonferroni correction for multiple comparisons. Seven of these taxa were only found in one area while four taxa were present in both areas but with significant differences in abundance: *Tracheleuglypha dentata* and *Arcella arenaria compressa* were more abundant in the non-imported soils and *Centropyxis orbicularis* and *Centropyxis aerophila sphagnicola* were more abundant in the imported soils (Table 1). Species

richness (permutation $t=-7.1$, $P<0.001$) and Shannon diversity (permutation $t=-5.0$, $P<0.001$) were significantly lower in imported soils (mean -51% and -33% respectively; Fig. 2). Overall test concentration did not significantly differ ($P=0.5$).

Compared to previously-established datasets (Fig. 3) our results for imported soils show significant difference from both the chernozem data (PERMANOVA $F_{(1,50)}=13.6$, $P<0.001$) and the Svalbard reference data ($F_{(1,38)}=8.8$, $P<0.001$). In the NMDS plot it is clear that there is more overlap with the non-imported soils from the neighbouring site than either reference data from elsewhere on Svalbard or chernozem soils in southern Russia (Fig. 3). However, testate amoeba assemblages of reference soils from Svalbard are 10% more similar to those of imported Pyramiden soils than those of reference chernozem soils (in terms of mean Bray-Curtis dissimilarity).

DISCUSSION

Several taxa were only identified in either imported or non-imported soils in our sampling. Of the taxa only identified in the imported soils, to our knowledge only *Diffflugia bicornis* has not previously been recorded in Svalbard (Balik, 1994; Beyens and Bobrov, 2016; Beyens and Chardez, 1995). The taxonomic status of *Diffflugia bicornis* is disputed with Mazei and Warren (2012) suggesting that *D. bicornis* and *Diffflugia bacilliarum* cannot be reliably distinguished and proposing the two be synonymised as *Diffflugia bacilliarum*. We are not aware of *D. bacilliarum* being recorded in either Svalbard or the Arctic region more generally (Beyens and Chardez, 1995). However this is a taxon relatively frequently recorded in other studies, although rarely with great abundance. *D. bacilliarum* is not included in our chernozem reference datasets and we are not aware of any other records from the steppe region, although it has been recorded elsewhere in Russia (Kur'ina, 2011; Rakhleeva, 2002). The literature suggests that the typical habitat of the taxon is freshwaters and minerotrophic wetlands (Lamentowicz et al., 2008; Patterson and Kumar, 2000; Song et al., 2014). As habitats similar to those in which the taxon is known to occur most frequently, are relatively widespread in Svalbard but rarer in the Eurasian steppe region where chernozem soils are found and it has not been recorded in the steppe region we consider it more probable that this is a previously-overlooked minor components of the indigenous Svalbard fauna rather than an introduced taxon. One further taxon, *Euglypha cristata* has been recorded in only one previous publication from Svalbard, but this record should perhaps be regarded with caution given it's age and general focus (Thor, 1930). *E. cristata* is a widespread Euglyphid testate amoeba which is not present in our chernozem reference data but has been widely recorded elsewhere, particularly in minerotrophic peatlands (Payne, 2011; Turner and Swindles, 2012) and has been recorded elsewhere in the Arctic (Beyens et al., 2009; Bobrov et al., 2013) as well as many other locations globally (Krashevskaya et al., 2007; Robinson et al., 2002). Similarly to *D. bicornis* it seems more likely that it is a relatively minor component of the Svalbard fauna than an introduction. It is notable that all the taxa identified only in one set of samples were rare occurrences and therefore may have been insufficiently abundant to be identified in both imported and non-imported soils.

Taxa which have been considered potential local (e.g. *Schoenbornia smithi* (Beyens and Chardez, 1997)) or regional (e.g. *Centropyxis gasparella* (Beyens and Bobrov, 2016)) endemics in the Arctic were not identified in the imported soils, but were also not identified in the nearby reference site. Taxa which appear to be quite abundant in chernozem soils were relatively rare in the Pyramiden imported soils. The samples of Mazei and Embulaeva (2008) and Mazei and Embulaeva (2009)

include considerable abundance of taxa with 'bowl-shaped' xenosome tests which are rare or absent in the Pyramiden soils and nearby sites (e.g. *Cyclopyxis kahli*, *Cyclopyxis eurystoma*, *Phryganella acropodia*, *Phryganella hemispherica*). Assemblages of imported soils here are more similar to reference data from Svalbard than to reference data from southern Russia. Taxa in the Svalbard reference site could conceivably also include introduced taxa if these were introduced into Pyramiden with the imported soils and then spread as far as the Petuniabukta site. However, there were similarly no taxa here which appear unexpected in this context; across both sampling areas the majority of taxa are known to be widely-distributed.

