Title

Saltwater intrusion history shapes the response of bacterial communities upon rehydration

Running Title

Saltwater exposure shapes bacterial community response

Authors

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Highlights

- Soil microbes may be impacted by saltwater intrusion (SWI).
- We simulated a SWI event and documented changes in bacterial community composition.
- Sites *with no* history of SWI did not respond as they are not pre-conditioned to respond to saltwater.
- Sulfate-reducing bacteria increased following saltwater treatment at sites with a history of SWI.
- Saltwater impacts bacteria causing a shift in cycling of essential nutrients.

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Abstract

Saltwater intrusion (SWI) can result in the loss of dominant vegetation from freshwater habitats. In northern Australia, sea level is predicted to rise by 17-50 cm by 2030-2070. This will exacerbate the impact of SWI, threatening Ramsar-listed habitats. Soil bacteria in these habitats play a significant role in biogeochemical cycling, regulating availability of essential nutrients such as nitrogen to vegetation. However, there is limited understanding as to how SWI will impact these soil bacteria. Floodplain soil samples were collected from the South Alligator River floodplain in Northern Australia from sites with contrasting histories of SWI. A SWI event was simulated over 7 d with treatments of saltwater and freshwater. Bacterial community composition before and after treatment were measured using next generation sequencing of bacterial DNA. Sites with no history of SWI showed no significant changes in community taxonomic composition following treatments, suggesting the community at these sites have broad functional capacity which may be due to their historic conditioning over many years. Sites with a history of SWI showed a significant response to both treatments. Following saltwater treatment, there was an increase in sulfate-reducing bacteria, which have an impact on carbon and nitrogen cycling. We suggest that the impact of SWI causes a shift in the soil bacteria which alters the community to one which is more specialised, with implications for the cycling of essential elements and nutrients.

Keywords

Floodplain, tropical, soil, microbes, biogeochemical cycling, salinity

Abbreviations

SWI – saltwater intrusion | FW – freshwater | SW – saltwater | PCR – polymerase chain reaction

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1. Introduction

Soil bacterial communities are some of the most plentiful and diverse on the planet with an estimated 2.6 x 10^{29} cells (Whitman et al., 1998; Lozupone and Knight, 2007). In wetlands and freshwater habitats, soil bacteria contribute greatly to biogeochemical cycling of key nutrients, such as nitrogen, phosphorus, sulfur and methane, and are an important sink for carbon (Fuhrman, 2009). These nutrients are essential to plant growth, and the soil bacterial community has an important role in regulating their availability. However, the composition and function of bacteria can be altered by abiotic changes, such as salinity (Horz et al., 2004; Lozupone and Knight, 2007; Jeffries et al., 2012). Under increased salinity regimes, bacterial communities display increases in carbon cycling and photosynthesis and decreases in phosphate and nitrogen cycling (Jackson and Vallaire, 2009; Jeffries et al., 2012; Cañedo-Argüelles et al., 2014).

Saltwater intrusion (SWI) has a significant effect on freshwater ecosystems (Mulrennan and Woodroffe, 1998; Long et al., 2012). The process involves saltwater moving into freshwater habitats due to a number of complex local features, including tidal influences, low altitude, sea-level rise, rainfall, boat traffic and the impact of feral animals (Mulrennan and Woodroffe, 1998; Petty et al., 2007; Hughes, 2010). This can result in the die-off of dominant vegetation and the loss of suitable habitat for aquatic and terrestrial organisms (Winn et al., 2006; Bowman et al., 2010). Grasses such as *Pseudoraphis spinescens* and *Hymenachne acutigluma,* which are a major component of the vegetation on freshwater floodplains, and *Melaleuca* species will potentially be lost due to SWI (Finlayson, 1991). The debris of these grasses left at the end of the wet season and the leaf litter from the *Melaleuca* species is rich in nitrogen, phosphorous and potassium and they are important contributors to elemental cycling on the floodplains (Finlayson, 1991; Finlayson et al., 1993). Thus, a loss of these grass and *Melaleuca* species causes a decrease in available nutrients such as nitrogen. Freshwater vegetation species in Kakadu National Park are predicted to decline at ≈3.7 psu (practical salinity units) while mangroves in Northern Australia prefer a moderate salinity range of 16 to 50 psu (Ball, 1998). These findings suggest that following SWI, there will potentially be a period of low vegetation and a decrease in nutrient availability on the floodplains.

