Environmental Microbiology (2008) **10**(9), 2223–2235 doi:10.1111/j.1462-2920.2008.01644.x

Responses of Antarctic soil microbial communities and associated functions to temperature and freeze–thaw cycle frequency

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Summary

Climatic changes will not only result in higher overall temperature, but also in greater variability in weather conditions. Antarctic soils are subjected to extremely variable conditions in the form of frequent freeze–thaw cycles (FTCs), but the importance of alteration in FTC frequency, compared with increases in average temperature and indirect vegetation-mediated effects on soil microorganisms, is still unknown. We therefore designed two complementary microcosm experiments using undisturbed soil cores from Signy Island (60°43′S, 45°38′W) in the maritime Antarctic. The experiments consisted of soil core incubations with or without the overlying vegetation at four different temperatures and six different FTC regimes. We assessed bacterial and fungal density and community structure, as well as the density of several key genes in microbial nutrient cycles using a combination of RNA- and DNA-based molecular fingerprinting and quantitative PCR approaches in addition to enzymatic activity assays. Results showed that bacteria were more affected by warming than by changes in FTC frequency. In contrast, fungal community structure and abundance were mostly influenced by FTC frequency, as well as the presence of vegetation cover. The relative densities of several bacterial gene families involved in key steps of the N-cycle were affected by FTCs, while warming had little or no effect. The FTCs and incubation temperature also strongly influenced laccase enzymatic activity in soil. In total, our results suggest that, in addition to climatic warming, increased climatic variability may also have a profound impact on Antarctic microbial communities. Although these effects are difficult to detect with assays of total bacterial community structure, they do become manifest in the analysis of key functional gene densities.

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Introduction

Terrestrial Antarctic environments exhibit low-complexity food-web structures, with a dominance of microbial processes in ecosystem functioning, thereby providing a relatively simplified system to disentangle the consequences of perturbations on soil microbial activities. The Antarctic Peninsula is also one of the regions in the world that has experienced the largest increase in temperature in recent decades (0.56°C per decade between 1951 and 2000) (Turner *et al.*, 2002). Predicted global warming will lead to longer growing seasons across this region, and extended macrophyte distributions have already been observed (Fowbert and Smith, 1994; Smith, 1994; Frenot *et al.*, 2005; Convey and Smith, 2006). However, relatively little information is available concerning the possible consequences of global warming on associated soil-borne microorganisms. Microbial responses to temperature are typically non-linear and, therefore, it has been suggested that changes in temperature and climatic variability might have disproportional effects on microbial communities and the functions for which they are responsible (Scherm and van Bruggen, 1994). Most climate change scenarios predict not only a general warming trend, but also an increased variability in weather conditions (IPCC, 2007), including alterations in precipitation and thawing patterns, which will lead to more variable soil conditions (Groffman *et al.*, 2001). Thus, predicted changes in temperature variability might have more profound consequences than increases in average temperature for Antarctic soil microorganisms, given the greater stress imposed by frequent temperature fluctuations as compared with gradual changes in average temperature (Vishniac, 1993).

Most of the microorganisms in Antarctic soils are believed to be cold-tolerant as opposed to cold-adapted (Kerry, 1990; Melick *et al.*, 1994; Zucconi *et al.*, 1996; Robinson, 2001). Consequently, it has been hypothesized

that the direct effects of increasing temperature on Antarctic soil-borne microorganisms will be less important than indirect effects, such as changes in vegetation density and other associated soil biophysical properties (Vishniac, 1993; Panikov, 1999). However, experimental evidence from a range of environments (including arctic and alpine systems) has suggested a variety of direct effects of warming on soil microbial communities (Zogg *et al*., 1997; Ruess *et al*., 1999; Deslippe *et al*., 2005; Rinnan *et al*., 2007) or associated nutrient cycling processes (de Klein and van Logtestijn, 1996; Maag and Vinther, 1996; Castaldi, 2000; Dobbie and Smith, 2001; Barnard *et al*., 2005). It is, however, still not known if such patterns also hold for microorganisms inhabiting more severe and isolated Antarctic soils (Convey, 2001).

In the Antarctic, freeze–thaw cycles (FTCs) are a common feature and changes in the frequency of such events could have substantial effects on the soil microbial communities and associated nutrient cycling functions. The freezing and thawing of soil have been shown to damage or destroy some microbial cells, releasing nutrients to surviving microbes, which are then highly active during periods of thaw (Skogland *et al*., 1988; Christensen and Tiedje, 1990). Extra substrate and nutrients can also be released via the physical disruption of soil aggregates because of frost action (Christensen and Christensen, 1991; Edwards and Cresser, 1992), resulting in altered microniches (Skogland *et al*., 1988). The penetration and effect of freezing and subsequent thawing depend on the level of insulation provided by vegetation and snow cover (Edwards and Cresser, 1992). The FTCs are therefore believed to play an important role in nutrient cycling in the Antarctic, not only because of the stress imposed on microbial communities, but also because they induce changes in exudation patterns of cryptogams (Tearle, 1987; Melick and Seppelt, 1992; Melick *et al*., 1994). It has been estimated that freeze–thaw events induce an annual release of >15% of the total organic matter of Antarctic cryptogams to the soil microbiota (Tearle, 1987), an input that is 10 times greater than that estimated via the decomposition of dead subsurface vegetation materials (Davis, 1986). Accordingly, the frequency of FTCs was identified as a potential important driving factor for the structure of the microbes involved in the C-cycle in Antarctic soils (Yergeau *et al*., 2007a). Several microcosm studies have already assessed the short-term (< 1 year) effects of few freeze–thaw events on Arctic, Antarctic and alpine soil microbial communities and associated functions (Wynn-Williams, 1982; Lipson *et al*., 2000; Larsen *et al*., 2002; Koponen *et al*., 2006), but many questions still remain unanswered, especially for Antarctic environments.

Previous field studies along an Antarctic latitudinal gradient have provided circumstantial evidence that both mean soil temperature and the frequency of FTCs can exert an impact on microbial community size, structure, diversity and functions (Yergeau *et al*., 2007a,b,c). The main purpose of the present study was to examine the influences of rising temperatures and of alterations in FTC frequency in inducing changes in soil-borne microbial community structure, functional gene densities and enzymatic activities. To achieve this goal, we designed two complementary microcosm experiments using carefully controlled climate conditions simulating features of predicted Antarctic climate scenarios. We used intact cores, with and without covering vegetation, from Signy Island, within the South Orkney Islands. Following incubation of soil cores at four different temperatures and six different FTC regimes, we assessed fungal and bacterial community density (using RNA- and DNA-based real-time PCR) and structure (using RNA- and DNA-based PCR-DGGE). Functional analyses were also carried out and involved DNA-based real-time PCR targeting genes for nitrite reductase (*nirS* and *nirK*), ammonia monooxygenase (*amoA*), assimilatory nitrate reductase (*nasA*), dinitrogenase (*nifH*) and nitrous oxide reductase (*nosZ*) as well as enzymatic activities assays for cellulase and laccase.

