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Review Paper

Fungal denitrification revisited – Recent advancements and future opportunities

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ABSTRACT

Fungi play a key role in the nitrogen cycle. Diverse fungi are known to reduce nitrate or nitrite to gaseous nitrogen oxides such as nitric oxide, nitrous oxide (N_2O), and dinitrogen via denitrification or co-denitrification (microbially mediated nitrosation), and to ammonium via ammonia fermentation (fungal dissimilatory nitrate reduction to ammonium). These processes could significantly contribute to the emission of N_2O from soils and the removal of nitrogen from nitrate and nitrite-contaminated environments. However, fungal N_2O production may not be necessarily related to their denitrification activity *sensu stricto* (i.e., reduction of nitrate or nitrite to gaseous N oxides for respiration): N_2O can be produced by partially abiotic processes. Therefore, fungi that can reduce nitrate or nitrite to N_2O should not be called denitrifying fungi instantaneously. Experiments should be carefully conducted to better discriminate fungal denitrification, co-denitrification, and chemo-denitrification. Various analytical tools have been developed and applied to clarify fungal denitrification and other nitrate/nitrite reduction processes, including the substrate-induced respiration-inhibition method, stable isotope analyses, and culture-dependent and -independent molecular and genomic approaches. In this mini-review, we overview fungal denitrification and other nitrate/nitrite reduction processes, discuss their environmental impacts, summarize recent advancements in the methods to study fungal denitrification, and provide insights on future research opportunities.

1. Introduction

Fungi play an essential role in the nitrogen (N) cycle. They can mineralize organic N and assimilate inorganic N to build their biomass. Some fungi can also reduce nitrate (NO_3^-) or nitrite (NO_2^-) to gaseous nitrogen oxides such as nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) via denitrification or co-denitrification, and to ammonium (NH_4^+) via ammonia fermentation (Shoun et al., 2012) (Fig. 1). However, their contribution to the N cycle in soil environments is still not well understood.

Fungal denitrification in particular receives recent attention primarily because they might contribute, in large part, to the emission of a greenhouse gas N_2O in soils (Hu et al., 2015; Yoon et al., 2019). Diverse fungi have been also shown to produce N_2O (Mothapo et al., 2015; Shoun et al., 1992). Development and applications of various stable isotope analyses and PCR targeting key genes for fungal denitrification such as fungal nitrite reductase gene (*nirK*) and cytochrome P450 nitric oxide reductase gene (*p450nor*) have also advanced our understanding of fungal denitrification in soil and other environments.

Recent studies, however, also suggest that fungal N_2O production may not be necessarily related to their denitrification activity *sensu stricto* (i.e., reduction of NO_3^- or NO_2^- to gaseous N oxides for respiration). Keusching et al. (2020) detected respiratory reduction of NO_3^- or NO_2^- to N_2O only in *Fusarium* strains, although other fungal strains tested also produced N_2O likely via partially abiotic processes. Co-denitrification has been also criticized as a partially abiotic process (microbially mediated nitrosation) (Spott et al., 2011; Phillips et al., 2016b). Analysis of >700 fungal genomes also raises questions about the involvement of cytochrome P450 Nor homologs in the detoxification of nitric oxide (and the production of N_2O) in various fungi (Higgins et al., 2018). Therefore, to better understand the ecology and impact of fungal denitrification in soil and other environments, it is necessary to carefully evaluate these potentially contrasting findings.

The purpose of this mini-review is to (1) overview fungal denitrification and other NO_3^-/NO_2^- reduction processes, (2) discuss their environmental impacts, (3) summarize recent advancements in the methods

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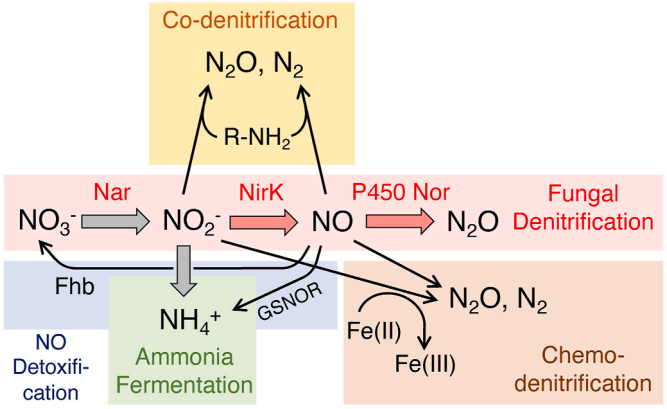