The Intergovernmental Panel on Climate Change predictions suggest increases of 17-50 cm by 2030-2070 (Stocker et al., 2013). This rise will amplify the occurrence of SWI in many areas, threatening the ecological function and maintenance of biodiversity in high-value

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wetlands. Because of the region's low topography, extensive areas in Northern Australia are susceptible to sea level rise (Hughes, 2010). Some areas have already undergone dramatic changes caused by SWI (Mulrennan and Woodroffe, 1998; Petty et al., 2007). On the Lower Mary River floodplains located adjacent to Kakadu National Park, more than 17,000 ha of freshwater habitat have been destroyed due to SWI (Mulrennan and Woodroffe, 1998; Bowman et al., 2010). This example provides a window into potential future impacts that predicted sea level rise scenarios could have on nearby World Heritage-Listed Kakadu National Park and its extensive range of Ramsar-listed freshwater habitats.

To investigate the impact of SWI on the soil bacterial community of these wetland systems, we simulated a lab-based SWI event on floodplain soils collected from sites with contrasting histories of SWI. Changes in bacterial community function and biogeochemical cycling is often indicated by changes in bacterial community composition (Reed and Martiny, 2013). Therefore, bacterial community composition was monitored before and after treatments with saltwater and freshwater.

2. Materials and methods

2.1. Study sites

The South Alligator River is located 220 km east of Darwin in the World Heritage-Listed Kakadu National Park, Northern Territory, Australia (Figure 1). It is a macro-tidal river 160 km in length with a tidal range of 5-6 m which extends 105 km up the river (Woodroffe et al., 1989). The floodplains flanking the river were previously covered with mangrove swamps up until 6,000 yr BP (before present) (Woodroffe et al., 1985, 1989). It was at this time that sea-level stabilised and the floodplains became the sedge and grass floodplain that exists today. The region is dominated by a tropical monsoonal climate, with a highly seasonal rainfall regime that defines two distinct seasons, the Dry and the Wet. Variation in rainfall, including rainfall intensity and the duration of the Wet season produces an immense change in the quantity of freshwater runoff transported across the catchment. The average annual rainfall of the region from Darwin to the Alligator Rivers is between 1,300 and 1,600 mm (Eliot et al., 2000). In contrast to this, very little rain falls during the Dry season months from May to September and this markedly affects the salinity structure of the river. The pronounced seasonality of the climate may be a significant factor in affecting regional vulnerability to saltwater intrusion (Woodroffe and Mulrennan, 1993).

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Figure 1 Location of study site

Location of South Alligator River floodplain, Kakadu National Park (A) with location in reference to Northern Territory, Australia inset. Sampling site with no history of saltwater intrusion (B) and sampling site with a history of saltwater intrusion (C) are shown enlarged.

Sites defined by Woodroffe et al., (1986) as lower floodplain were selected with different histories of SWI. Site 7 (12°37'19.95"S, 132°29'22.25"E) had a history of SWI as indicated by tidal creek extension and mangrove encroachment around the site since the 1950s (Cobb et al., 2007). Soil salinity of replicates at this site was 5.62 +/- 0.24 psu. Site 10 (12°33'5.57"S, 132°27'29.23"E) had no history of SWI and soil salinity was 0.25 +/- 0.04 psu, which is typical of freshwater habitats throughout the region (Cobb et al., 2007).

2.2. Sample design and collection

Replicate soil samples (n=3) were collected from sites with contrasting histories of SWI in August 2012 (Figure 1). Replicate samples were collected within a 1 x 1 m quadrat from each site using a shovel. Quadrat sites were chosen with no to minimal vegetation cover to reduce variation. Samples of 100 g were collected from the top 2 cm layer and stored in zip-lock bags. These were held at 4°C during collection and transit and placed at -20°C on return to the laboratory 24-48 h later.

