# Evaluating restoration trajectories using DNA metabarcoding of invertebrates and their associated plant communities

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**Abstract**

Invertebrate communities provide many critical ecosystem functions (e.g. pollination, decomposition, herbivory and soil formation), and have been identified as indicators of ecological restoration. Unfortunately, invertebrates are often overlooked in restoration monitoring because they are time-consuming to survey, often require rare taxonomic expertise, and there are many undescribed species. DNA metabarcoding is a tool to rapidly survey invertebrates and can also provide information about plants with which those invertebrates are interacting. Here we evaluate how invertebrate communities may be used to determine ecosystem trajectories during restoration. We collected ground-dwelling and airborne invertebrates across chronosequences of mine-site restoration in three ecologically different locations in Western Australia, and identified invertebrate and plant communities using DNA metabarcoding. Ground-dwelling invertebrates showed the clearest restoration signals, with communities becoming more similar to reference communities over time. These patterns were weaker in airborne invertebrates, which have higher dispersal abilities and therefore less local fidelity to environmental conditions. Invertebrate community recovery was most evident in ecosystems with relatively stable climax communities, while the trajectory in the Pilbara, with its harsh climate and unpredictable monsoonal flooding, was unclear. Plant assay results indicate invertebrates are foraging locally, providing data about interactions between invertebrates and their environment. Thus, we show how DNA metabarcoding of invertebrate communities can be used to evaluate likely trajectories for restoration. Testing and incorporating new monitoring techniques such as DNA metabarcoding is critical to improving restoration outcomes, and is now particularly salient given the ambitious global restoration targets associated with the UN decade on Ecosystem Restoration.

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## Abstract

Invertebrate communities provide many critical ecosystem functions (e.g. pollination, decomposition, herbivory and soil formation), and have been identified as indicators of ecological restoration. Unfortunately, invertebrates are often overlooked in restoration monitoring because they are time-consuming to survey, often require rare taxonomic expertise, and there are many undescribed species. DNA metabarcoding is a tool to rapidly survey invertebrates and can also provide information about plants with which those invertebrates are interacting. Here we evaluate how invertebrate communities may be used to determine ecosystem trajectories during restoration. We collected ground-dwelling and airborne invertebrates across chronosequences of mine-site restoration in three ecologically different locations in Western Australia, and identified invertebrate and plant communities using DNA metabarcoding. Ground-dwelling invertebrates showed the clearest restoration signals, with communities becoming more similar to reference communities over time. These patterns were weaker in airborne invertebrates, which have higher dispersal abilities and therefore less local fidelity to environmental conditions. Invertebrate community recovery was most evident in ecosystems with relatively stable climax communities, while the trajectory in the Pilbara, with its harsh climate and unpredictable monsoonal flooding, was unclear. Plant assay results indicate invertebrates are foraging locally, providing data about interactions between invertebrates and their environment. Thus, we show how DNA metabarcoding of invertebrate communities can be used to evaluate likely trajectories for restoration. Testing and incorporating new monitoring techniques such as DNA metabarcoding is critical to improving restoration outcomes, and is now particularly salient given the ambitious global restoration targets associated with the UN decade on Ecosystem Restoration.

## Introduction

Fauna are often overlooked in restoration monitoring in favor of vegetation (Cross, Tomlinson, Craig, Dixon, et al. 2019; Ruiz-jaen and Aide 2005; Gomes Borges et al. in press), with the general assumption that they will naturally recolonize an area with the return of plant communities (Palmer, Ambrose, & Poff, 1997). However, this is not always the case (Cristescu, Rhodes, Frére, & Banks, 2013), and understanding the recovery of fauna is important because they play a vital role in many ecosystem function including pedogenesis, seed dispersal, pollination and nutrient cycling (Bronstein, Alarcón, & Geber, 2006; Catterall, 2018; Hunter, 2001; Ness, Bronstein, Andersen, & Holland, 2004). Recently, greater attention has been paid to fauna to both assess and facilitate ecological restoration (Catterall 2018; Cross, Bateman, and Cross 2020; Majer 2009).

Invertebrates are of particular interest as they have long been used as indicators of ecosystem recovery in both aquatic and terrestrial systems (Andersen et al. 2002; Andersen & Sparling, 1997; Folgarait, 1998; Majer, 2009). They are sensitive to disturbances and are essential for ecosystem function (Folgarait, 1998; Rosenberg, Danks, & Lehmkuhl, 1986), not to mention being numerous, easy to capture, and incredibly diverse (Gaston, 1991). Because studies tend to target particular groups of arthropods, responses to restoration are mixed, depending on the target taxa (Cristescu, Frère, & Banks, 2012). Some of the variation in responses to restoration among different arthropod classes may be attributed to dispersal ability. For example, beetles with high dispersal abilities are able to recolonize more quickly than millipedes in a regenerating forest (Magura et al., 2015). Along with dispersal ability, changes in community composition during restoration (Andersen et al. 2002; Majer 2009) have been attributed to a shift from generalist r-strategist species, which thrive in disturbed and unpredictable environments, to K-selected species, which require predictable, and favorable environments (Majer 1989). As such, invertebrate communities may be used to evaluate restoration trajectories of recovery or convergence, where the objective and expectation is *directional*change in composition towards a reference community (Mcdonald et al. 2016; Suding and Gross 2006). However, in harsher ecosystems that are often naturally unpredictable, the lower diversity and selection of A (adversity)-strategists (Southwood 1977; Dunlop et al. 1985; Majer 1989) may make directional changes during restoration less likely.

Despite being excellent indicators of ecosystem change, the high diversity within invertebrate communities makes it particularly difficult to identify invertebrate specimens, often requiring many expert person-hours

from multiple taxonomists specializing in different invertebrate taxa (Majer 1983). This process is costly and time consuming, and dependent on taxonomic expertise that is dwindling worldwide (Pearson, Hamilton, & Erwin 2011; Majer et al. 2013). Additionally, many invertebrate taxa are cryptic (Smith, Fisher, & Hebert, 2005) or have yet to be identified, especially in Australia with its high degree of endemism (Austin et al., 2004; Rix et al., 2015) and where as much as 75% of arthropod diversity is undescribed (Austin et al., 2004; Yeates, Harvey, & Austin, 2003). Consequently, most studies looking at invertebrate responses to restoration have targeted particular taxa either because they have previously shown to be good bioindicators (Andersen et al. 2002), or they are threatened and of legal and conservation value (i.e. Lepidoptera) (Majer, 2009).