Results

Two experiments were performed to examine the effects of global warming on Antarctic soils. In the first experiment, 24 soil cores with overlying vegetation were incubated for 6 months at four different constant temperatures (3°C, 5°C, 7°C or 15°C), simulating higher average temperatures. In the second experiment, 60 soil cores from Signy Island were used with or without overlying vegetation and submitted to six different FTC frequencies (0, 0.25, 1, 2, 3, 4 cycles per week) for a period of 3 months, thus simulating changes in climate variability. Following these experiments, several soil, microbiological and functional parameters were assessed as detailed below.

Soil factors and enzymatic activities

One of the most striking observations was that the soil characteristics of the different replicates were highly variable, resulting in large error bars (Figs 1 and 2). However, even with this high background variability, there were several significant effects of the experimental treatments. Soil pH showed no obvious decrease or increase with incubation temperature (Fig. 1), although some treatments had significantly different pH. While FTC frequency did not affect soil pH, there was a nearly significant effect of vegetation presence, with vegetated cores having higher pH (Fig. 2). The effect of FTC frequency on soil NO_3 content was also significant (Kruskal–Wallis ANOVA), but there was no effect of FTC frequency on soil NH_4

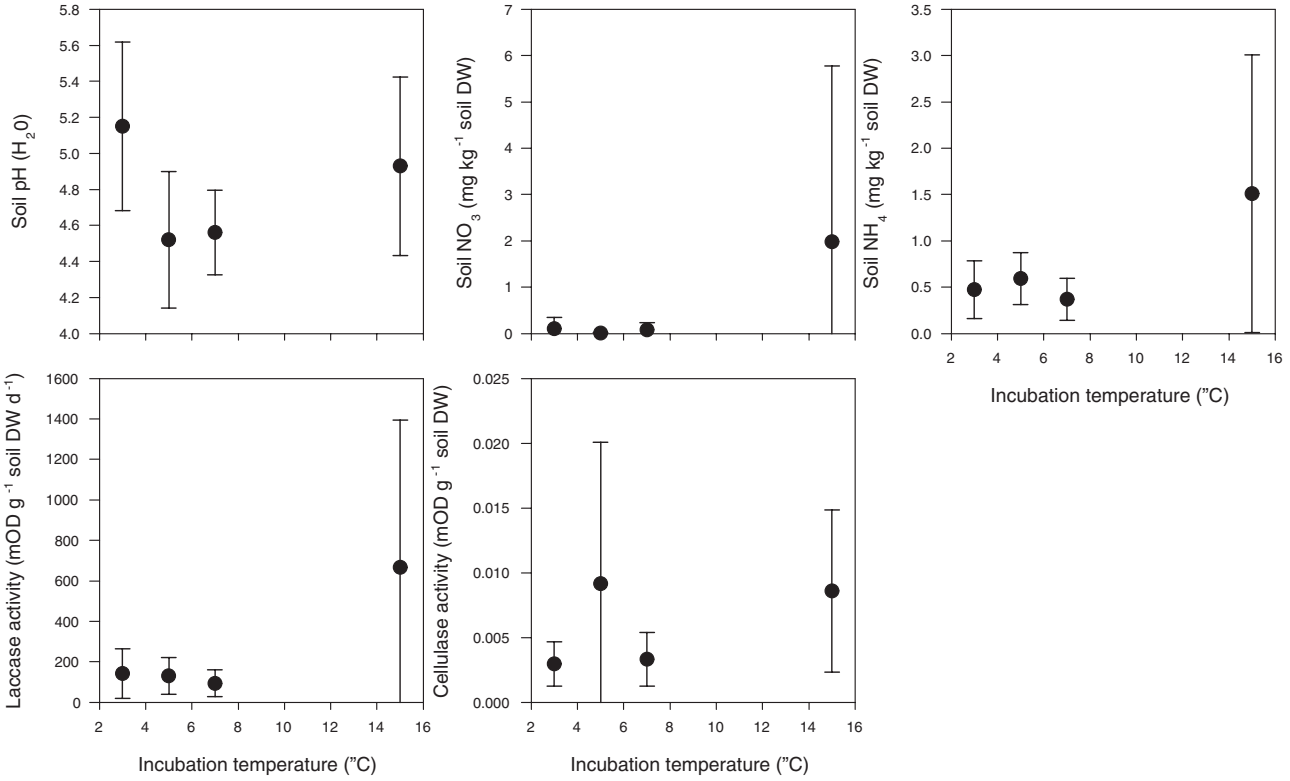

Fig. 1. Average soil pH, NO_3 and NH_4 contents, cellulase and laccase activities for soil cores incubated at different temperatures. Each data point represents the average measurement of six individual replicate cores with error bars representing the standard deviation.

(Fig. 2). Soil cores not submitted to any FTC had the highest amount of NO_3 and NH_4 both for vegetated and unvegetated cores. Vegetation presence did not significantly affect soil NO_3 and NH_4. For the constant incubation temperature experiment, some soil cores incubated at 15°C had extremely high amounts of NO_3 and NH_4, but high variability between replicates cores caused these differences to be insignificant (Fig. 1). Laccase activity in soil was significantly influenced by the incubation temperature and the frequency of FTCs (Figs 1 and 2). According to Tukey HSD *post hoc* tests, there was significantly more laccase activity in cores incubated at 15°C than in cores incubated at all other lower temperatures. In the case of FTC, the greatest difference was between the laccase activity of cores subjected to two or four cycles per week and the activity of cores not submitted to any FTCs. Cellulase activity showed a similar response (Figs 1 and 2), with increasing activity at higher temperature or lower FTC frequency, but this was not significant. The only significant factor for cellulase activity was the presence of vegetation.

Bacterial community size and structure

DNA and RNA simultaneously extracted from soil samples were used to assess bacterial community size and structure across the different treatments (Fig. 3A and B). DNA-based analyses provided information about community size and structure, while RNA-based analyses provided a rough proxy for active community size and structure, although this might not hold at the low growth rates typical of these soil environments (Kowalchuk *et al.*, 2006). The effect of incubation temperature on bacteria was only significant when assessing community structure at the RNA level and this also held for community size estimates. As seen in Fig. 3B, the bacterial community structures based upon 16S rRNA were quite different for the different treatments, and the cores incubated at 15°C had a greater 16S rRNA density. No such effect of incubation temperature was observed at the DNA level (Fig. 3A), and a similar lack of significance on community structure was observed using DNA-based terminal restriction fragment length polymorphism analyses (data not shown).