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2.3. Experimental conditions

Samples were homogenised using a mallet to breakdown large components of the sample. Large, obvious components of the soil such as roots, and rocks were removed. Each replicate was divided into two portions of approximately 25-30 g. These were transferred into sterile plastic containers measuring 10 x 15 x 8 cm. A saltwater solution was made to reflect the water salinity of the South Alligator River of 34 psu using synthetic sea salt (Aqua One, AU) and sterile Milli-Q water. Sterile Milli-Q water was used as the freshwater treatment. Each replicate was rehydrated by adding 500 ml of treatment to the containers. Containers were placed into an incubator set at 29°C, based on the average annual temperatures for the months December to February (Bureau of Meteorology, 2013). Sub-samples were collected from each replicate before the treatment application and again following seven days exposure; yielding 24 samples (see Figure S1, Table S1).

2.4. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from 5 g of soil sample using the PowerMax DNA Kit (MoBio, USA) following the manufacturer's protocol. Prior to extraction, soil samples were homogenised to a fine powder using a mortar and pestle. Following extraction, samples were concentrated using Zymo DNA Clean and Concentrator Kit (Integrated Sciences, AU). DNA quality was determined by separation on a 1 % agarose gel by electrophoresis with a molecular weight standard 1 Kb Plus DNA Ladder (Invitrogen, AU) and viewed under UV trans-illumination (Biorad, AU). DNA quantity was determined using NanoDrop 2000c (Thermo Fisher Scientific Inc., USA).

Oligonucleotide barcoded primers were used to PCR amplify a ~600 base pair (bp) product spanning the V4 to V9 hyper variable region of the 16S rRNA gene on a thermal cycler. The oligonucleotide primers included 454 Life Science's (Roche Diagnostics, USA) adaptor sequence (shown in lowercase) 563F, 5'-ccatctcatccctgcgtgtctccgactcag-AYTGGGYDTAAAGNG-3' (Claesson et al., 2010) and 1046R, 5'-cctatcccctgtgtgccttggcagtctcag-CGACAGCCATGCANCACCT- 3'(Sogin et al., 2006). Forward primer sequences contained barcode sequences (Parameswaran et al., 2007) (Table S1). PCR amplification reactions were carried out individually for each sample using the FastStart High Fidelity PCR System (Roche Diagnostics) and all steps were performed according to the manufacturer's protocol. Cycling conditions were as follows: an initial denaturation step at 94°C for 2 min; 30 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. Negative

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DNA controls were included in each PCR batch. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Aus.). The quality and quantity of purified PCR products was determined as reported above for DNA. PCR products were sent to the Australian Genome Research Facility (AGRF, Aus.) for pyrosequencing on the GS FLX+ System (454 Life Sciences).

2.5. Sequence processing and taxonomic assignment

Initial quality control measures, used to ensure sequence fidelity, included: the removal of short sequences (< 100 bp), removal of any sequence not perfectly matching the 5' primer, and removal of any sequence containing an unresolved nucleotide. Sequence data were analysed using the Mothur v.1.30.1 suite of programs (Schloss et al., 2009). Sequences were aligned to the SILVA database v 115 (Pruesse et al., 2007) and those that did not align were removed. Alignments were trimmed so that all sequences covered the entire alignment length. Sequences were clustered to 97% similarity. Representative operational taxonomic units (OTUs) were identified using the SILVA taxonomy tool.

2.6. Data analysis

The final OTU dataset was trimmed to remove singletons (OTUs which occurred only once in one sample). Square-root transformed abundance data were used to generate a resemblance matrix using the Bray-Curtis similarity algorithm (Bray and Curtis, 1957). Similarities between sample groups were visualised using Principal Co-ordinates Analysis (PCOA). In visualisations sample MP35 was identified as an outlier and removed from future analyses.