Some of the difficulties associated with invertebrate monitoring can be reduced using DNA metabarcoding to provide taxonomic assignments. This process uses high-throughput sequencing to determine invertebrate diversity from small barcoding regions of the genome (Beng et al., 2016; Ji et al., 2013; Yu et al., 2012). Compared to morphological identification, where each specimen has to be identified individually, DNA metabarcoding has been shown to be accurate, reliable, and faster than conventional morphological methods (Beng et al., 2016; Ji et al., 2013). As an added benefit, the sequencing data can be readily stored and analyzed by a third party, such as regulators (Fernandes et al., 2018). Although abundance estimates using DNA metabarcoding are often skewed by primer bias (Elbrecht & Leese, 2015) or DNA extraction method (Majaneva et al., 2018), presence/absence data has been used to demonstrate arthropod responses to restoration post mining (Fernandes et al., 2019) and land-use change (Beng et al., 2016).

One of the advantages of DNA metabarcoding is its ability to detect not only invertebrate diversity and composition but also provide functional data by identifying the organisms they have been interacting with (Jurado-Rivera et al., 2009; Pornon et al., 2016). In the case of arthropods, previous studies suggest that DNA from arthropod samples should be able to identify which plant species pollinators have visited (Pornon et al., 2016) and which plant species they have consumed (Jurado-Rivera et al., 2009). However, these studies have hitherto not been undertaken in a restoration context, so the utility of such approaches for restoration monitoring is unknown. Presumably, assessing these communities can illustrate the interaction between invertebrates and plants during restoration. However, since the invertebrates may carry plant DNA from outside the restoration area (van der Heyde et al., 2020a), they may not necessarily have high fidelity to local conditions.

Our earlier work has explored the use of DNA metabarcoding of ground-dwelling invertebrates to monitor mine site restoration (Fernandes et al., 2019); however, this study used a single reference site per mine and the results were spatially auto-correlated as older sites were closest to the reference sites. Here we use two spatially separated reference sites per mine, two trap types that capture ground dwelling and airborne invertebrates, and study sites in multiple locations with different climates and ecosystems. This study evaluates whether we can use DNA metabarcoding of invertebrates to evaluate restoration trajectories (convergence to reference communities) in restored sites. We have four hypotheses:

i) Ground dwelling invertebrates will show recovery trajectories better than airborne invertebrates because with lower dispersal abilities they better reflect local environmental conditions.

ii) Ecosystems with stable climax communities demonstrate trajectories of recovery more clearly than less diverse, climatically harsher unpredictable ecosystems.

iii) The plants associated with invertebrates will not show trajectories of recovery as well as invertebrates because plant DNA may be sourced from outside the site area.

iv) Metabarcoding provides functional information by indicating how invertebrate communities are interacting with the plants in and around restoration sites

## Materials and Methods

### Study Sites

Restoration and reference sites were sampled from three locations up to 1000 km apart in Western Australia, namely: Swan Coastal Plain (SCP); Jarrah Forest (JF); and Pilbara (PB). There was consistency in restoration approaches, soil type, climate and site aspect of the sites within each location. At each location, sites of different restoration age were sampled along with two spatially separated reference sites (Figure 1, see Figure S1 for maps). At all three locations, we sampled at least two sites less than 9 years old (Young), and at least two sites older than 9 years (Older). These sites are previously described in van der Heyde et al. (2020b), and briefly below. At all locations two reference sites were selected on the basis of the following criteria: similarity to ecosystems that are the target of restoration efforts, proximity to restoration sites, similarity in slope and aspect, and spatially separate from each other to account for variation in reference communities.

The Coastal Plain has a warm-summer Mediterranean climate with mild cool wet winters; mean minimum temperature 12.8°C, mean maximum 24.7°C, and with 757 mm mean annual rainfall (Australian Bureau of Meteorology). This location is part of the broader region of south-western Australia, a globally recognized biodiversity hotspot (Myers et al., 2007). The mine is located on the silicaceous Bassendean dunes, with high acidity and low water-holding capacity (Dodd & Heddle, 1989; McArthur, 1991). The ecosystem is referred to as Banksia woodland after the dominant tree species, *Banksia attenuata* and *B. menziesii* . Other trees include less dominant *Eucalyptus todtiana* and *Nuytsia floribunda.* The understory consists of woody species of Myrtaceae, Ericaceae, Proteaceae, and non-woody species in Anthericaceae, Stylidiaceae, Cyperaceae, and Haemodoraceae (Trudgen, 1977). In October 2018, we sampled eight sites at a Hanson Construction Materials sand quarry in Lexia (31.76 °S, 115.95 °E), with two reference sites and restoration sites 1, 3, 7, 11,14, 22 years old. The sites have been restored with the aim of returning mined areas to the surrounding native Banksia woodlands. All restoration was done by Hanson and previous mine owners and included direct transfer of fresh topsoil, ripping, and seeding with native plant species. Plant species richness and density tended to be higher in restoration than reference sites, and percent cover has increased with restoration age and is highest in reference sites (Benigno, Dixon, & Stevens, 2013).

The second location in the Jarrah (*Ecalyptus marginata* ) forest is also part of the Southwest Australia Global Biodiversity hotspot (Myers et al., 2007) and has a similar hot-summer Mediterranean climate; mean minimum temperature of 8.6°C, mean maximum of 23.7°C, and 668.9 mm annual mean rainfall (Australian Bureau of Meteorology). The lateritic soils are nutrient poor and high in gravel with surfaces rich in iron and aluminum (McArthur, 1991). The vegetation is dominated by *E. marginata;* other common trees are *E. patens,* and *E. wandoo* . The understory consists of sclerophyllous shrubs from several families including Anthericaceae, Fabaceae, Asteraceae, Proteaceae, Dasypogonaceae, and Myrtaceae (Havel, 1975). We sampled six sites from the bauxite mine which is now run by South32 (32.96°S, 116.48°E) in October 2018; two reference sites and restoration sites 2, 6, 11, and 20 years old. All restoration was undertaken by South32 or the previous mine owners. After mining the landscape was shaped using waste material and gravel. Fresh topsoil was directly transferred from newly mined areas to the restoration area and supplemented with stockpiled topsoil as needed. The sites were then ripped, seeded with over 100 native species, recalcitrant plants (mostly grasses) were planted, and a one-time treatment of superphosphate is applied (Data from South32). Reference and restoration sites are dominated by Myrtaceae and Fabaceae species. Total cover has increased with age of restoration to similar cover percentages of reference sites (Data from South32).