The FTC frequency had a significant effect on the number of 16S rRNA genes and a nearly significant effect on the amount of 16S rRNA detected (Fig. 4A and B). The number of 16S rRNA genes was highest in cores submitted to one FTC per week and lowest in cores submitted to two FTCs per week (Fig. 4A), while 16S rRNA were more abundant in cores that were not submitted to any FTCs (Fig. 4B). The community structure at the DNA level was

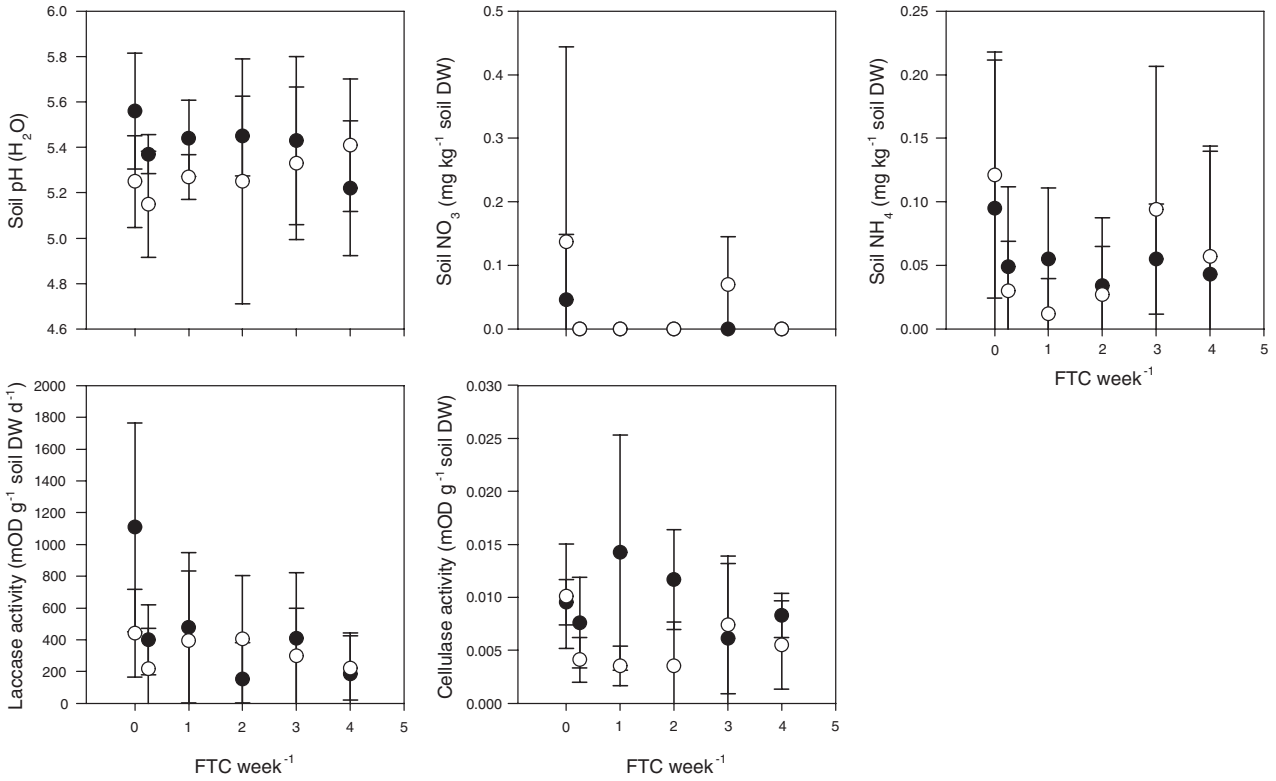

Fig. 2. Average soil pH, NO_3 and NH_4 contents, cellulase and laccase activities for soil cores submitted to different frequency of freezing and thawing. Each data point represents the average measurement of five individual replicate cores with error bars representing the standard deviation. ●: vegetated cores, ○: unvegetated cores.

not significantly affected by FTCs, but there was a nearly significant effect of FTCs on community structure at the RNA level, although this last effect is not clearly visible in the two dimensions represented in the ordination of Fig. 4B. The presence of vegetation and the interaction term (vegetation presence × number of FTC) showed no significant effects on any of the bacterial community indicators measured.

Fungal community size and structure

As for bacteria, fungal communities were assessed using DGGE and real-time PCR on simultaneously extracted DNA and RNA (Figs 3 and 4). Changes in incubation temperature did not lead to any significant changes in fungal community structure (for both DNA and RNA) or in the abundance of 18S rRNA genes and 18S rRNA (Fig. 3C and D). However, there was a nearly significant influence of the frequency of FTCs on the fungal community structure at the DNA level, and this is visible in Fig. 4C, where the fungal community not subjected to any FTCs was clearly separated from all the other treatments on the first axis of the ordination. Although not significant, a similar response was visible for the fungal community structure at the RNA level (Fig. 4D). Cores not subjected to any FTCs were also generally harbouring more 18S rRNA genes and 18S rRNA than other cores (Fig. 4C and D). Vegetation presence had a significant effect on the community structure at the RNA level and a nearly significant effect on the number of 18S rRNA genes (data not shown). The vegetated cores harboured significantly (one-way ANOVA) more 18S rRNA genes regardless of the FTC frequency [average copies per g of soil (dry weight): vegetated, 4.53×10^7; unvegetated, 2.83×10^7].

N-cycle functional gene abundances

As found in previous analyses of environmental samples (Henry *et al.*, 2006), a large proportion of samples showed no detectable amplification product when targeting mRNA of N-cycle functional genes, even if the corresponding genes could be readily detected via PCR assays targeting DNA. We therefore focussed on functional genes at the DNA level.