Bacterial community diversity was expressed using the log(e) Shannon's diversity index (H') (Shannon and Weaver, 1949). The contribution of OTUs to the average dissimilarity between sites was calculated using a similarity percentages procedure (SIMPER), which identifies OTUs that are characteristic of bacterial community structure (Clarke and Gorley, 2006). Characteristic communities for sites were visualised using a heatmap in the *gplots* package (Warnes et al., 2009) in R version 3.0.2 (Ihaka and Gentleman, 1996).

Differences in bacterial community composition between sample sites were tested using a three factor Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001). Each test was done using 9,999 permutations under Type III sum of squares (SS) and a reduced model to generate a permutated F statistic (*F*) and *p-value* (*P*). In the case of a

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significant interaction term, pair-wise *a posteriori* tests for all combinations of factors were conducted using the t-statistic. Because of the small number of replicates, results were considered significant where *p-value* = < 0.025. Unless stated otherwise, all statistical tests were performed using the software PRIMER-E v6 (Clarke and Gorley, 2006).

3. Results and discussion

Replicate samples from sites clustered closely together (Figure 2). Bacterial community composition of sites with contrasting histories of SWI differed significantly before treatment (PERMANOVA Pairwise Tests: t = 6.84, P_{perm}=<0.01, Table 1; Figure 2). Sites with a history of SWI had a greater abundance of OTUs from the genus *Desulfobacterium*, a known sulfate reducer (Widdell and Bak, 1992) (Figure 3). Sulfate-reducing bacteria (SRB) respond to higher concentrations of sulfate (SO_4^{2-}) (Capone and Kiene, 1988) obtaining energy through the reduction of SO_4^{2-} to H_2S (hydrogen sulfide) (Edmonds et al., 2009). In turn, this can result in an increase in the production rate of carbon dioxide (Chambers et al., 2011) and ultimately a loss in soil organic carbon (Weston et al., 2010). Sulfate concentrations are naturally higher in saltwater compared with freshwater (Stumm and Morgan, 1995) and therefore a change in the bacterial community to one with a greater abundance of SRBs suggests a response to this increase in sulfate. The phylum Chloroflexi, including *Ignavibacterium* and *Sphaerobacter*, and the phylum Acidobacteria were also more abundant at sites with a history of SWI (Figure 3). Members of these groups have a role in carbon and nitrogen cycling (Freeman et al., 2009; Ward et al., 2009; Hug et al., 2013). In particular, the Acidobacteria are typically associated with low carbon (Fierer et al., 2007) (Figure 3) and their presence at sites with a history of SWI could suggest a negative impact on soil carbon availability.

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Table 1 Differences in bacterial community composition grouped by SWI histories, treatments and time

Source of variation	df	SS	F	P
Hi	1	31992	46.797	<0.001***
Tr	1	2320.3	3.394	<0.001***
Ti	1	4808.2	7.034	<0.001***
Ti x Hi	1	4202.6	6.148	<0.001***
Ti x Tr	1	2090.6	3.058	<0.001***
Hi x Tr	1	2019.1	2.954	<0.001***
Ti x Hi x Tr	1	1901.4	2.781	<0.001***
Pair-wise tests			**T**	**P**
Hi SWI, Hi No SWI			6.841	<0.001***
Within level Hi SWI: FW, SW			0.730	*0.735*
Within level Hi No SWI: FW, SW			1.055	*0.399*
Within level Hi SWI with Tr FW: B, A			2.846	*0.008***
Within level Hi SWI with Tr SW: B, A			3.029	*0.006***
Within level Hi No SWI with Tr FW: B, A			1.528	*0.108*
Within level Hi No SWI with Tr SW: B, A			1.220	*0.276*

PERMANOVA conducted on bacterial community composition between sample replicates to generate a permutated F statistic (*F*) and permutated p-value (*P*) with calculated degrees of freedom (df) and sums of squares (SS) noted. *P-values* given in italics were obtained using Monte Carlo samples from the asymptotic permutation distribution. Pair-wise *a posteriori* tests between factors were conducted using the t-statistic (*t*). Significance level: ***$P \leq 0.001$, **$P = 0.01$, *$P = 0.025$. Factors are: history (Hi) of saltwater intrusion (SWI) or no saltwater intrusion (No SWI); treatment (Tr) of saltwater (SW) or freshwater (FW); and, time (Ti) of before (B) or after (A).