The third location, the Pilbara, is in north-western Australia. The Pilbara has a hot, arid climate with most rainfall occurring in summer, and associated with cyclonic activity (McKenzie, van Leeuwen, & Pinder, 2009) causing unpredictable flooding. Temperatures have a mean minimum of 15°C and mean maximum of 30.6 °C, with 263.8 mm mean rainfall (Australian Bureau of Meteorology). The unfavourable conditions and large variation in yearly rainfall are thought to select for a wide range of r-and A-strategist invertebrates (Majer, 1989). Soils are acidic stony loams with low fertility, which support open woodlands of snappy gum (*E. leucophloia* ) over hummock grasses (*Triodia wiseana, T. basedowii, T. lanigera* ) and low *Acacia* shrubs (McKenzie et al., 2009). The Pilbara is a significant mining region and accounts for 39% of global iron ore

production (Government of Western Australia, 2019). We sampled six sites at a BHP iron ore mine (22.84 °S, 118.95 °E) in September 2018, with two reference sites and restoration sites 4, 7, 11, and 15 years old. Restoration was conducted by the mine owners; landscapes were reformed and stockpiled topsoil (average age 10 years) was applied and then ripped. Restoration areas tended to have higher coverage of woody shrubs (*Acacia* ), while reference sites and older restoration areas have more hummock grasses (*Triodia* ). Restoration areas also had invasive species such as buffel grass (*Cenchrus ciliaris* ) and kapok bush (*Aerva javanica* ), which were absent in reference sites (Data from BHP).

**Sample Collection**

At each site we collected 10 invertebrate samples, five from vane traps and five from pitfall traps (n=200). Each vane trap sample included the contents of a yellow and blue vane trap with 150 mL of ethylene glycol and was left on the site for 7 days. Each pitfall trap sample included the contents of four pitfall traps (4 cm diameter, 12 cm deep with ethylene glycol as a capture fluid), and was also left in the field for 7 days Pitfall traps were spaced 10 m apart in a square around the vane traps in the center for each sample point.

**Sample Processing**

For DNA extraction, we first rinsed off the ethylene glycol with de-ionized water using 20-µm sieves that were sterilized in bleach and under UV light between every sample. Samples were then homogenized using a TissueLyser (Qiagen) for 2 min in 30 sec increments at 30/s in 50mL falcon tubes with 4 steel balls (4 mm diameter). 400 µL of the homogenate was digested overnight and the DNA extracted using the DNeasy Blood and Tissue kit (Qiagen) on the QiaCube Connect automated platform (Qiagen). The final elution volume was 200 µL, and extraction controls (blanks) were carried out for every set of extractions. Quantitative PCR (qPCR) was run on neat extracts and a 1/10 dilution to see if samples exhibited inhibition, and to determine optimal DNA input for PCR for each sample to maximize input relative to any inhibitors (Murray, Coghlan, & Bunce, 2015). Two assays were used in this study to target invertebrate and plant diversity. The invertebrate assay used the primers fwhF2/fwhR2n (Vamos, Elbrecht, & Leese, 2017) to amplify a 205 bp section of the cytochrome c oxidase I (COI) region. For plants we used the trnlc/h primers (Taberlet et al., 2007) which targets the chloroplast trnL (UAA) intron

The qPCRs were run on a StepOne Plus (Applied BioSystems) real-time qPCR instrument with the following conditions: 5 min at 95°C, 40 cycles of 95°C for 30s, 30s at the annealing temperature (50°C for invertebrates, 52°C for plants) and 45s at 72°C, a melt curve stage of 15s at 95°C 1 min at 60°C and 15s at 95°C, ending with 10 min elongation at 72°C. The PCR mix for quantitation contained: 2.5 mM MgCl2 (Applied Biosystems, USA), 1× PCR Gold buffer (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4 mg/ml bovine serum albumin (Fisher Biotec, Australia), 0.4 µmol/L forward and reverse primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies, USA). Extraction control and non-template controls were included in qPCR assays.

After optimal DNA input was determined by qPCR, each sample was assigned a unique combination of multiplex identifier (MID) tags for each primer assay. These MID tags were incorporated into fusion tagged primers, and none of the primer-MID tag combinations had been used previously in the lab to prevent cross contamination. Fusion PCRs were done in duplicate and to minimize PCR stochasticity, the mixes were prepared in a dedicated clean room before DNA was added. The PCRs were done with the same conditions as the standard qPCRs described above. Samples were then pooled into approximately equimolar concentrations to produce a PCR amplicon library that was size-selected to remove any primer-dimer that may have accumulated during fusion PCR. Size selection was performed (150-450bp) using a PippinPrep 2% ethidium bromide cassette (Sage Science, Beverly, MA, U.S.A). Libraries were cleaned using a QIAquick PCR Purification Kit (Qiagen, Germany) and quantified using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). Sequencing was performed on the Illumina MiSeq platform using the 300 cycle V2 as per manufacturer's instructions.

### Sequencing analysis

Sequences were demultiplexed using a demultiplex function in the "insect" package (Wilkinson et al., 2018) on the R 3.5.3 platform (R Core Team, 2018). Further sequence processing was performed in R using the "DADA2" package (Callahan et al., 2016) where sequences were quality filtered, the error rates were estimated, and the sequences were dereplicated. The error rates were then used in the sample inference stage to remove sequences likely to be errors and leave Amplicon Sequence Variants (ASV). These ASVs are equivalent to zero radius operational taxonomic units (ZOTUs) in usearch (Edgar, 2016). The sequence table was then constructed and chimeras removed. Taxonomy was determined using the Basic Local Alignment Search Tool (blastn) on a high-performance cluster computer (Pawsey Supercomputing Centre) to search against the online reference database GenBank (*https://www.ncbi.nlm.nih.gov/genbank/*). Invertebrate sequences were also searched against and arthropod COI reference sequences extracted from the Barcode of Life Database (BOLD:*https://www.barcodeoflife.org*), because there are reference sequences that are found uniquely on one of the two databases. We used MEGAN (Huson et al., 2007) to assign taxonomy with a minimum support of 205.