Despite the lack of clear evidence for regionally endemic taxa our results do clearly demonstrate that testate amoeba assemblages of imported soils in Pyramiden differ from those of indigenous Svalbard soils in both nearby locations sampled at the same time and previously-established data from elsewhere in the archipelago. Imported soil assemblages were less diverse than those of adjacent near-natural sites and differed in terms of community composition, but it is not clear that these differences relate directly to the soil translocation. Imported assemblages were also more variable, perhaps a consequence of differing land-uses within the settlement. The pattern of species differences does not imply an obvious cause for the differences between imported and non-imported soils but it is plausible that this may relate to factors such as differences in vegetation, compaction and soil pollution, as well as soil origin (Coulson et al., 2015). Perhaps more surprising than the difference in assemblage, however, is their general similarity. The two most abundant taxa: *Trinema lineare* and *Euglypha laevis* were the same in both Pyramiden soils and the reference site. Both sets of assemblages were dominated by small taxa with idiosome tests and filose pseudopodia with a smaller proportion of larger Centropyxid taxa. Such idiosome taxa may have important roles in silica cycling (Puppe et al., 2014). Arcellidae and Hyalospheniidae were rare in both soils and the list of species is dominated by very common taxa well-known from soils. The assemblage structure was similar, with considerable overlap in ordination space (Fig. 1). Given their different origins, different vegetation and different land uses the assemblages of these soils can arguably be considered surprisingly similar. It seems probable that since importation the testate amoeba assemblages of the imported soils have adapted to conditions in Svalbard and the soils have been colonised by locally-present taxa.

The lack of demonstrably introduced testate amoeba taxa and apparent acclimation of the assemblage composition contrasts with the situation in invertebrates. In the same imported Pyramiden soils Coulson et al. (2015) identified three mesostigmatid mite taxa and two collembola taxa previously unknown on Svalbard. At least four of these taxa are known to occur in chernozem soil regions and these are very likely to represent introductions. There were also very substantial differences in microinvertebrate assemblages between imported soils and reference sites with oribatid mites notably very scarce in Pyramiden. In Barentsburg, another Russian settlement on Svalbard with imported soils, Coulson et al. (2013) identified eleven species of invertebrate previously-unknown to Svalbard with many of these species probably introduced. Again, there appeared to be very strong differences in assemblage structure compared to near-natural reference sites (Seniczak et al., 2014). There may be several causes of this disparity between testate amoebae and invertebrate metazoa. While it is clear that some testate amoeba taxa do show restricted distributions (Heger et al., 2011), most taxa are very widely distributed and this may be particularly the case in the relatively impoverished Arctic fauna (Beyens and Bobrov, 2016). Although there are some candidates for regional endemism, most taxa found in most studies in the Arctic have wide

distributions and are widely known beyond the region (Beyens and Bobrov, 2016; Beyens and Chardez, 1995). Micro-invertebrates by contrast have much stronger biogeographic patterning with many taxa most likely restricted to either the Arctic or southern Eurasia (although data is limited for many groups). It is probable that initial differences between the imported chernozem soils and indigenous Svalbard soils were much stronger in terms of micro-invertebrate assemblages than testate amoeba assemblages. A further factor is likely to be generation time. Under laboratory conditions testate amoeba can have generation times as little as a few hours (Beyens and Meisterfeld, 2001), considerably less than any invertebrates and testate amoeba assemblages can be expected to react more quickly to environmental change. It seems likely that in this case sufficient time has elapsed for the testate amoeba assemblage to substantially adapt to the conditions of Svalbard.