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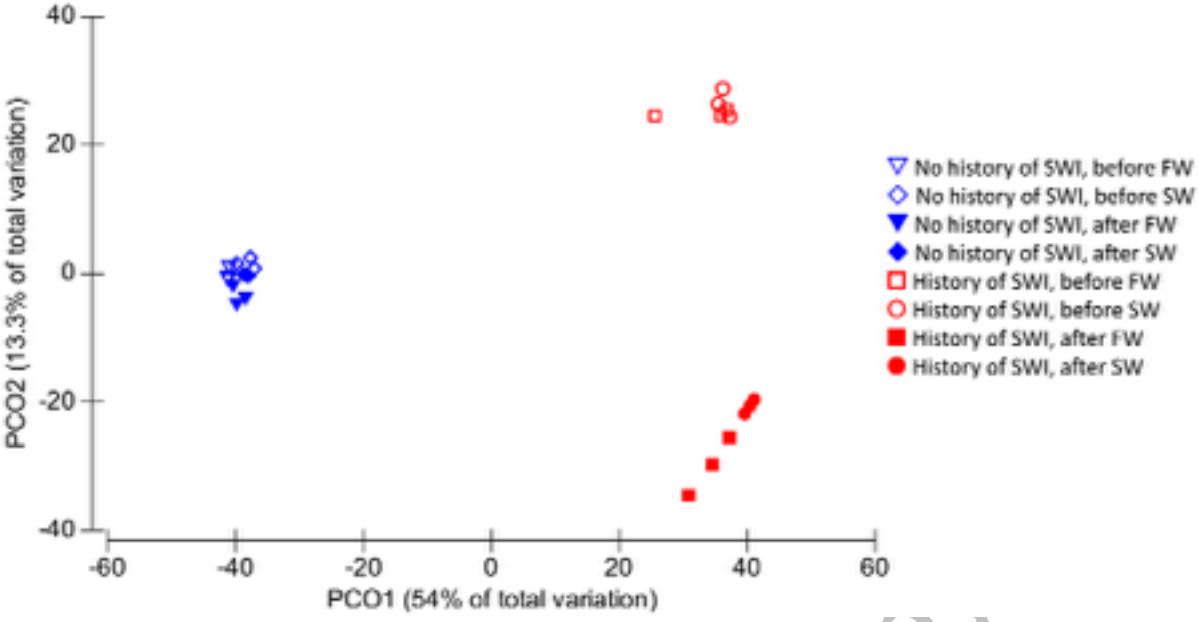

Figure 2 Bacterial community response following treatment and time at sites with different histories of saltwater intrusion

Principal co-ordinates analysis (PCO) displays the data matrix on axes which explain the most variation. Each data point represents the bacterial community at replicate floodplain soil sites with and without a history of saltwater intrusions (SWI) before and after treatment with saltwater (SW) or freshwater (FW).