Similar to observations for soil analyses, there was also large variation between replicates in the density of functional genes. Changes in incubation temperature did not induce any significant changes in the abundance of the different N-cycle genes examined (data not shown). However, the frequency of FTC had a significant influence

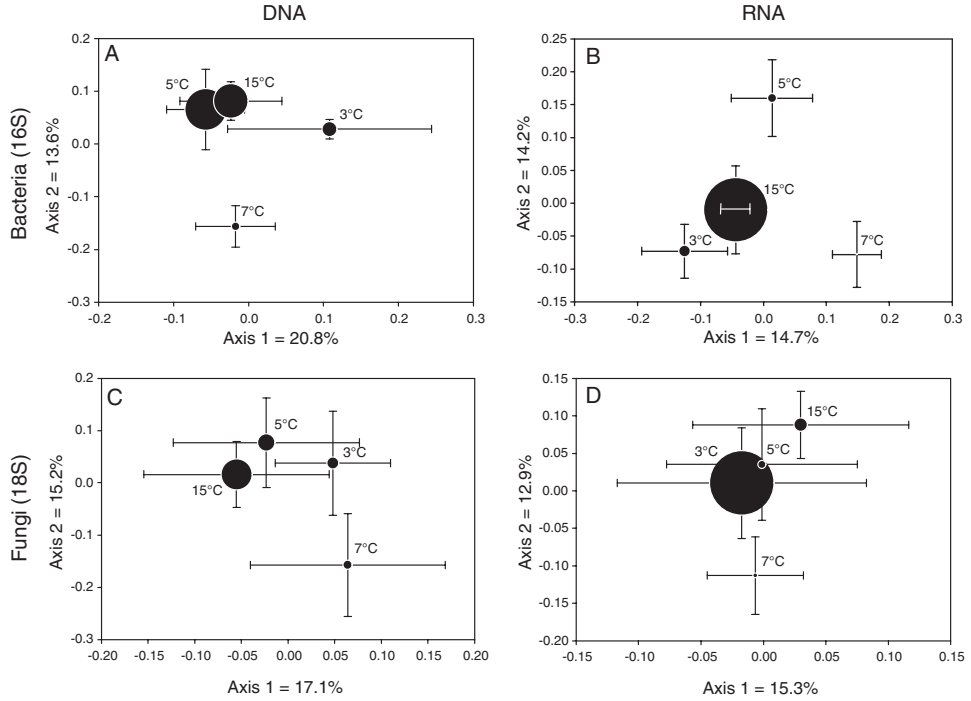

Fig. 3. Principal co-ordinate analysis of DGGE patterns made with (reverse transcription-) PCR-amplified ssu rRNA genes or rRNA using bacterial 16S- or fungal 18S-specific primers for soil cores incubated at 3°C, 5°C, 7°C or 15°C. The position of the point is the mean position of six replicate cores and the associated errors bars represent the standard error of the mean position. The sizes of the points are proportional to the average copy number of 16S or 18S rRNA genes or rRNA per g of soil (dry weight) for six replicate cores as assessed by (reverse transcription-) real-time PCR on DNA or RNA.

on *nirS* gene abundance, and this effect was highly dependent on the presence of vegetation, yielding a significant FTC × vegetation cover interaction effect (Fig. 5). The copy number of *nirS* genes increased with increasing frequency of FTCs when the vegetation cover was removed, but decreased with increasing FTCs frequency when the vegetation was left intact (Fig. 5). An almost identical trend was recorded for *nifH* genes (Fig. 5), with the interaction term also being significant in ANOVA tests. The *nasA* gene numbers were also influenced in a similar fashion, but with an interaction term that only showed a nearly significant trend, as a result of the large variation of gene numbers in the vegetated cores (Fig. 5). *amoA* and *nirK* gene numbers were not significantly influenced by any factors in ANOVA tests, but the general trends in the data set (Fig. 5) were similar to the ones observed for *nirS*, *nifH* and *nasA*. Interestingly, *nosZ* gene copy numbers showed a completely different trend (Fig. 5), being only significantly influenced by the presence of vegetation.

Discussion

One of the most striking features of our data set was the large variation between replicates, indicating large spatial variation in the site sampled. The cores were all sampled in neighbouring and similarly vegetated environments, and it was expected that the variation between cores would be small, as previously reported (Yergeau *et al.*, 2007b). One would also expect the no-FTC control (constantly incubated at 10°C) from the FTC frequency experiment to act similarly to the 7°C and 15°C treatments from the constant incubation temperature experiment. However, this was clearly not the case, suggesting pronounced year-to-year variability as the samples in these experiments were gathered in successive years. Following the results presented here, it appears that this spatial and temporal variation might have a strong overriding effect on some of the responses of soil microorganisms to global warming. Despite the large variation observed, several potentially important consequences of warming and changes in FTC frequency could be detected within the soil-borne microbial communities.

General effects of FTC frequency and temperature on bacterial communities

Freeze–thaw events typically led to initial decreases in bacterial biomass followed by a respiratory burst, which can be explained by assuming that a fraction of the bacteria are killed, and that the surviving cells utilize the liberated nutrients (Skogland *et al.*, 1988; Edwards and

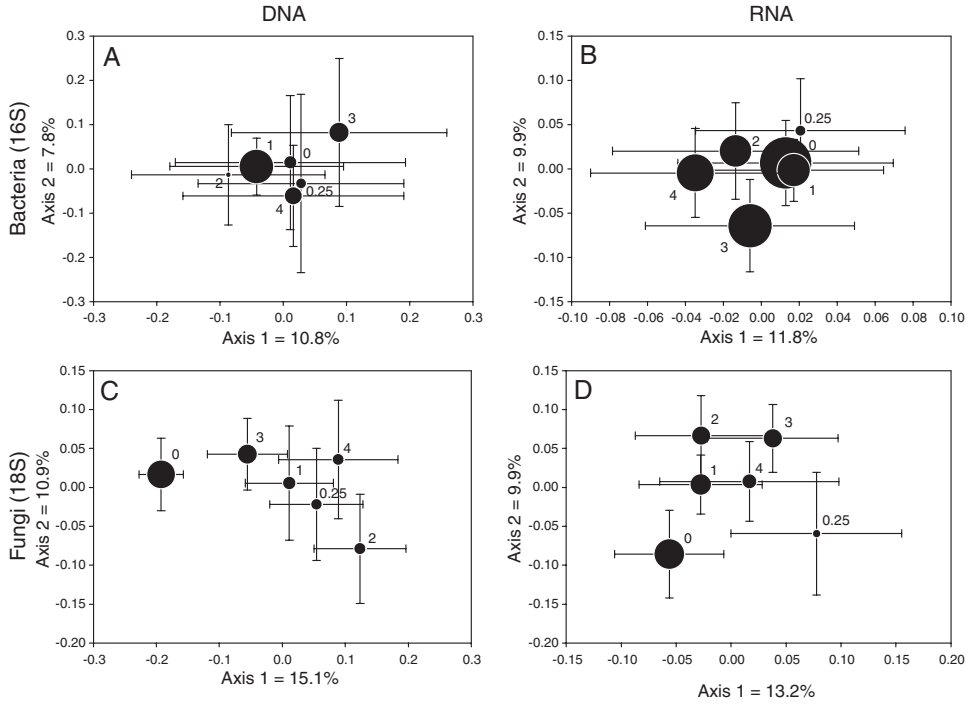

Fig. 4. Principal co-ordinate analysis of DGGE patterns made with (reverse transcription-) PCR-amplified ssu rRNA genes or rRNA using bacterial 16S- or fungal 18S-specific primers for soil cores submitted to 0, 0.25, 1, 2, 3 or 4 FTCs per week. Different vegetation treatments were combined for the purpose of this analysis. The position of the point is the mean position of 10 replicate cores and the associated errors bars represent the standard error of the mean position. The sizes of the points are proportional to the average copy number of 16S or 18S rRNA genes or rRNA per g of soil (dry weight) for 10 replicate cores as assessed by (reverse transcription-) real-time PCR on DNA or RNA.