### Statistics

All statistics were run using R 3.5.3 (R Core Team, 2018). Samples with low sequencing depth were removed and ASVs that were present in the extraction controls were removed from the dataset (Figure S2). We selected ASVs in the phylum 'Arthropoda' for the invertebrate assay and 'Plantae' for the plant assay. Copy numbers in each sample were filtered to a minimum of 0.05% within sample abundance. We verified there was no correlation between sequencing depth and ASV richness before continuing. Read counts were transformed to presence/absence to reduce the effects of biases (Elbrecht & Leese, 2015; Majaneva et al., 2018). Spatial autocorrelation was tested using the Mantel test in the 'ade4' package in R (Mantel, 1967). Three criteria were examined to determine if communities showed a trajectory of recovery or convergence to the reference community. First, community composition should be different between younger restoration, older restoration, and reference sites. This was visualized using Non metric multidimensional scaling (NMDS), based on presence/absence ASV table and with Bray-Curtis dissimilarity. The 'ordiellipse' function from the 'vegan' R package was used to draw ellipses showing the 95% confidence interval of the group (Oksanen et al., 2018). Differences between restoration ages were tested using permutational multivariate analysis of variance (PERMANOVA). Second, establishing a restoration trajectory requires *directional*change; we expect that restored communities become more similar to reference communities over time. Replicates at each site were pooled and the similarity between each site and the reference sites was calculated. This relationship was tested using linear models separately for each assay and location. Third, we expect that the proportion of 'reference' ASVs, that is, ASVs that were found in reference sites, would increase over time. This relationship was tested using a simple linear model. For all three, we tested the SCP data with and without the extra two sites (7 years and 11 years) to ensure that any comparisons of trajectory between the locations were fair. This analysis is based on the prediction of changing composition from r- or A- to K-strategists and provides additional information about whether the patterns in community similarity to reference communities is driven by compositional changes, or richness. Finally, to understand the taxa associated with restoration and reference sites, we ran a multipattern analysis for each site using the R package 'indicspecies' (De Caceres & Legendre, 2009).

## Results

In total, we generated 14,780,759 quality-filtered invertebrate sequences from 196 samples with a minimum of 3,000 reads/sample. Out of 5862 initial ASVs, 2635 belonged to the phylum Arthropoda. The remaining ASVs were either unidentified or fungi, and only made up 23.7% of the read count. In the plant assay, we generated 13,441,527 filtered plant sequences from 197 samples with a minimum of 5600 sequences/sample. From the initial 511 plant ASVs, 381 remained post filtering and these accounted for 87.8% of the sequences. Overall, there were fewer ASVs in the Pilbara compared to the Coastal Plain or Jarrah, especially in the

pitfall traps where the Pilbara had 17-32% fewer invertebrate ASVs (Table S1)

### Community Composition

Invertebrate diversity in the vane traps was dominated by Hymenoptera, Coleoptera, Diptera, Hemiptera, and Lepidoptera. Some of these (Hymenoptera, Coleoptera, and Hemiptera) also made up most of the diversity in the pitfall traps, along with Collembola and Araneae. Collembola were largely absent from the Pilbara, which had more Orthoptera ASVs. The majority (67%) of invertebrate ASVs could not be identified beyond order level. However, 99% of plant ASVs could be identified to family level. Plant diversity in the SCP and Jarrah forest was dominated by Myrtaceae, Fabaceae, Dilleniaceae, and Proteaceae, while in the Pilbara the richest families were Fabaceae, Poaceae and Malvaceae (Figure 2). Because of the poor taxonomic assignments, we confined our considerations to ASVs for our subsequent analyses.

There were significant differences in community composition between younger restoration, older restoration and reference sites in all locations for both trap types and assays (Figure 3, PERMANOVA, alpha=0.05). Similarly, the pairwise analysis showed all restoration ages were significantly different from one another (alpha=0.05), with the exception of the plant community in the Pilbara samples, where the reference samples were not significantly different from the younger (vane) or the older (pitfall) restoration samples (Table S2). The Mantel tests showed significant spatial autocorrelation in the invertebrate communities from pitfall traps but not the vane traps (alpha=0.05, Table 1). Similarly, the spatial correlation with community dissimilarity was lower in the plant sequences compared to the invertebrate assay (Table 1).

### Similarity to reference communities

The invertebrate communities showed clear directional changes (increasing similarity to reference over time) in the pitfall traps from the Coastal Plain and the Jarrah forest (Figure 4). This trajectory was less evident (in the SCP) or entirely absent in the vane traps (in the JF, PB). There were no observed directional changes in invertebrate community composition in the Pilbara. The results from the plant communities were different. In the Coastal Plain, there was a significant relationship between similarity to reference communities and age of restoration in the vane traps, but not the pitfall traps. The directional change in plant communities occurred in both the pitfall and vane trap samples in the Jarrah, but was significant only in the pitfall traps. Similarly, the plant communities became more similar to reference communities in the Pilbara pitfall traps, while there was not a relationship in the vane traps (Figure 4).

### Proportion of "reference" associated ASVs

Only the invertebrate communities from the pitfall trap samples from the Coastal Plain and the Jarrah forest showed significant increases in the proportion of 'reference' ASVs over time. For plant sequences, only pitfall traps in the Pilbara showed increasing 'reference' ASVs over time (Figure 5). Overall, the vane traps had a higher proportion of ASVs that were shared with reference samples than pitfall traps. This was true for both the invertebrate assay (49.4% vs 22.2% 'reference' ASVs) and the plant assay (78.6% vs 59.7% 'reference' ASVs). There was also a higher proportion of shared ASVs in the plant assay compared to the invertebrate assay (Figure 5). Between the two reference sites, there was variation in the number of ASVs shared with each other. The pitfall traps in the Pilbara only had 3 ASVs shared between the two reference sites (average of 1.2 $\pm$0.4ASVs per sample). The amount of shared ASVs was higher between the Coastal Plain and Jarrah pitfall traps (10 and 8 respectively).

### Multipattern Analysis

Across the three locations, there were 82 invertebrate ASVs with significant association (alpha =0.05) with younger restoration (<9 years), older (>9 years), reference sites, or a combination (Table S3). Of these, 44 were assigned to family, 16 to genus, and only 3 to species level. This includes the ant *Iridomyrmex sanguineus* , which was associated with younger restoration in the Pilbara and the ant *Monomorium rothsteini* , associated with reference sites in the Pilbara. Most Coleoptera (12/16) were associated with older restoration

or reference sites and 13 of those were from vane trap samples. Apidae ASVs were found mainly in the younger restoration vane traps. For the plant assay, there were 59 ASVs with significant association (Table S4), 52 of which were assigned to family, 21 to genus, and 8 to species level. Species included *Petrophile squamata* (Proteaceae),found in older restoration in the Jarrah, and *Duperreya commixta*(Convulvulaceae), found in vane traps of reference sites in the Pilbara (Table 2). Most Fabaceae ASVs (13/14) were associated with younger restoration in the Coastal Plain and Jarrah forest.