'Accidental experiments' provide unique opportunities to address critically important questions in biological science (HilleRisLambers et al., 2013). In the case of Pyramiden's imported soils the opportunities are clear: the soil translocation was of a very large-scale and across a very large distance to a very different environment. This is a remarkable 'experiment' in the large-scale response of a soil biological system to dramatic environmental change and the potential for species introduction. The Pyramiden instance also benefits from the time which has elapsed since the translocation, around thirty years at the time of sampling, allowing a much longer temporal scope than most deliberate experiments. However accidental experiments also suffer issues which can complicate analyses and confound interpretation of results. Accidental experiments are by their nature un-replicated and often un-repeatable. 'Treatments' can be confounded by other variables, establishing valid controls can be difficult and there is frequently no baseline data from which to assess subsequent impacts (HilleRisLambers et al., 2013; Wiens and Parker, 1995). These are all important issues in the Pyramiden case and constitute constraints on what can be determined in this study. Notably, the reference samples here do not represent a perfect control. Urban soils are likely to be more disturbed, more exposed to contaminants, lower in organic matter and more compacted relative to natural sites (De Kimpe and Morel, 2000; Gregory et al., 2006). Differences between our treated and control sites may therefore not relate solely to the origin of the soils but also to their subsequent treatment. This is an unavoidable situation. While non-imported soils are present within Pyramiden itself these also do not provide secure controls for the effect of soil origin because the imported soils were managed differently from other soils, including planting with grass seed, manuring, grazing and restricting access by residents (Coulson et al., 2015). The possibility for taxonomic confusion must also be considered in any study of testate amoebae as much of the taxonomic literature is old, criteria for differentiating some species are unclear and much data is produced by inexperienced analysts (Mitchell and Meisterfeld, 2005; Payne et al., 2011). The situation is particularly acute when attempting to compare datasets produced by different analysts (Payne et al., 2011) and in different climatic zones with the possibility for phenotypic plasticity (Mulot et al., 2017). Future molecular analyses may ultimately remove some of the taxonomic uncertainty inherent in microscopic analyses, but the latter currently have the crucial advantage of a large body of established reference data from around the world.

While our results fail to provide convincing evidence for species translocation such negative data remain important. Although testate amoebae do provide convincing examples of regional endemism, these examples are a small proportion of the total and most discussion has been restricted to a very few taxa (e.g. *Apodera vas* (Smith and Wilkinson, 2007)). Most taxa identified in

most locations around the world have very wide distributions and most probably do occur globally where the correct environmental conditions are present. Although species introductions due to human activity may well occur, our data suggest they may be rare.

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FIGURE CAPTIONS

Figure 1. Non-metric multi-dimensional scaling ordination on Bray-Curtis dissimilarity of (a) relative abundance and (b) concentration data for testate amoebae from imported and non-imported soils.

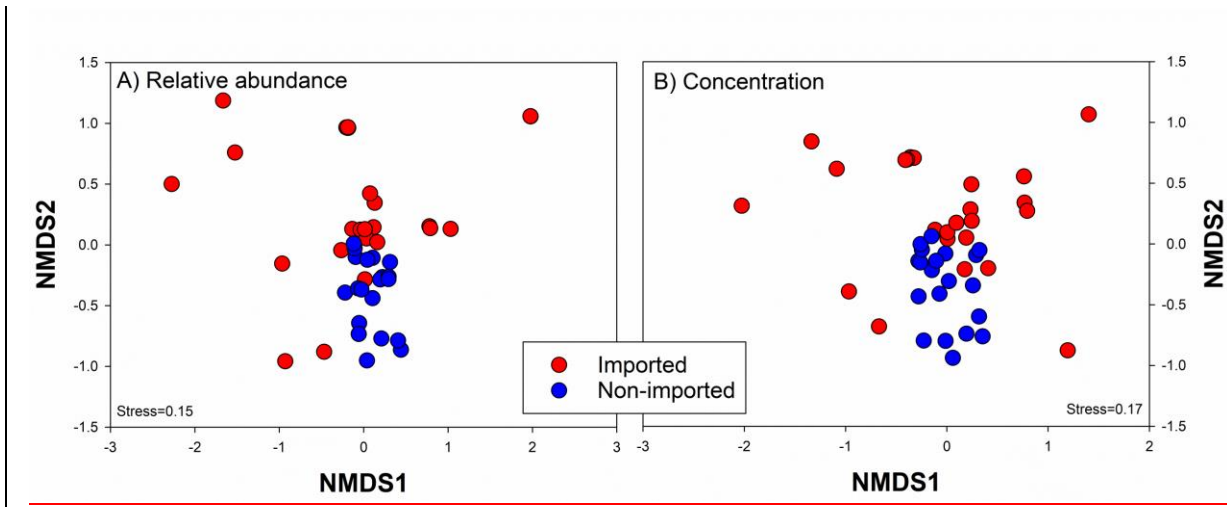


Figure 2. Diversity of imported and non-imported soils in terms of Shannon diversity index and taxon richness. Box-plots show median (central line), first and third quartiles (box) and tenth and ninetieth percentiles ('whiskers'). Differences between imported and non-imported soils are significant ($P < 0.05$).