Cresser, 1992). The physical disruption of soil aggregates due to frost action can also release nutrients (Christensen and Christensen, 1991; Edwards and Cresser, 1992). In the longer term, repeated FTCs might be expected to lead to changes in bacterial community composition as a result of the combined selective pressures of freeze–thaw stress and use of liberated substrates. There is normally a rapid response after the first FTC, but this often diminishes in intensity after subsequent cycles (Morley *et al*., 1983; Skogland *et al*., 1988; Walker *et al*., 2006), leading to little or no bacterial response after several FTCs (Lipson *et al*., 2000; Koponen *et al*., 2006). In line with this reasoning, we observed no clear directional trends in general bacterial responses to FTC frequency. Although bacterial community size and structure were affected by FTCs, these parameters were not more similar in soils submitted to similar numbers of FTCs. Some bacteria are highly resistant to FTCs (Morley *et al*., 1983; Walker *et al*., 2006), and it would be expected that a large proportion of bacteria in maritime Antarctic soils, as used in our microcosms, would be highly resistant to frequent FTC. Indeed, previous adaptation to frequent FTCs may help explain the relative unresponsiveness of bacteria to FTCs in our microcosms. It should be noted that our 3 month experiment probably allowed for sufficient time for bacterial communities to adapt to, and recover from, any initial changes induced by the novel freeze–thaw regimes imposed.

In contrast, when incubating cores at different temperatures, significantly more bacterial rRNA were detected in cores incubated at 15°C when compared with cores at all other temperatures. In soils from temperate environments, Zogg and colleagues (1997) also found that the major difference in bacterial community structure was visible at higher temperatures (between 15–25°C) and smaller differences were observed between soils incubated at 5°C and 15°C. Interestingly, the optimum temperature for germination and growth of many Antarctic macrophyte taxa is in the range of 10–20°C (with 15°C being optimal for most species, Kennedy, 1996). Warming of a few degrees (in the range of what is predicted in the next hundred years for the region) did not provoke any significant changes in bacterial density and community structure in our microcosm experiments, and similar results were previously reported for Arctic (Larsen *et al*., 2002) and Antarctic (Bokhorst *et al*., 2007) soil microcosms. It is important to note, however, that in a study of Arctic soils, more than a decade of warming of a few degrees was necessary before significant responses of the microbial biomass were observed (Rinnan *et al*., 2007). Thus, it could also be that our relatively short-term experiment (6 months)

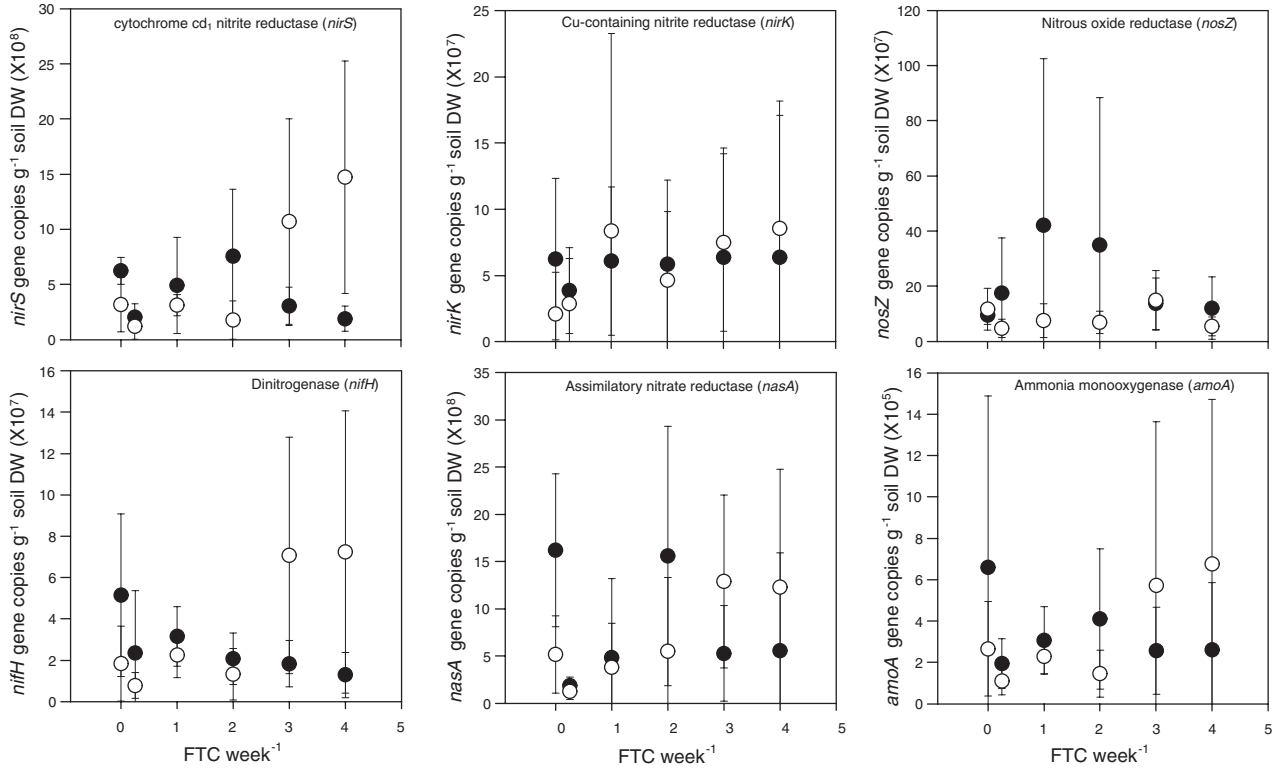

Fig. 5. Number of copies of different N-cycle genes in soil cores submitted to different FTC frequencies as measured by real-time PCR. Each data point represents the average measurement of five individual replicate cores with error bars representing the standard deviation.
●: vegetated cores; ○: unvegetated cores.

did not provide sufficient time to allow bacterial community shifts to become manifest.