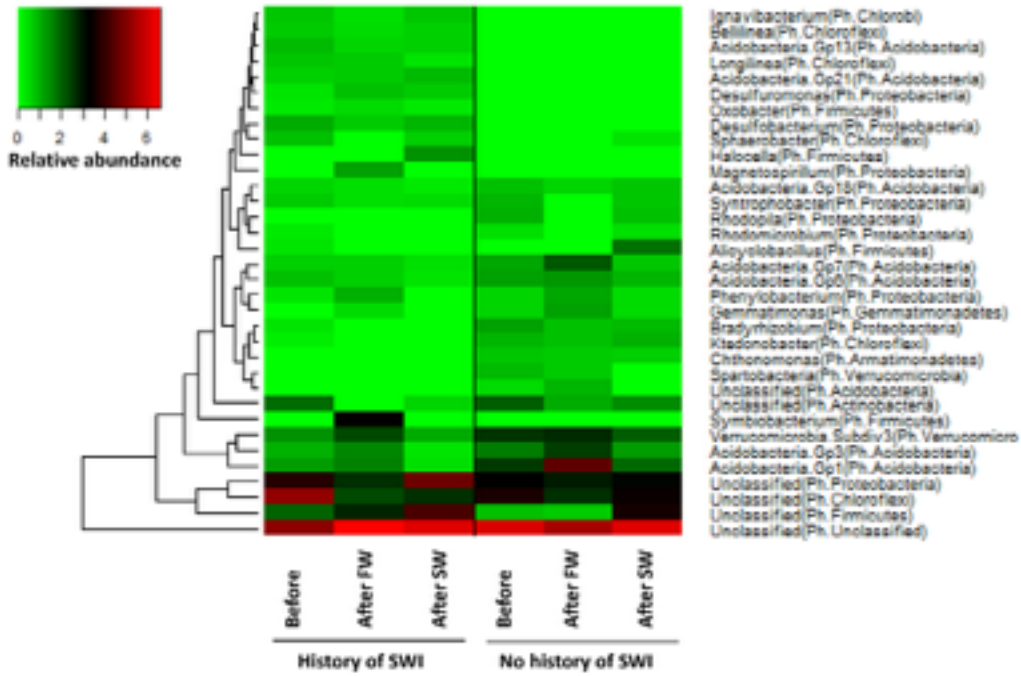

Figure 3 Bacterial community response following treatment of freshwater and saltwater before and after 7 d from sites with contrasting histories of SWI

Data were generated using SIMPER to display the dominant 50% of bacterial genera observed in each treatment group. Bacterial communities are grouped by history of saltwater intrusion (SWI) before and after 7 d treatment with either freshwater (FW) or saltwater (SW).

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Replicate samples from the sites with a history of SWI showed significant changes in bacterial community composition following freshwater and saltwater treatment (Pairwise Tests: t = 2.85, 3.03, P_{perm}=<0.01, <0.01, respectively, Table 1, Figure 2). No significant effect was measured following treatments to replicate samples from the site with no history of SWI (Table 1, Figure 2). For sites with a history of SWI, there was a significant reduction in bacterial community diversity following rehydration for both treatments. However the reduction was not as pronounced for replicate samples from the site with a history of SWI (Figure 4). Since rehydration causes osmotic stress (McKew et al., 2011), cell lysis may be partly responsible for the measured reduction in diversity. Many of the OTUs detected after rehydration were not detected in the 'before' samples, suggesting they maintain very low numbers during desiccation and respond when conditions are favourable. Members of the genera *Halocella* and *Desulfuromonas* increased following saltwater treatment (Figure 3) and are commonly isolated from sites with high salinities (Vos et al., 2009; McBeth et al., 2013). Members of the genus *Desulfuromonas*, like *Desulfobacterium,* are known SRBs and increase in response to sulfate in saltwater (Widdell and Bak, 1992). Members of the genus *Halocella* have haloadaptation strategies (Oren, 2008) and along with other species may be able to take advantage of the altered conditions.