Fig. 1. Transformation of various inorganic nitrogen compounds in fungi and related environments. Fungal denitrification, ammonia fermentation, and nitric oxide (NO) detoxification are biological reactions, whereas co-denitrification (microbially mediated nitrosation) and chemo-denitrification are, at least in part, abiotic reactions. The key reactions in fungal denitrification are shown as red arrows. Legends: Nar, nitrate reductase; NirK, fungal nitrite reductase; P450 Nor, cytochrome P450 nitric oxide reductase; Fhb, NO detoxifying flavohemoglobins; GSNOR, *S*-nitrosoglutathione (GSNO) reductase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to study fungal denitrification, and (4) provide insights on future research opportunities.

2. Fungal denitrification and other NO_3^-/NO_2^- reduction processes

2.1. Fungal denitrification

Denitrification is a microbial respiration process in which NO_3^- or NO_2^- is reduced to NO, N_2O, and/or N_2 in a stepwise manner (Zumft, 1997). Some denitrifiers lack nitrous oxide reductase (Nos), thereby releasing N_2O as their final product. Electrons taken from electron donors such as organic carbon (C) flow through the electron transport chain and are used to generate a proton gradient across a membrane for ATP synthesis. Nitrogen oxides (e.g., NO_3^-, NO_2^-, N_2O) can be used as the terminal electron acceptor for denitrification.

While the production of N_2O from *Fusarium* species was first reported about 50 years ago (Bollag and Tung, 1972), the detailed mechanism of their N_2O production was unclear until Shoun and Tanimoto (1991) discovered fungal nitric oxide reductase in *Fusarium oxysporum*. They found that one of the cytochromes P450 of *Fusarium oxysporum* was overexpressed under anoxic conditions and functioned as a nitric oxide reductase (Nor) (Shoun et al., 1989; Shoun and Tanimoto, 1991). Fungal NO reductase is structurally different from bacterial NO reductase (Park et al., 1997; Hino et al., 2010). In addition, unlike the bacterial NO reduction system, fungal NO reductase is not directly associated with the membrane-bound electron transport chain. Instead, the enzyme is water soluble and localized in both mitochondria and cytosol, and receives electrons directly from NADH ($2\ NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+$) (Shoun et al., 2012; McQuarters et al., 2014). Therefore, fungi likely receive benefits by oxidizing NADH to maintain cellular metabolism under anoxic conditions (Shoun et al., 2012; Shimizu, 2018). These dissimilarities between fungal and bacteria Nor indicate that fungal P450 Nor might have evolved independently of the bacterial Nor system.

Denitrifying fungi also possess copper-containing nitrite reductase (NirK). Fungal NirK is associated with the mitochondrial respiratory chain and plays a role in the synthesis of ATP (Kobayashi and Shoun, 1995; Kobayashi et al., 1996), which appears similar to bacterial nitrite reductases. While in bacterial denitrification, there are two types of nitrite reductases, copper-containing NirK type and cytochrome cd_1-containing NirS type (Zumft, 1997; Jang et al., 2018), only NirK type nitrite reductase has been identified in fungi (Higgins et al., 2018). All fungal NirK sequences identified thus far are monophasic and closely related to bacterial NirK, indicating that fungi most likely acquired NirK by endosymbiotic gene transfer (EGT; Timmis et al., 2004) from proto-mitochondrion (Kim et al., 2009).