General effects of FTC frequency and temperature on fungal communities

The effects of changing temperatures on soil fungi are less well studied than for bacteria. However, fungi are believed to be less negatively affected by freeze–thaw events as compared with bacteria (Sharma *et al*., 2006). They may be relatively frost-resistant and often well-equipped to grow on the types of organic matter released after freeze–thaw events (Wynn-Williams, 1982). However, it has been reported that fungal propagule numbers can be effectively reduced by freezing and thawing (Skogland *et al*., 1988). Here, we observed nearly significant changes in fungal community structure at the DNA level when soil cores were submitted to contrasting FTC frequencies. The main difference was between fungal communities that were not subjected to any FTCs and the rest of the treatments (Fig. 4C). We also observed that vegetation presence influenced fungal community structure (at the RNA level) and size (at the DNA level) in our study. These patterns were observed only for fungi and not for bacteria which might be related to the fact that fungi are thought to be more related to substrate availability than bacteria (Wardle, 2002). Indeed, freezing and thawing are known to induce large changes in exudation patterns of cryptogams, and as little as one cycle per month could be enough to change the availability of C-compound in soil (Tearle, 1987; Melick and Seppelt, 1992; Melick *et al*., 1994). The presence of vegetation is also important for the type of organic matter that will be available. Vegetation presence has previously been reported to influence several microbial community functions (bacterial and fungal) related to the C-cycle (Yergeau *et al*., 2007a), and we previously reported that Antarctic fungal community structure and density were largely affected by the presence of vegetation cover, in a location-specific manner (Yergeau *et al*., 2007b). In contrast to the strong effects of FTC and vegetation presence, no effect of incubation temperature was observed on any of the fungal parameters examined.

Effects of temperature and FTC frequency on specific soil functions

Incubation temperature did not have any significant effects on the density of any of the N cycle-related functional genes (*nirS*, *nirK*, *nosZ*, *amoA*, *nasA*, *nifH*)

assessed in our study. This lack of response is in line with previous results, which failed to detect significant or consistent changes in N-cycle process rates following soil warming of a few degrees in different environments (Jonasson *et al*., 1999; Schmidt *et al*., 1999; Shaw and Harte, 2001). This non-responsive trend was also observed for the associated functional communities (Deslippe *et al*., 2005) and functional genes (Horz *et al*., 2004). Although our results suggest that these microbial functions may also be relatively unresponsive to average temperature increases in Antarctic systems, additional studies would be necessary to determine if this holds across other terrestrial Antarctic habitats. It should also be stressed that we were only able to look at functional genes at the DNA level, and that analyses of the mRNAs coded by these genes could have provided further insights about the effects of temperature. One way to overcome this limitation might be to amplify selectively functional genes mRNAs, but this supplementary step can also bring more biases.

In contrast to simple average temperature treatments, different FTC regimes influenced the density of some N cycle-related genes directly or in interaction with vegetation presence. Several studies have reported that FTCs can increase expression of denitrifying genes (Sharma *et al*., 2006), as well as increase N_2O production (Koponen *et al*., 2006; Sharma *et al*., 2006), denitrification and mineralization (DeLuca *et al*., 1992; Edwards and Cresser, 1992). The impacts of freezing and thawing were highly dependent on the presence versus absence of vegetation cover, with the two treatments often yielding opposite patterns for functional gene abundances. These differences could stem from the protective effects of vegetation on soil microbes, the increases in soil water content caused by vegetation presence (not shown) or the release of organic matter from the vegetation. Microorganisms are generally more tolerant to gradual changes than to abrupt changes in temperature, and freezing and thawing rates can be modulated by the level of insulation provided by the vegetation cover (Edwards and Cresser, 1992). Dry soils have also been shown to freeze more rapidly than wet soils (Edwards and Cresser, 1992). A similar interactive effect of temperature and soil water content on ammonia-oxidizing bacteria was previously observed, with temperature increases having opposite effects at low and high soil water contents (Horz *et al*., 2004). Furthermore, as stated above, FTCs induce changes in exudation pattern of cryptogams (Tearle, 1987; Melick and Seppelt, 1992; Melick *et al*., 1994), and this could also have modulated the response of N-cycle bacteria to FTCs.

Laccases are present exclusively in fungi and higher plants (except for one bacterial species, Thurston, 1994; Mayer and Staples, 2002), so it can be assumed that this function in our cores was principally carried out by fungi. Although the cores examined here are essentially devoid of lignin, laccases are believed to be important players in decomposition processes. Indeed, laccase genes were previously reported to be present at high densities in a range of Antarctic soils (including Signy Island, Yergeau *et al*., 2007a), which was hypothesized to be related to the presence of a wide range of phenolic compounds that can be suitable for this broad-specificity enzyme. Freeze–thaw treatments had a significant effect on laccase activities when taking both vegetated and unvegetated cores into account. There was significantly higher activity in the unfrozen controls when compared with the four FTCs per week treatment. Significant increases in enzymatic activity were also observed at the highest incubation temperature. Thus, the effect of FTCs may also partly be explained by the decreasing average temperature associated with increasing the frequency of frozen periods in our experiment. Interestingly, a similar trend was observed for the general fungal community structure and size, where the main difference seemed to be between the unfrozen controls and all the remaining cores (Fig. 4C and D).

Fungi are believed to be the main cellulose decomposers in the Antarctic, as compared with the Arctic where bacteria are believed to dominate this process (Walton, 1985). Thus, it was not surprising that patterns of cellulase activity across the treatments were also reflected in the general fungal community. In line with previous studies in the Antarctic (Walton, 1985; Bokhorst *et al*., 2007; Yergeau *et al*., 2007a), we found that the main factor affecting cellulase activity was the presence of overlying vegetation. Furthermore, high cellulase activity was generally found in cores submitted to high incubation temperatures or to low FTC frequencies. A similar positive relationship between cellulase and temperature in Antarctic soils was reported previously (Pugh and Allsopp, 1982; Kerry, 1990; Yergeau *et al*., 2007a). Generally, the enzymatic assays described here predict an increase in decomposition rates in Antarctic soils following global warming.

Conclusions and perspective

The results of our microcosm studies generally showed that fungi and bacteria respond differently to changes in soil temperature regimes. Fungal parameters were only influenced by the freeze–thaw regime and by the presence of vegetation, whereas bacterial parameters were more consistently influenced by changes in temperature. This conclusion is in good agreement with one of our previous studies that monitored both bacteria and fungi along an Antarctic latitudinal gradient, including Signy Island, which concluded that fungi were less affected than bacteria by latitude (thus temperature), but more by the

presence and composition of the vegetation cover (Yergeau *et al*., 2007b).

Interestingly, even though bacterial communities were affected by incubation temperature, no concomitant changes were recorded in associated functional genes. Inversely, although no consistent changes were observed in the structure of the total bacterial community following freezing and thawing, some of the associated functional genes were affected. This indicates that studies looking only at broad parameters of bacterial communities following climate change might overlook important changes in key nutrient cycle functions. In contrast, the changes in laccase and cellulase activities were well mirrored by general fungal community parameters.