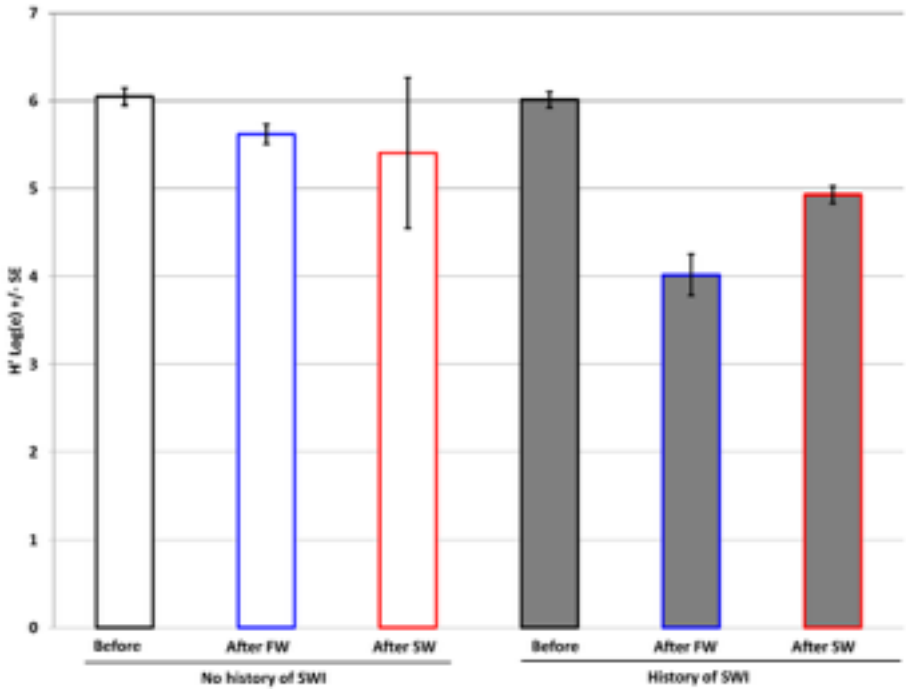

Figure 4 Bacterial community diversity response following treatment of freshwater and saltwater before and after 7 d

Replicate data were standardised and averaged. Community diversity is represented by the Shannon's diversity index loge ± standard error (SE).

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The response following FW and SW rehydration at sites with no history of SWI was less marked than sites with a history of SWI and this could reflect the lack of pre-exposure to variables like elevated sulfate and therefore an absence of bacteria such as SRBs. These and other halo-tolerant bacteria were key drivers of the changes measured at sites with a history of SWI where the changes were more dramatic following the rehydration treatments. While change in bacterial community composition may reflect change at the functional level (Reed and Martiny, 2013), this is not always the case (Edmonds et al., 2009; Berga et al., 2012). Since we measured bacterial composition, it's possible that a survey of gene function might prove more informative if in fact the undisturbed (no history of SWI) community composition did not change in our experiments because it had greater functional diversity, which did not necessitate compositional change.

Although salinity between sites didn't differ substantially (0.25 to 5.62 psu) compared to the river salinity of 34.0 psu, there was a measurable difference in bacterial community composition at sites with and without a history of SWI. For some vegetation in the region, a salinity of 3.71 psu is the limit of sensitivity (Cobb et al., 2007). The changed bacterial community at the site with a history of SWI may indicate a similar sensitivity to salinity. Changes in biochemical pathways of bacterial communities have been shown to occur within hours or days of water chemistry changes (Edmonds 2009). An increase in salinity causes dramatic changes to the soil microchemistry; the influx of sulfate can cause a reduction in carbon and nitrification activity as a result of chemical and bacterial changes (Rysgaard et al., 1999). The changes occur due to a number of inter-related factors including osmotic stress and shifts in elemental cycling (Fierer et al., 2003; Edmonds et al., 2009). As these changes persist in the environment for longer periods, those bacteria that favour the new habitat conditions are likely to thrive and out-compete other species until a different community exists. For vegetation and soil dwelling flora the changes in salinity affect the internal metabolism of the organism (Cheeseman, 1988). The distribution of salt-tolerant vegetation is not governed by one single factor (Silvestri et al., 2005) and the soil bacterial community and their effect on soil nutrient availability and micro-chemistry are factors worthy of consideration. The encroachment of saltwater into freshwater systems causes a cascade of changes in the soil microenvironment, one which bacteria have a significant role. Changes to the soil bacterial community has implications for the biogeochemical cycling of the system, which has implications for the availability of essential elements and nutrients that foster freshwater habitats.