NirK and P450 Nor are likely the key enzymes for fungal denitrification. In addition to these enzymes, NO detoxifying flavohemoglobins (Fhb), which oxidize NO to NO_3^-, and ubiquinone-dependent formate dehydrogenase (UQFdh) are also involved in fungal denitrification in *Fusarium* (Uchimura et al., 2002; Kim et al., 2010). Some denitrifying fungi also possess dissimilatory nitrate reductase for NO_3^- reduction to NO_2^-. The dissimilatory nitrate reductase of *Fusarium oxysporum* is localized within the mitochondrion and is closely related to bacterial respiratory nitrate reductase (NarG type) (Uchimura et al., 2002), similar to the NirK case. However, some denitrifying fungi (e.g., *Fusarium lichenicola*, formally known as *Cylindrocarpon tonkinense*) lack dissimilatory nitrate reductase. They most likely use assimilatory nitrate reductase to convert NO_3^- to NO_2^- (Watsuji et al., 2003; Shoun et al., 2012), although this process was not experimentally verified in a recent study (Keuschnig et al., 2020). Interestingly, the gene for periplasmic nitrate reductase (*napA*) was much more frequently detected than *narG* on the genomes of most fungi that have NirK or P450 Nor (Higgins et al., 2018), suggesting the potential involvement of NapA-type nitrate reductase in fungal denitrification similar to bacterial denitrification system. In addition, Nos has not been identified in fungi (Higgins et al., 2018), although the production of $^{30}N_2$ from ^{15}N-labeled NO_3^- in some denitrifying fungi was reported (Aldossari and Ishii, 2021). As a result, the end product of fungal denitrification is believed to be mostly N_2O (Yoon et al., 2019).

Although the expression of fungal denitrification can occur in low O_2 conditions, it is reported that a minimal amount of oxygen is still required (Zhou et al., 2001). Under such conditions, both oxygen respiration and denitrification can occur simultaneously (Zhou et al., 2001; Shoun et al., 2012). However, respiratory NO_3^- reduction can occur even after O_2 is depleted in *Fusarium* strains (Keuschnig et al., 2020). In addition, some fungi are reported to denitrify in the absence of O_2 (Aldossari and Ishii, 2021; Phillips et al., 2016a).

2.2. Co-denitrification (microbially mediated nitrosation)

Co-denitrification is a (semi-)microbial process in which a hybrid N_2 or N_2O species is formed by combining the N in NO_2^- or NO and other N compounds such as amines, imines, or azides (Tanimoto et al., 1992). N_2 or N_2O is produced as the product of co-denitrification depending on the form of the organic N used: amines generate N_2, whereas imines or azides form N_2O (Shoun et al., 2012). However, co-denitrification has been criticized as a partially abiotic process (see section 2.5) and is also called as microbially mediated nitrosation (BioNitrosation) (Spott et al., 2011; Phillips et al., 2016b). In the BioNitrosation mechanism, NO_2^- or NO formed by biotic reaction can abiotically react with other N species (e.g., amines) to form N–N bonding (e.g., N_2 and N_2O). Although BioNitrosation has been reported to occur in various bacteria (Spott et al., 2011), it may be possible that the fungal denitrification system is more prone to BioNitrosation (i.e., more NO_2^- or NO could be available for abiotic reactions than the bacterial denitrification system). This should be experimentally tested in the future. Nonetheless, co-denitrification (BioNitrosation) can produce N_2 gas, and therefore, can reduce the emission of N_2O, a potent greenhouse gas.

When ^{15}N-labeled NO_3^- is used, fungi can produce $^{29}N_2$ via co-denitrification (Aldossari and Ishii, 2021; Tanimoto et al., 1992; Shoun et al., 2012). Because the production of $^{29}N_2$ is frequently used as an indication of the occurrence of anaerobic ammonium oxidation (anammox) (Oshiki et al., 2016), ^{15}N tracer experiments should be carefully designed to differentiate the contribution of fungal

co-denitrification and anammox (See Section 4.2 for more details).

2.3. Ammonia fermentation (dissimilatory nitrate reduction to ammonium)

Ammonia fermentation, or fungal dissimilatory nitrate reduction to ammonium (DNRA), is another form of fungal respiration in which NO_3^- is reduced to NO_2^- and then to NH_4^+ under anaerobic condition (Zhou et al., 2002; Shimizu, 2018). The process is similar to bacterial DNRA (Tiedje, 1988); however, in fungal ammonia fermentation, aNar, and assimilatory nitrite reductase (aNir) are involved (Takasaki et al., 2004). In ammonia fermentation, the reduction of NO_3^- is coupled with the oxidation of ethanol or acetate via substrate-level phosphorylation (Zhou et al., 2002; Takasaki et al., 2004). During this process, a small amount of N_2O can be also produced (Stief et al., 2014).

Fungal ammonia fermentation is poorly studied compared with fungal (co)denitrification (Gleason et al., 2019). Further research is needed to understand the physiology, ecology, and impacts of fungal ammonia fermentation in soil environments. In the following sections, we focus mostly on fungal denitrification and co-denitrification.