The results outlined here demonstrated that following global warming, changes in FTC frequency and increases in average temperature might have strong influences on Antarctic soil-borne microbial communities. Vegetation was also reported to influence significantly many microbial parameters, highlighting the potential for indirect effects of global warming on soil microbes.

Experimental procedures

Soil core sampling and characteristics of sampling site

Eighty-four 10-cm-diameter, 10- to 20-cm-deep soil cores with overlying vegetation were taken in January 2005 and January 2006 from moss patches (dominated by *Chorisodontium aciphyllum*) on Signy Island, South Orkney Islands, maritime Antarctic (60°43′S, 45°38′W). Cores were frozen (–20°C) within 24 h and kept frozen in the dark until used in the experimental set-up. Depth of all cores was adjusted to 10 cm before experimental use. Because of logistical constraints, cores could not be kept free from air spores for transportation and, consequently, no special efforts in that sense were made in the experimental set-up.

Habitat conditions in moss patches on Signy Island were previously reported (Bokhorst *et al*., 2007; Yergeau *et al*., 2007b). Average soil temperature (5 cm depth) from January 2004 to January 2006 was –1.8°C and soils at that depth experienced on average 145 FTCs per year. Two different experiments were set up to test the effects of increasing temperature and increasing frequency of FTCs. The former experiment aimed at reproducing the average conditions experienced during the growing season. On Signy Island, this period extends from October to April (6 months) and the average soil temperature (5 cm depth) for that period in 2004–2005 was 1.2°C, with a maximum of 16.1°C and a minimum of –10.0°C. During this period, the day length varied from ~12–21 h.

Most of the FTCs occurred during two 3 month periods from February to May (autumn) and from October to December (spring). Following Henry (2007), we aimed at co-ordinating the season for which FTCs were simulated with the sampling date. As logistical constraints did not allow for early spring sampling at Signy Island, we aimed at reproducing autumn conditions. For that period, conditions in the field were as follows: from the beginning of February to the end of April 2005, soils (at a 5 cm depth) experienced 42 FTCs for an average of around 15 FTCs per month. During this period, average soil temperature 5 cm below the surface was 1.1°C with a minimum of –5.6°C, a maximum of 15.8°C and an average daily temperature variation of 4.1°C. The day length during this period varied between ~9 and ~18 h.

Constant temperature microcosms

For this experiment, 24 cores sampled in January 2005 were used. Six replicate soil cores per temperature were placed in water baths at four different temperatures (3°C, 5°C, 7°C and 15°C). The baths were all kept in the same growth chamber with an air temperature of 10°C with a day length of 20 h [at a photosynthetically active radiation (PAR) light intensity of 200 μmol m^{-2} s^{-1}] and a relative humidity of 80%. The soil cores were watered every week, by giving 100 ml of water to each core. After 6 months of incubation, soil was sampled from the centre of the core using an alcohol-scrubbed knife. These samples, weighing around 20 g, were frozen (–20°C) within 1 h from sampling and kept until used for molecular analyses (within a week). The remainder of the soil cores was used for analysis of physical and chemical properties.

Variable FTC frequency microcosms

For this experiment, 60 cores sampled in January 2006 were used. Ten soil cores were submitted to six different FTC frequency treatments (none, 1 $month^{-1}$, 1 $week^{-1}$, 2 $week^{-1}$, 3 $week^{-1}$ and 4 $week^{-1}$) for a duration of 12 weeks, resulting in a total of 0, 3, 12, 24, 36 or 48 FTCs respectively. For each FTC treatment, the covering vegetation of half the cores was removed using a knife (unvegetated treatment), while the other half was left intact (vegetated treatment), resulting in five replicate cores per treatment. Cores were incubated in a climate room at 10°C with a day length of 15 h (at a PAR light intensity of 200 μmol m^{-2} s^{-1}) and a relative humidity of 80%. To simulate a FTC, cores were frozen at –15°C during the night-time (for 9 h) and then completely thawed during daytime (for 15 h) at 10°C in the climate room. Within each week, FTC treatments were applied during four consecutive days, and the cores were left untouched for the remainder of the week. Soil cores were watered and sampled as described above.

Soil chemical analyses and enzymatic activities

Soil analyses for NO_3, NH_4^+ and pH were performed via established standard protocols (Carter, 1993). Enzymatic activities (laccase and cellulase) were assessed in water extracts from 4 g of soil (fresh weight) as previously described (Yergeau *et al*., 2007a).

Nucleic acid extractions

Soil samples were frozen at –20°C and extracted within a week, but RNA degradation still could have occurred. This might have affected our RNA-based analyses, but it is

unlikely to have introduced biases between samples, as all samples were submitted to the same conditions. To homogenize samples that consisted of plant parts from the overlying vegetation, litter and soil, samples were ground in liquid nitrogen using a mortar and pestle. Nucleic acids were then extracted from a 500 mg subsample after disruption by bead beating in a CTAB buffer and subsequent phenol-chloroform purification as described in Yergeau and colleagues (2007b). Mortars, pestles, glassware and beads were baked for at least 2 h at 180°C and all solutions were treated with diethyl pyrocarbonate [0.3% (v/v), overnight incubation at 37°C] to inactivate RNAses. RNA samples were generated by subjecting a portion of the total nucleic acids extraction to treatment with RNAse-free DNAse I (Qiagen, Venlo, the Netherlands).

Molecular analyses

Reverse transcription was carried out with random hexamer primers using a RevertAid First Strand cDNA Synthesis kit (Fermentas, St.Leon-Rot, Germany), following the manufacturer's instructions. All recommended controls for reverse transcription (including a 'no enzyme' control) were negative, ensuring that there was no DNA contamination in RNA samples. PCR amplification and subsequent DGGE analysis of the soil DNA or of the reverse-transcribed soil RNA were carried out using bacterial 16S rRNA gene- and fungal 18S rRNA gene-specific primers as described in Yergeau and colleagues (2007b). Real-time PCR was carried out on soil DNA or reverse-transcribed soil RNA (only for bacterial 16S and fungal 18S rRNA) using an ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) as previously described (Yergeau *et al*., 2007a), using primers and annealing conditions summarized in Table 1. Known template standards were made from whole-genome extracts from pure bacterial isolates (see Table 1). Some of the samples and all standards were assessed in at least two different runs to confirm the reproducibility of the quantification.

Statistical analyses

The two experiments were treated separately in all statistical analyses. DGGE gel banding patterns (Figs S1 and S2) were analysed using the Image Master 1D program (Amersham Biosciences, Roosendaal, the Netherlands). The resulting binary matrices were exported and used in statistical analyses as 'species' presence–absence matrices. Ordination of samples was carried out using principal co-ordinate analysis (PCoA) based on Jaccard's similarity index in P. Legendre's R package (Casgrain and Legendre, 2001). For the presentation of the results, the positions of the different replicate samples were averaged and, in the case of the FTC experiment, the different vegetation treatments were also averaged together.