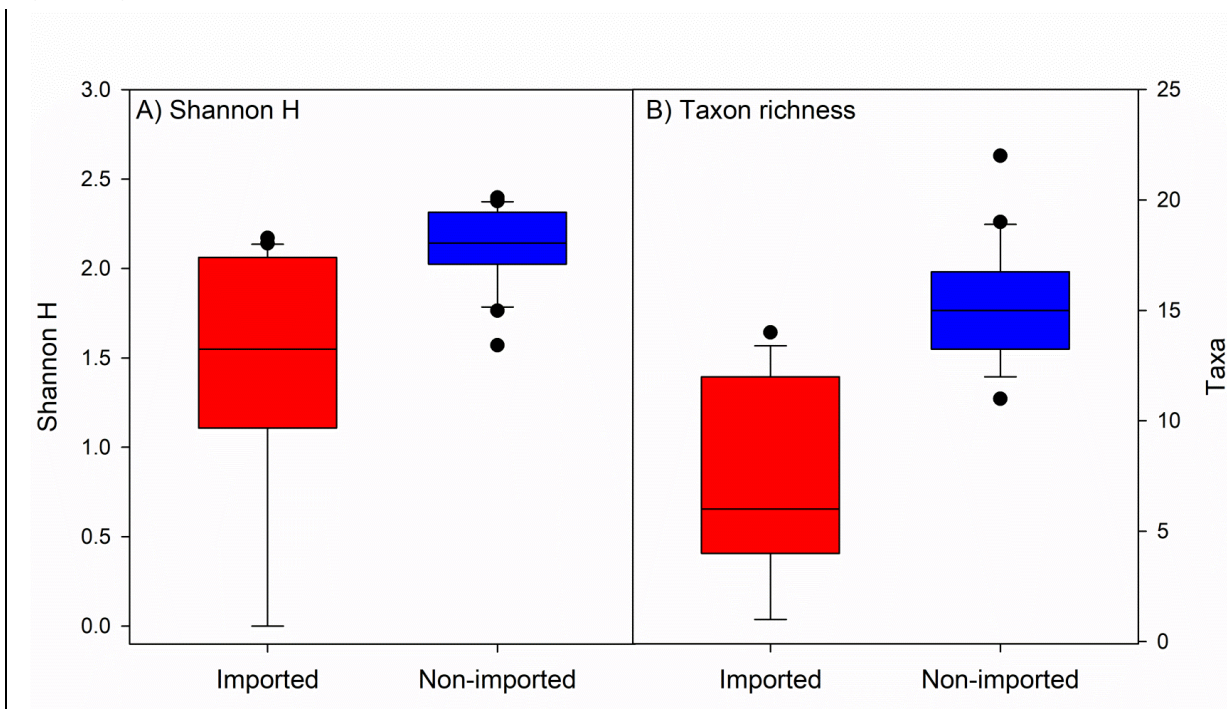


Figure 3. Comparison of testate amoeba data from Pyramiden (imported and non-imported soils) with reference data from elsewhere on Svalbard (Barentsburg region) and Chernozem soils in southern Russia.