2.4. Production of NO and N_2O as a result of fungal non-respiratory processes

By definition, denitrification is a microbial respiratory process (Zumft, 1997); however, the production of NO and N_2O by some fungi could be unrelated to their respiration. Nitric oxide is used as a signaling molecule in plants and is involved in the regulation of variety of processes, including defense against bacterial and fungal pathogens (Martínez-Medina et al., 2019). While plants likely use NO to produce nitrosasive stress to combat fungal infections, pathogenic fungi use NO as a signaling molecule to infect plants (Cánovas et al., 2016). Some fungal pathogens also produce NO when infecting plants. Ding et al. (2020) reported that genes related to NO production and detoxification are activated at an early stage of infection of a plant pathogen *Fusarium graminearum* to plant roots (Ding et al., 2020).

It is vital for fungi to maintain the level of NO because high NO levels can be toxic to cells. This can be achieved by using their NO detoxification mechanisms. While flavohemoglobin NO dioxygenase (Fhb) converts NO to NO_3^-, cytochrome P450 Nor converts NO to N_2O (Cánovas et al., 2016). In addition, the levels of NO can be maintained by *S*-nitrosoglutathione (GSNO) reductase, which converts GSNO, an *S*-nitrosylated form of NO, to NH_4^+ and other less toxic compounds (Cánovas et al., 2016). As discussed in more detail in Section 2.5 below, NO can be abiotically produced. Fungi may have to deal with these abiotically produced NO in addition to those produced by fungi and plants by using their NO detoxification mechanisms; however, it remains unclear if and how P450 Nor is involved in the NO detoxification in fungi.

2.5. Confusion with abiotic NO_3^-/NO_2^- reduction and N_2O/N_2 production reactions

One of the major criticisms on fungal (co-)denitrification is the potential involvement of abiotic reactions during fungal NO_3^-/NO_2^- reduction and N_2O/N_2 production reactions (i.e., chemodenitrification and nitrosation). Chemodenitrification is the abiotic reduction of nitrogen oxide, especially NO_2^- and NO to N_2O and N_2 (Zhu-Barker et al., 2015). Nitrosation is the abiotic production of N_2 and N_2O from nitroso compounds (e.g., NO_2^-) or NO and amine compounds (see Section 2.2 above). Chemodenitrification is facilitated under acidic or metal-rich conditions (Zhu-Barker et al., 2015; Ishii et al., 2016). Soil organic matter can also promote chemodenitrification (Wei et al., 2017). Chemodenitrification can be a significant source of N_2O in environments where Fe^{2+} or soil organic matter is abundantly present (Wankel et al., 2017; Buessecker et al., 2019; Otte et al., 2019; Wang et al., 2020).

Although the abiotic reduction of NO_3^- to NO_2^- coupled with the oxidation Fe^{2+} can be slow at anoxic and circumneutral pH condition (Buresh and Moraghan, 1976; Choi and Oh, 2020), NO_2^- and NO are highly reactive and can be abiotically reduced at much faster rates (Kampschreur et al., 2011; Benaiges-Fernandez et al., 2020; Chen et al., 2020). Therefore, it is possible that NO_2^- produced as the results of fungal NO_3^- reduction can be abiotically reduced to NO and N_2O. Therefore, N_2O produced during the incubation of fungal strains does not necessarily originate from biological denitrification *sensu stricto* (Keuschnig et al., 2020). More research is needed to distinguish N_2O produced via fungal denitrification from those produced via abiotic processes (see section 4.2 below for further discussion) and establish a standard protocol to confirm fungal denitrification ability (See Section 4.3 below).

3. Environmental impacts of fungal denitrification and NO_3^-/NO_2^- reduction

3.1. Emission of N_2O

As stated above, N_2O can be produced by fungal denitrification and NO_3^-/NO_2^- reduction reactions, which is of great concern because N_2O is a potent greenhouse gas and a stratospheric ozone layer destructor (Ravishankara et al., 2009; Hu et al., 2015; Yoon et al., 2019). Numerous studies have suggested the large contribution of fungi to N_2O production in various soil environments. In many cases, the contribution of fungi to soil N_2O emission is greater than that of bacteria (e.g., Laughlin and Stevens, 2002; Laughlin et al., 2009; Marusenko et al., 2013; Chen et al., 2014; Wei et al., 2014; Rex et al., 2018). These findings are based on the substrate-induced respiration-inhibition (SIRIN) method, stable isotope analyses, and/or culture-dependent and -independent analyses. These technologies have their unique strengths and weaknesses in detecting fungal denitrification signatures, which is discussed further in Section 4 below.