## Discussion

Terrestrial invertebrate fauna are key indicators of ecosystem change (Andersen et al., 2002; Majer, 2009; Majer, Brennan, & Moir, 2007), and in this study, we show that even with limited taxonomic identification, DNA metabarcoding of invertebrate samples can be used to rapidly assess complex biological interactions and establish restoration trajectories. These trajectories of community recovery were more evident in stable climax ecosystems and in ground-dwelling invertebrates with lower dispersal ability than airborne invertebrates. Examining plant diversity associated with invertebrate samples also showed some indications of directional changes in community composition and indicates that invertebrates are likely foraging locally.

### Ground-dwelling vs airborne invertebrate

Vane traps do not show the same local fidelity as pitfall traps and, as expected, tend to have weaker indications of community recovery (Figure 3, Figure 4). Vane traps capture airborne invertebrates, often pollinators (Hall, 2018), and can trap organisms that may come from more than 1.8 km away (Jha & Dick, 2010) while species caught by pitfall traps have more limited dispersal (Majer, 1980; Ness et al., 2004; Ward, New, & Yen, 2001). This would also explain the greater proportion of shared taxa in the vane traps compared to the pitfall traps (Figure 5), and the greater spatial correlation in pitfall trap samples (Table 1). Beyond the differences in attraction distance of the traps, our results also suggest quicker recolonization of airborne invertebrates as evidenced by the number of 'reference' associated taxa is similar to reference sites within a few years (Figure 5, SCP, PB). Variation in dispersal abilities is important as those with more mobility are able to recolonize areas more quickly (Magura et al., 2015) and from greater distance (Knop, Herzog, & Schmid, 2011). Fortunately, there is no sign of thermophilic or other barriers (Cranmer, McCollin, & Ollerton, 2012; Tomlinson et al., 2018) preventing invertebrates from accessing and using restoration sites. Because of their more sedentary nature, ground-dwelling invertebrates are good indicators of organisms that are likely reproducing in situ, while airborne invertebrates can indicate the forage support and attractiveness of a site. Our findings indicate that invertebrate communities are demonstrating an ability to recover without intervention following the establishment of plant communities. This conforms with the 'Field of Dreams' hypothesis which posits that if suitable habitat can be re-established, species will colonize it, leading to the restoration of function (Palmer et al., 1997). However, this is dependent on the presence of source populations. In this study, all sites were near remnant vegetation that could act as a taxa pool; in cases of isolated restoration sites, it may be more difficult to evaluate restoration trajectories using invertebrate communities.

### Stable vs unpredictable ecosystems

The r/K selection theory is a predictive model for life history strategies that vary from r selected (high fecundity, short lifespan, small bodies, opportunistic, high dispersal) in unpredictable environments, to K-selected (low fecundity, long lifespan, large bodies, low dispersal) in unpredictable environments (Pianka 1970). In ecological succession and restoration, it is expected that systems are dominated by r-selected species initially as they take advantage of the disturbance, followed by a shift to K-selected species as the system develops towards a stable climax community (Majer, 1989). This concept is developed further by Southwood (1977) and Greenslade and Greenslade (1983) as a 'habitat template', which condenses the variety of habitats onto two axes equivalent to their favourableness and predictability. As well as explaining the conditions for r- and K-strategists, this template introduces a third adversity or A-selection strategy, which is selected

for in environments that are very unfavourable and not always predicable. Such environments, including the Pilbara, support lower diversities of organisms with lower interaction between species (Greenslade and Greenslade 1983). In this study, we classified taxa based on whether they were found in reference sites as a proxy for selection strategy, since there was inadequate information to classify them based on taxonomic identification. As expected, in older restored sites we recorded significant increases in the proportion of 'reference' taxa with time in both the Jarrah and Coastal Plain (Figure 5), which shows a directional change in community composition toward that of the reference community.

The Pilbara location, which has a more unpredictable and harsher climate, did not show a similar trajectory of community recovery. Dunlop et al. (1985), and to a lesser extent, Fletcher (1990), observed that ant richness rapidly recovered in young Pilbara rehabilitation, but, similar to our results, the species composition remained different between natural and restored sites. In the Pilbara, the main factors driving compositional turnover in terrestrial fauna are regolith/soil and landform/hydrogeologic, as well as climate (Gibson et al. 2015). All were factors that were shared between Pilbara restored and reference sites. Here, the structure of the revegetation rapidly came to resemble the structure of the original predominantly grassland habitat (see Figure 1), which is in marked contrast to the situation at the other two locations. In that regard, the reference areas may provide conditions that are as unpredictable and unfavorable as the areas under restoration; and compared with the other two regions, they are also less rich in species. Thus, recolonization of Pilbara sites may be more stochastic and less influenced by selection pressures than in the Coastal Plain and Jarrah forest. However, there was a particularly low proportion of shared 'reference' taxa overall in the Pilbara pitfall traps (Figure 5), so ecosystem recovery is far from complete.

**Plants associated with invertebrate samples**

Generally, directional changes in community composition were less evident in the plant diversity associated with invertebrate samples (Figure 3, Figure 4). This was expected, as we hypothesized that the signal would be diluted because invertebrates can carry plant DNA from outside the study area (van der Heyde et al., 2020a). The lack of abundance or behavior data is a commonly acknowledged limitation of DNA metabarcoding (Elbrecht & Leese, 2015; Elbrecht, Peinert, & Leese, 2017; Fernandes et al., 2018; Lim et al., 2016). However, the clear difference in plant assay community composition between restoration sites (Figure 3) indicates that the invertebrates are interacting with plants locally on the restoration site, rather than only passing through. Plant sequences reflected some site characteristics, generating a greater richness of Fabaceae ASVs in younger restoration sites observed to have high cover of *Acacia* shrubs (Data from BHP, South32). While some plant DNA may originate from debris falling into traps, there is also evidence that these are plants that were ingested or otherwise visited by invertebrates. For example, plants in the family Goodeniaceae require insect pollination (Jabaily et al., 2012; Keighery, 1980). While there are virtually no Goodeniaceae ASVs in the pitfall traps (PB and JF), they are present in most sites in vane traps (PB and JF, Figure 2). Unfortunately, we cannot identify which invertebrates are interacting with which plants. This would require isolating invertebrates and extracting DNA from each species separately, for example by extracting DNA from the pollen loads (Bell et al., 2017; Pornon et al., 2016). Alternatively, DNA from flowers has also been used to identify probable pollinators (Thomsen & Sigsgaard, 2019). However, these methods require species-specific sampling and therefore far more samples and greater costs. We argue that using bulk arthropod samples is a cost, time and resource efficient method that allows researchers to gain an informative snapshot of the invertebrate community and the plants they are interacting with.