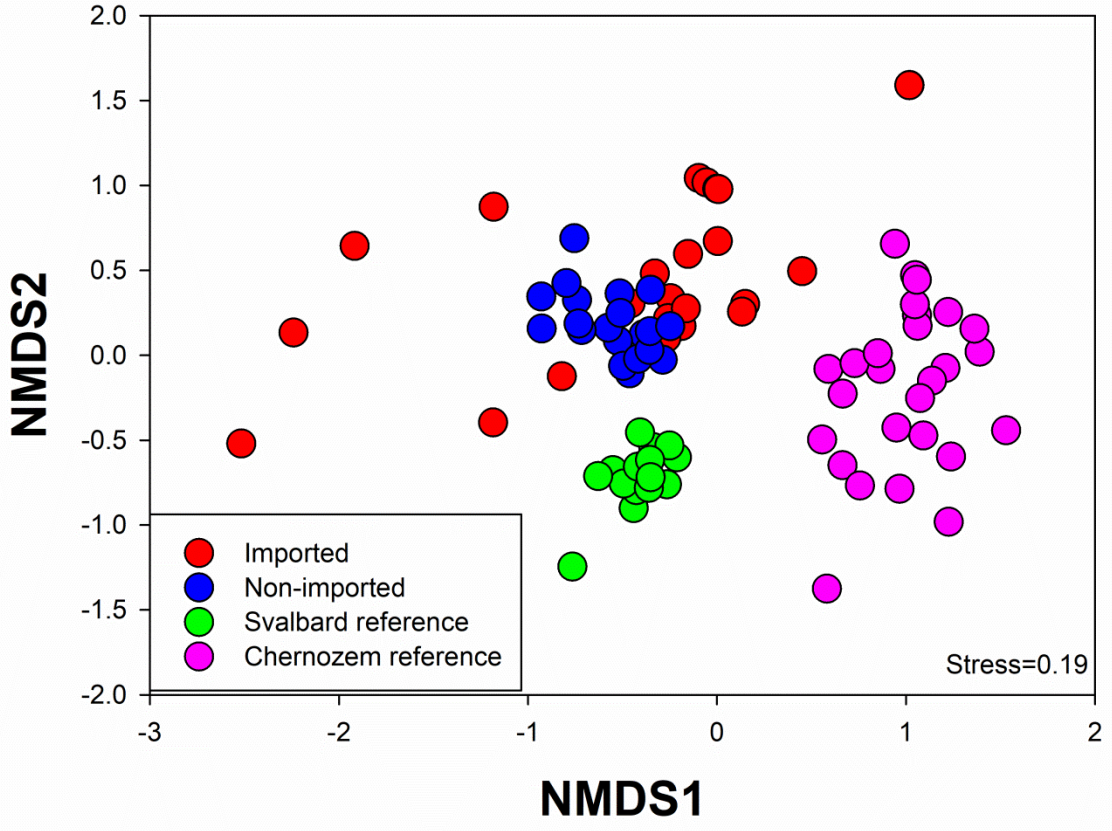


Table 1. Abundance of testate amoeba taxa in imported and indigenous soils. Taxa marked with an asterisk show significant ($P < 0.05$) difference between imported and non-imported soils in separate testing (permutation t-test).

Taxon	Relative abundance (%)	
	Imported	Non-imported
<i>Arcella arenaria compressa</i>	0.06	1.24*
<i>Arcella arenaria sphagnicola</i>	0.00	0.21
<i>Arcella catinus</i>	0.00	0.14
<i>Arcella rotundata stenostoma</i>	0.00	0.41*
<i>Assulina muscorum</i>	1.22	1.59
<i>Assulina seminulum</i>	0.00	0.35
<i>Centropyxis aculeata</i>	0.00	0.35
<i>Centropyxis aerophila</i>	6.77	10.91
<i>Centropyxis aerophila sphagnicola</i>	7.62	2.62*
<i>Centropyxis cassis</i>	3.60	4.07
<i>Centropyxis constricta</i>	0.37	0.28
<i>Centropyxis ecornis</i>	0.00	0.07
<i>Centropyxis gibba</i>	0.12	0.00
<i>Centropyxis orbicularis</i>	13.11	3.45*
<i>Centropyxis sylvatica</i>	5.91	2.76
<i>Centropyxis platystoma</i>	0.12	0.00
<i>Corythion dubium</i>	0.12	0.21
<i>Cryptodiffugia sacculus</i>	0.00	0.55
<i>Diffflugia bicornis</i>	0.06	0.00
<i>Diffflugia globulosa</i>	0.00	0.14
<i>Diffflugia penardi</i>	0.30	1.80
<i>Diffflugia pyriformis</i>	0.00	0.07
<i>Euglypha compressa</i>	0.00	0.48*
<i>Euglypha compressa glabra</i>	0.00	1.80*
<i>Euglypha cristata</i>	0.37	0.00
<i>Euglypha laevis</i>	13.05	17.61
<i>Euglypha rotunda</i>	6.95	5.73
<i>Euglypha strigosa</i>	0.00	0.14
<i>Euglypha simplex</i>	0.37	0.28
<i>Euglypha tuberculata</i>	0.30	1.17
<i>Heleopera petricola</i>	0.00	1.24*
<i>Nebela bohémica</i>	0.00	0.83*
<i>Nebela collaris</i>	0.00	0.07
<i>Phryganella acropodia</i>	3.11	2.69
<i>Plagiopyxis callida</i>	4.21	5.04
<i>Plagiopyxis declivis</i>	0.12	0.00
<i>Plagiopyxis penardi</i>	4.51	2.69
<i>Tracheleuglypha dentata</i>	0.24	1.04*
<i>Trinema complanatum</i>	0.06	0.14
<i>Trinema enchelys</i>	0.30	0.00
<i>Trinema lineare</i>	26.52	27.42

<i>Trygonopyxis arcula</i>	0.24	0.41
<i>Trygonopyxis arcula minor</i>	0.24	0.00