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Supplementary material

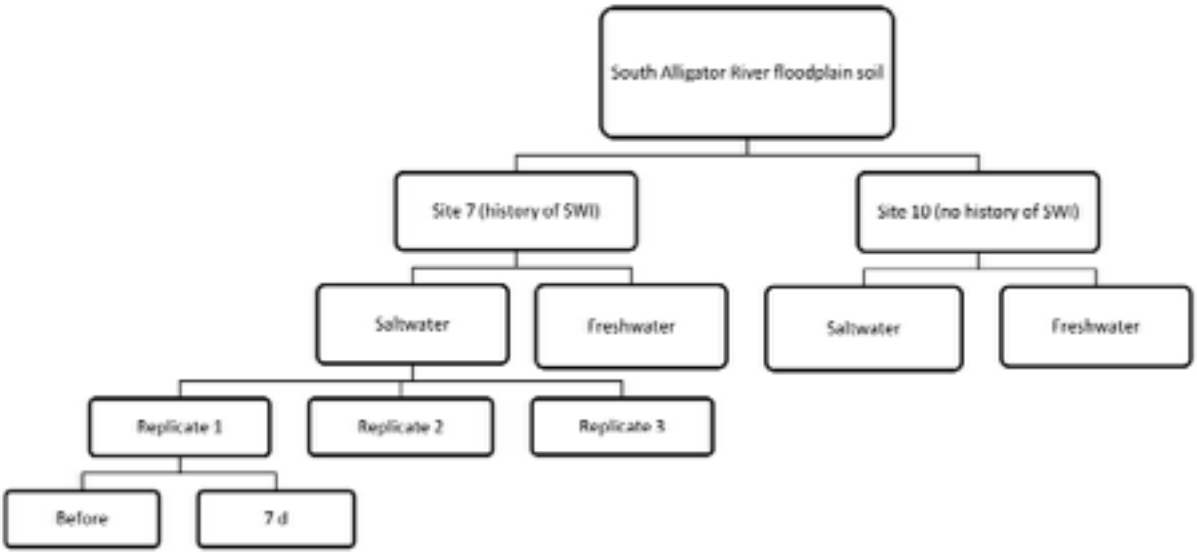

Figure S1 Sample design
Experimental design and treatments for sites with and without a history of saltwater intrusion (SWI).

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Table S1 Sample and site information and primer barcode

Sample ID	Site ID	Time	History of SWI	Treatment	Soil and water salinity (psu)	Primer barcode
MP1	7.1	Before	Yes	FW	5.86	ACGAGTGCGT
MP2	7.2	Before	Yes	FW	5.62	ACGCTCGACA
MP3	7.5	Before	Yes	FW	5.38	AGCACTGTAG
MP4	10.3	Before	No	FW	0.22	ATCAGACACG
MP5	10.4	Before	No	FW	0.23	ATATCGCGAG
MP6	10.5	Before	No	FW	0.29	CGTGTCTCTA
MP13	7.1	Before	Yes	SW	5.86	CGTAGACTAG
MP14	7.2	Before	Yes	SW	5.62	TACGAGTATG
MP15	7.5	Before	Yes	SW	5.38	TACTCTCGTG
MP16	10.3	Before	No	SW	0.22	TAGAGACGAG
MP17	10.4	Before	No	SW	0.23	TCGTCGCTCG
MP18	10.5	Before	No	SW	0.29	ACATACGCGT
MP19	7.1	After 7 d	Yes	FW	0.89	ACGCGAGTAT
MP20	7.2	After 7 d	Yes	FW	1.25	ACTACTATGT
MP21	7.5	After 7 d	Yes	FW	4.05	ACTGTACAGT
MP22	10.3	After 7 d	No	FW	0.16	AGACTATACT
MP23	10.4	After 7 d	No	FW	0.20	AGCGTCGTCT
MP24	10.5	After 7 d	No	FW	0.23	AGTACGCTAT
MP31	7.1	After 7 d	Yes	SW	61.06	TACAGATCGT
MP32	7.2	After 7 d	Yes	SW	66.94	TACGCTGTCT
MP33	7.5	After 7 d	Yes	SW	65.02	TAGTGTAGAT
MP34	10.3	After 7 d	No	SW	71.33	TCGATCACGT
MP35*	10.4	After 7 d	No	SW	65.54	TCGCACTAGT
MP36	10.5	After 7 d	No	SW	91.36	TCTAGCGACT

Sample identification (ID) for sites collected from the South Alligator River floodplain with and without a history of saltwater intrusion (SWI). Salinities are recorded from the soil for samples MP1-MP18 and in the overlaying water following treatment for samples MP19-MP36. Sample MP35 was noted as an outlier an removed from analyses.

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