Various factors can influence the N_2O emission in soil environments, including the concentrations of N oxides (e.g., NO_3^- and NO_2^-), soil moisture content (which affects soil redox potential and O_2 availability), organic matter contents, temperature, and pH (Hu et al., 2015). These factors also influence the activity of fungi and their N_2O production. For example, fungi were identified as the main contributor to the N_2O emission in a cropland field soil amended with organic fertilizers, especially after precipitation events (Wei et al., 2014). High NO_3^- concentration and low O_2 availability in these conditions likely promoted fungal denitrification (Wei et al., 2014). Organic carbon content could also influence fungal N_2O emissions in soils (Ma et al., 2017; Huang et al., 2021). Temperature can also impact the N_2O producing communities in soils. N_2O producing fungi are more tolerant to elevated temperature (40 °C) than bacteria (Xu et al., 2017). Fungal contribution to the N_2O production can also vary by pH. The contribution of fungi to the emission of N_2O can increase in more acidic conditions (Huang et al., 2021), which is in agreement with the higher fungi-to-bacteria ratios in low-pH soils (Rütting et al., 2013; Chen et al., 2015). However, the effect of pH may be soil dependent as Herold et al. (2012) reported no significant difference in the fungal N_2O production rates in soils with different pH ranging from 4.2 to 6.6. In addition to fungal denitrification, fungal co-denitrification can significantly contribute to the production of N_2O in soils (Selbie et al., 2015; Rex et al., 2018), especially when NO_2^- concentration and soil moisture content are high (Clough et al., 2017).

Recently, inhibitors for the fungal denitrification process were identified (Matsuoka et al., 2017). These inhibitors can bind to nitrite reductase and thereby significantly reduce the enzymatic activity in *Fusarium oxysporum*. The use of fungal denitrification inhibitors has the potential to decrease the N_2O emission from soils, which should be tested in the future.

3.2. Application for nitrate removal

Fungal denitrification has a strong potential for biotechnology application, especially for N removal. For example, fungi play a crucial role in degrading woodchips to provide C and electrons for denitrification in woodchip bioreactors, a promising technology to remove NO_3^- leached from agricultural fields (Schipper et al., 2010). Some fungi can also grow well and reduce NO_3^- at low temperatures (Aldossari and Ishii, 2021), which is important because the temperature of nitrate-rich agricultural drainage could be low (<5 °C) (Jang et al., 2019a). In addition, some fungi can reduce NO_3^- to gaseous N under aerobic conditions (i.e., aerobic denitrification) (Zhang et al., 2018; Cheng et al., 2020; Yao et al., 2020). Furthermore, the ability of fungi to extend their hyphae would allow them to be better retained (compared to bacteria) in bioreactors filled with solid media (e.g., woodchips). This is important when bioaugmentation is used to enhance N removal because the effects of bioaugmentation with bacterial denitrifiers did not last long most likely due to the washout of inoculated bacteria (Feyereisen et al., 2018). Indeed, the inoculation of nitrate-reducing fungi has been shown to enhance N removal in a woodchip bioreactor (Yao et al., 2020). Although the production of N_2O by fungal denitrification can be a potential problem, this can be mitigated by using N_2O-reducing biofilters (Yoon et al., 2017).