Importantly, as this study was conducted in the spring/early summer, we cannot confirm whether the same patterns exist throughout the year. Seasonality affects invertebrate communities (Santorufo, Van Gestel, & Maisto, 2014; Shimazaki & Miyashita, 2005), plant communities, and especially the interaction between the two (CaraDonna et al., 2017; Rico-Gray et al., 1998). A previous study conducted during autumn (April) in the Coastal Plain sites using pitfall traps also detected directional changes in invertebrate communities (Fernandes et al., 2019), but no differences in plant communities generated from pitfall traps (Unpublished). In the spring there is more new plant growth and flowering, resulting in more invertebrates that use those resources (Clark & Dallwitz, 1974; Herrera, 1988). This study offers preliminary testing of consistency in

restoration patterns across space, but not temporally within or between years.

## Conclusion

We have demonstrated the use of high throughput sequencing of invertebrate samples to establish restoration trajectories. Defining the likely trajectory of a restored site is important as it enables the definition of success criteria, and the required time scales for restoration monitoring. We show that trajectories towards reference ecosystems were more evident in ground dwelling invertebrates in stable climax ecosystems. Despite the lack of abundance data, metabarcoding can indicate functional ecosystem recovery by showing how the invertebrates are interacting with the plant community. Understanding restoration trajectories using DNA metabarcoding will require additional research to determine the effects of seasonal variation, and consistency of patterns across multiple years and different ecosystems. It is important to remember that ecosystems are dynamic, so determining whether sites have been fully restored depends heavily on the selection of appropriate reference sites to capture the natural variation in the reference ecosystem. The Bonn Challenge goal to restore 350 million $km^2$ of degraded terrestrial ecosystems by 2030 (Suding et al., 2015) means we must ensure that we get the best value from the considerable financial investment required to meet these ambitious global restoration targets. Testing new monitoring techniques such as DNA metabarcoding and evaluating where they are beneficial is critical to potentially incorporating them in restoration projects and improving restoration outcomes.

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## Data Accessibility

Sequencing and sample data and is available at the Dryad Digital Repository:*https://doi.org/10.5061/dryad.q573n5tgw*

## Author Contributions

MvH conducted the study and wrote the manuscript. MvH, PN, MB, NW, and GW-J were involved in the experimental design. Samples were collected and processed by MvH; molecular and bioinformatics work was performed by MvH; all data was analyzed and processed by MvH; statistical analysis was done by MvH; the manuscript was edited by all authors.

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**Tables and Figures**

Table Results of the Mantel test showing the correlation between spatial distances and community dissimilarity. Results for the samples separately, and pooled (sites) are shown. SCP-Swan Coastal Plain, JF-Jarrah Forest, PB-Pilbara.

| | | | Samples | Samples | Sites | Sites |
|---|---|---|---|---|---|---|
| Trap | **Assay** | **Location** | r | p | r | p |
| Pitfall | Invertebrate | JF | 0.161 | 0.001 | 0.189 | 0.205 |
| | | PB | 0.308 | 0.002 | 0.566 | 0.018 |
| | | SCP | 0.375 | 0.001 | 0.349 | 0.116 |
| | Plant | JF | 0.069 | 0.064 | -0.1858 | 0.706 |
| | | PB | -0.0118 | 0.508 | -0.203 | 0.838 |
| | | SCP | -0.077 | 0.785 | -0.2365 | 0.789 |
| Vane | Invertebrate | JF | 0.131 | 0.011 | 0.237 | 0.104 |
| | | PB | 0.114 | 0.082 | -0.084 | 0.548 |
| | | SCP | -0.117 | 0.851 | -0.074 | 0.555 |
| | Plant | JF | 0.0466 | 0.147 | -0.314 | 0.916 |
| | | PB | 0.233 | 0.007 | 0.079 | 0.319 |
| | | SCP | 0.115 | 0.125 | -0.359 | 0.874 |

Table Taxa of interest, based on general observations of the data and indicator species analysis

| Taxa of interest | Name | Reason |
|---|---|---|
| *Melophorus* | Australian genus of ant | Associated with younger restoration sites in both the SCP and PB. |
| *Iridomyrmex sanguineus* | Northern meat ant | Associated with younger restoration sites in PB. *Iridomyrmex* speci |
| Hemiptera | Order of sucking insects | Higher richness in younger restoration in the JF pitfall traps. Hemip |
| Apidae | Family of bees | Found primarily in the younger restoration sites in JF. Newly restor |
| Julida - *Ommatoiulus* | Portugese millipede | Invasive detritivore species found in great abundance in the SCP, pa |

| Taxa of interest | Name | Reason |
|---|---|---|
| Fabaceae | Legume family | ASVs in this family are strongly associated with younger restoration |
| *Goodenia microptera* | Narrow-winged Goodenia | An insect pollinated species found predominantly in the vane traps c |
| *Anigozanthos* | Kangaroo paw | Associated with younger restoration in SCP pitfall traps. These grow |