Multivariate test of significance of the effect of experimental treatments on DGGE patterns was carried out using distance-based redundancy analysis (db-RDA) (Legendre and Anderson, 1999). First, the binary matrices coming from DGGE pattern analyses were transformed using PCoA as detailed above, but without averaging the positions of the different replicates. Then, all the axes (representing 100% of the variation in the data set) were used as 'species' data in RDA in Canoco 4.5 (ter Braak and Šmilauer, 2002), with dummy binary-coded treatment variables being the only environmental variable included in the analysis. The different DGGE gels were entered as covariables and treated as experimental blocks limiting the permutations. The significances of each treatment were tested with 999 permutations.

Real-time PCR, soil data and enzymatic activities were transformed to a dry-weight basis and were subsequently analysed using ANOVA in Statistica 7.1 (Statsoft, Tulsa, OK).

Table 1. Primers and real-time PCR conditions used in this study.

Target	Enzyme	Primers	Annealing temperature (°C)	Standard	Reference
Ribosomal genes					
Bacterial 16S	–	Eub338/Eub518	53	Unidentified 16S clone	Fierer *et al*. (2005)
Fungal 18S	–	Fung5f/FF390r	48	Unidentified 18S clone	Lueders *et al*. (2004)
Functional genes					
nifH	Dinitrogenase (EC 1.18.6.1)	nifHF/nifHRb	TD 65–50	*Burkholderia* sp.	Rosch and Bothe (2005)
amoA	Ammonia monooxygenase (EC 1.13.12.-)	amoA-1F/ amoA-2R-TC	57	*Nitrosomonas europaea*	Nicolaisen and Ramsing (2002)
nasA	Assimilatory nitrate reductase (EC 1.7.1.1)	nas964/nasA1735	59	*Pseudomonas fluorescens*	Allen *et al*. (2001)
nirK	Cu-containing nitrite reductase (EC 1.7.2.1)	F1aCu/R3Cu	57	*Pseudomonas* sp.	Throbäck *et al*. (2004)
nirS	cd_1-containing nitrite reductase (EC 1.7.2.1)	cd3aF/R3cd	57	*Pseudomonas fluorescens*	Throbäck *et al*. (2004)
nosZ	Nitrous oxide reductase (EC 1.7.99.6)	nosZF/nosZ1622R	TD 65–53	*Pseudomonas fluorescens*	Throbäck *et al*. (2004)

TD, touchdown PCR, with a decrease in annealing temperature of 1°C per cycle from the first temperature until the second temperature is reached.

Averages and standard deviations for all data are provided as supplementary material (Tables S1–4). Appropriate normalizing transformations were performed as required (mostly log or square root transformation). One-way ANOVA was used to test the effect of incubation temperatures while factorial ANOVA was used to test the effects of FTC frequency, vegetation and their interaction. In a few extreme cases (NH_4, NO_3 and cellulase activity), data could not be transformed to reach the assumption of parametric ANOVA and non-parametric Kruskal–Wallis ANOVA was carried out instead. Results were considered to be significant at $P < 0.05$ but, as there was large variation between replicates, we also reported effects with *P*-values between 0.05 and 0.10 as nearly significant.

Acknowledgements

This study was supported by NWO Grant 851.20.018 to R. Aerts and G.A.K. E.Y. was partly supported by a Fonds Québécois pour la Recherche sur la Nature et les Technologies Postgraduate Scholarship. Stef Bokhorst and Merlijn Janssens are gratefully acknowledged for sampling soil cores at Signy Island. Wiecher Smant, Gregor Disveld, Barbara Drigo and Sachie Ogawa are thanked for helping with soil analyses, experimental set-up and maintenance of growth chambers. Kevin Newsham, Hans van Veen and Johan Leveau provided useful comments on a previous version of this manuscript. We acknowledge Pete Convey and the British Antarctic Survey for providing logistical support for soil sampling and insightful discussions. NIOO-KNAW Publication #4307.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Average and standard deviation ($n = 6$) for soil parameters and enzymatic activities for microcosm submitted to different incubation temperatures.

Table S2. Average and standard deviation ($n = 5$) for soil parameters and enzymatic activities for vegetated and unvegetated microcosm submitted to different frequencies of freeze–thaw cycles.

Table S3. Average and standard deviation ($n = 6$) for the density of 16S and 18S rRNA genes and rRNA and functional genes related to the N-cycle for microcosm submitted to different incubation temperatures.

Table S4. Average and standard deviation ($n = 5$) for the density of 16S and 18S rRNA genes and rRNA and functional genes related to the N-cycle for vegetated and unvegetated microcosm submitted to different frequencies of freeze–thaw cycles.

Fig. S1. DGGE gels based on fungal 18S or bacterial 16S rRNA genes or rRNA for the constant temperature microcosms. Samples were loaded randomly across gels. For all gels: 3°C incubation treatment: lanes 9, 10, 18, 24, 25, 26; 5°C incubation treatment: lanes 6, 7, 17, 20, 21, 29; 7°C incubation treatment: lanes 2, 3, 4, 13, 14, 28; 15°C incubation treatment: lanes 5, 11, 12, 19, 22, 27; bacterial or fungal marker: lanes 1, 8, 15, 16, 23, 30. X indicates lanes that were left empty and that are not taken into account in lane numbering.

Fig. S2. DGGE gels based on fungal 18S or bacterial 16S rRNA genes or rRNA for the freeze–thaw cycle microcosms. Samples were loaded randomly across gels. For all gels: 0 FTC per week, vegetated: lanes 13, 15, 27, 45, 63; 0 FTC per week, unvegetated: lanes 11, 21, 30, 44, 48; 0.25 FTC per week, vegetated: lanes 8, 16, 19, 41, 60; 0.25 FTC per week, unvegetated: lanes 5, 7, 33, 36, 59; 1 FTC per week, vegetated: lanes 3, 4, 14, 37, 54; 1 FTC per week, unvegetated: lanes 31, 50, 51, 57, 64; 2 FTCs per week, vegetated: lanes 6, 18, 62, 65, 66; 2 FTCs per week, unvegetated: lanes 9, 22, 32, 53, 61; 3 FTCs per week, vegetated: lanes 17, 25, 28, 34, 56; 3 FTCs per week, unvegetated: lanes 10, 26, 38, 39, 55; 4 FTCs per week, vegetated: lanes 2, 29, 40, 43, 67; 4 FTCs per week, unvegetated: lanes 20, 42, 49, 52, 68; bacterial or fungal marker: lanes 1, 12, 23, 24, 35, 46, 47, 58, 69.

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