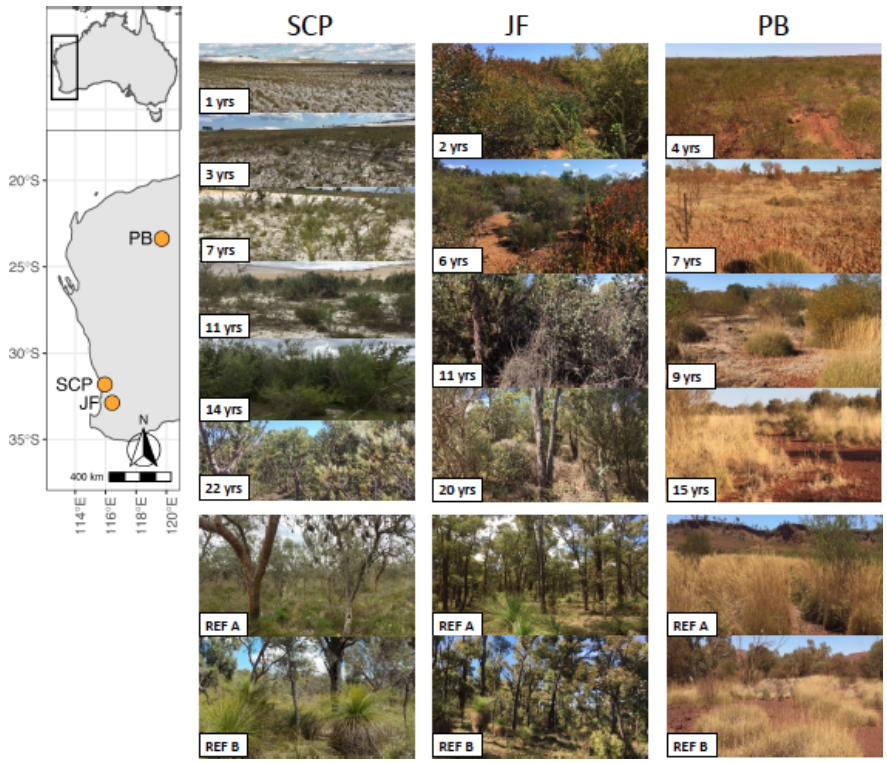


Figure Chronosequences of mining restoration where invertebrate samples were collected. Restoration sites shown with the number of years restoration from 1 to 22 years. Reference sites shown below. SCP-Swan Coastal Plain, JF-Jarrah Forest, PB-Pilbara.

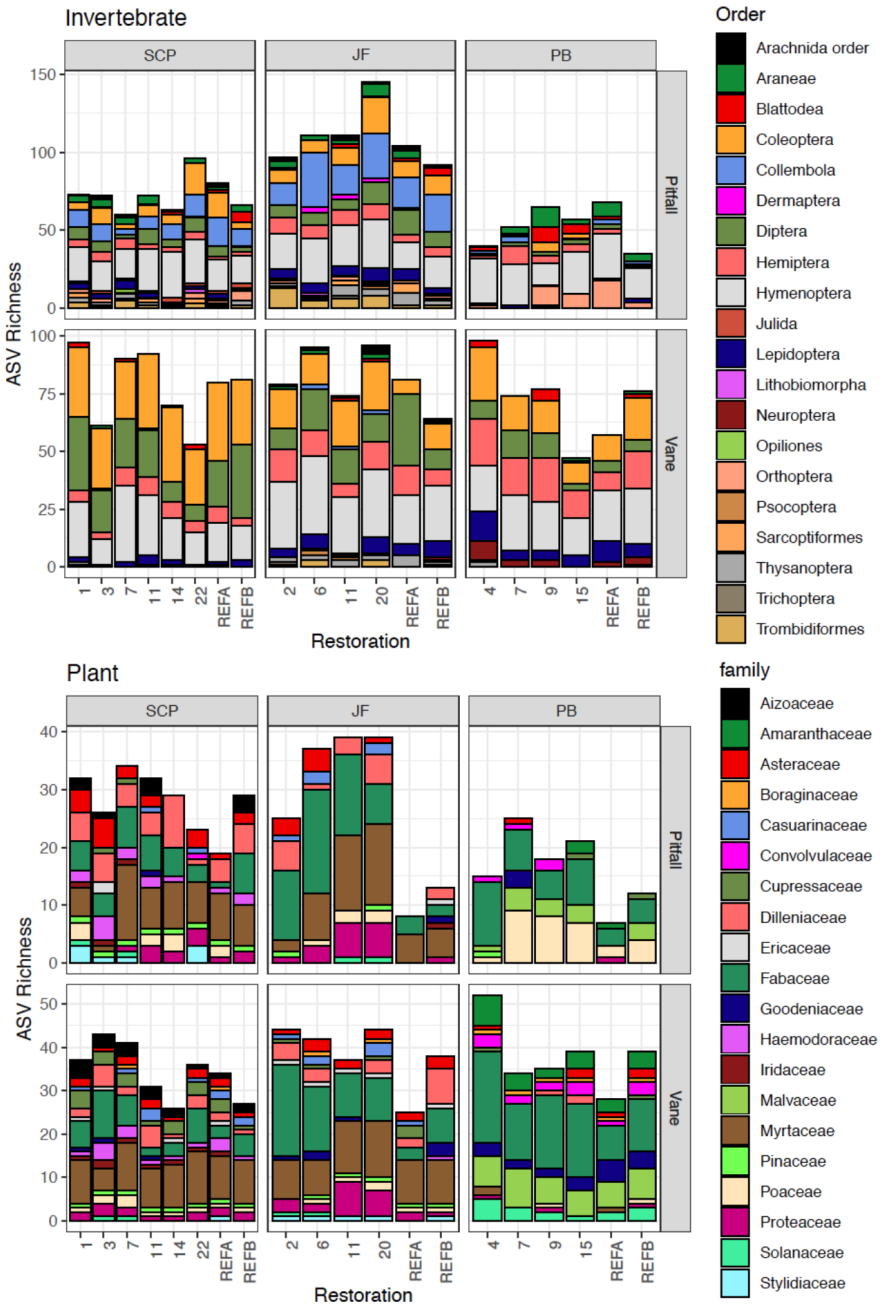


Figure Composition of invertebrates (above) and plant (below) communities detected from pitfall and vane traps. Shows the number of ASVs in each order (invertebrates) or family (plants) at all restoration and reference sites.

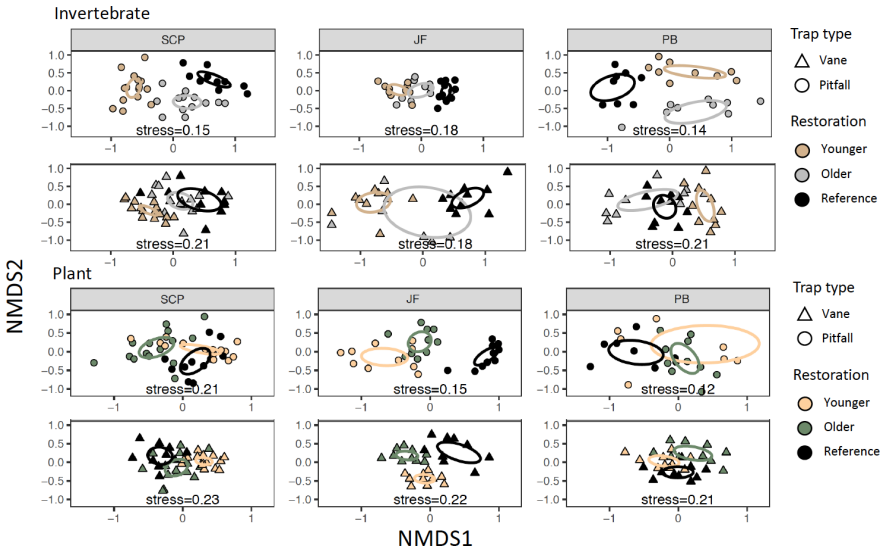


Figure NMDS ordinations or Invertebrate and plant communities in restoration and reference sites. Ellipses were drawn using 'Ordiellipse' in the vegan R package and indicate 95% confidence interval of the group. PERMANOVAs were significant for all facets (alpha=0.05).

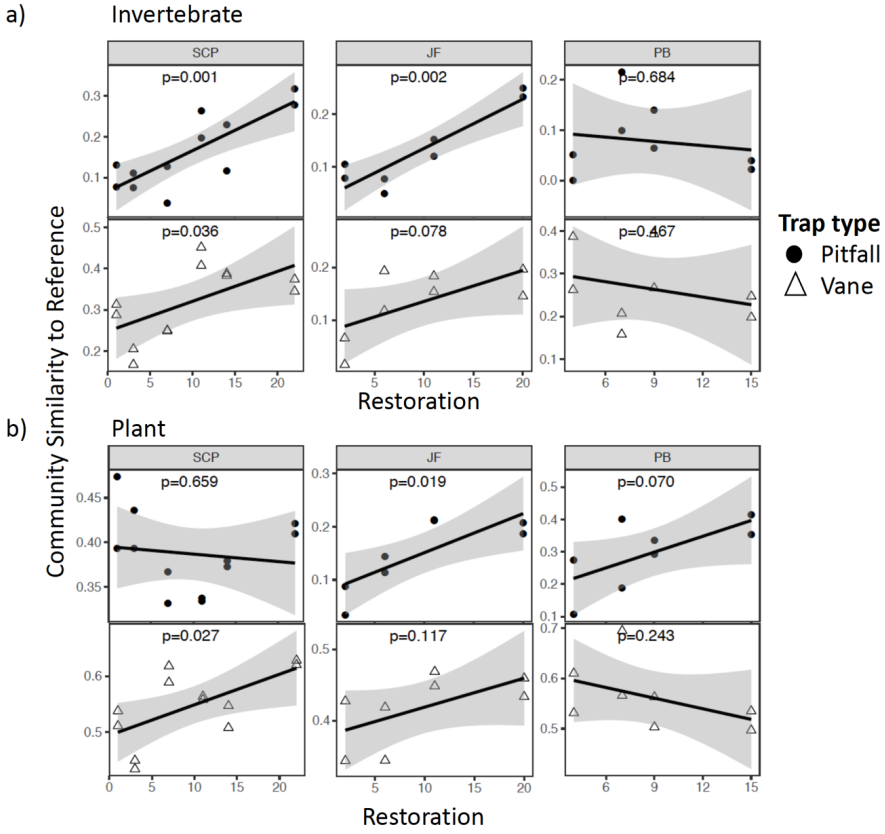


Figure Similarity (Bray-Curtis) of restoration sites of different ages (years) to communities in reference sites. Lines indicate linear models with 95% confidence interval shown with shading. P-values for the linear models

shown for each plot. Removing the two extra sites in the SCP (7,11 years) did not change the relationships or the significance of the models, with the exception of invertebrate communities from vane traps (from p=0.036 to p=0.053)

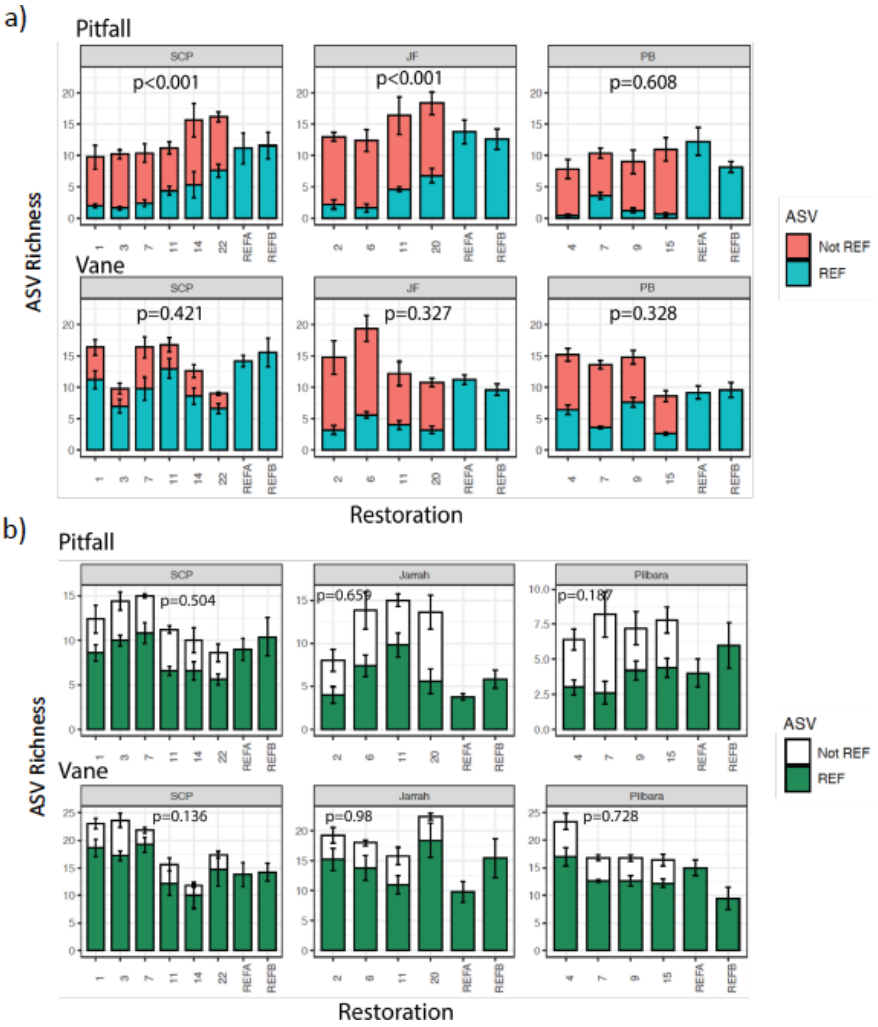


Figure ASV richness in the different sites, separated into ASVs that were present in reference sites and those not found in reference sites. Separated into a) invertebrate and b) plant communities. P-values indicate the significance of the relationship between the proportion of reference ASVs and age of restoration (years).