4. Methods for studying fungal denitrification and NO_3^-/NO_2^- reduction

4.1. Substrate-induced respiration-inhibition (SIRIN) method

Both bacteria and fungi as well as abiotic processes (e.g., nitrosation and chemodenitrification) can contribute to the reduction of NO_3^-/NO_2^- to gaseous products (NO, N_2O, and N_2). To mitigate N pollution and prevent N_2O production, it is important to identify the pathways of NO_3^- reduction. For this purpose, the substrate-induced respiration-inhibition (SIRIN) method originally developed by Anderson and Domsch (Anderson and Domsch, 1973, 1975) is frequently used. In the SIRIN method, bacterial and fungal activities are inhibited using bactericides (e.g., streptomycin, oxytetracycline, and bronopol) and fungicides (e.g., cycloheximide and captan), respectively. By comparing the amount of N_2O or N_2 produced in the presence of bactericides and fungicides, we can estimate the contribution of fungi and bacteria, respectively, to the production of N_2O/N_2. Based on the SIRIN method, fungi have been identified as a significant contributor to the N_2O productions in various environments such as grassland, agricultural, and forest soils (e.g., Laughlin and Stevens, 2002; Laughlin et al., 2009; Chen et al., 2014; Wei et al., 2014; Rex et al., 2018), although recent research done with the combination of the SIRIN method and stable isotope analysis suggest limited fungal contribution (Rohe et al., 2020a).

Although the SIRIN method has been widely used, it also has several limitations. One of the major criticisms of the SIRIN method is related with the insufficient inhibition of the target organisms as well as the inhibition of non-target organisms by the bactericides/fungicides used. For example, only about 60% of bacterial respiration was inhibited by streptomycin in a fungus-free soil (Velvis, 1997). When such inefficient bacteria inhibitors are used, the contribution of fungi to nitrate reduction may be overestimated (Ladan and Jacinthe, 2016). Some inhibitors could also influence non-target organisms. For example, cycloheximide, which is commonly used to inhibit protein synthesis in fungi, may also inhibit some bacteria such as ammonia oxidizing bacteria when concentration is high (>2.5 mg/g soil) (Castaldi and Smith, 1998). Similarly, the fungicides captan and benomyl can also inhibit the growth of bacteria (Rousk et al., 2009). The effectiveness of antibiotics can be also influenced by environmental conditions (e.g., pH; Bååth and Anderson, 2003) and time after application. For example, cycloheximide can be degraded immediately after applied to soil (Badalucco et al., 1994). Therefore, it is essential to conduct a preliminary experiment to determine appropriate inhibitors and their concentrations (Pan et al., 2019). It is also important to include appropriate controls (e.g., bacterial + fungal inhibitors, no inhibitors) to evaluate the occurrence of abiotic NO_3^-/NO_2^- reducing reactions.

Future research may include the use of more specific bacterial and fungal denitrification inhibitors. For example, recently developed inhibitors for bacterial (Bardon et al., 2014, 2016) and fungal denitrification processes (Matsuoka et al., 2017) might be useful for the SIRIN method. Because the fungal denitrification inhibitor specifically inhibits fungal dissimilatory nitrite reductase (Matsuoka et al., 2017), fungal non-denitrifying NO_3^-/NO_2^- reducing reactions (e.g., fungal NO_3^- reduction followed by abiotic NO_2^- reduction) are likely not inhibited. Therefore, it would be possible to evaluate the contribution of fungal denitrification *sensu stricto* separately from other fungal NO_3^-/NO_2^- reducing reactions.

4.2. Stable isotope analyses

4.2.1. Natural abundance of N and O isotopes

N_2O can be produced as an end or intermediate product of denitrification (i.e., reduction of NO; done by bacteria/archaea and fungi) and as a by-product of nitrification (i.e., oxidation of NH_2OH; done by bacteria and archaea). N_2O can be also abiotically produced (i.e., chemo-denitrification), especially when NO_2^- or NH_2OH are present under acidic or metal-rich conditions (Heil et al., 2014; Wang et al., 2020). Analysis of the natural abundances of stable isotopes in N_2O has been widely used to identify the sources of N_2O production pathways, including the measurements of isotope ratios of bulk N_2O ($\delta^{15}N^{bulk}$ and $\delta^{18}O$, where $\delta=(((R_{sample}/R_{standard})-1) \times 1000)$ in units of ‰ and $R = {}^{15}N/{}^{14}N$ or ${}^{18}O/{}^{16}O$) and the intramolecular distribution of the ^{15}N isotopes in the central (α) and peripheral (β) positions in asymmetric N_2O molecule (^{15}N site preference [SP] $= \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$) (Baggs, 2008; Ostrom and Ostrom, 2017; Yu et al., 2020). Traditionally, a gas chromatography isotope ratio mass spectrometry (GC-IRMS) has been used to measure $\delta^{15}N$ and $\delta^{18}O$ as well as the N_2O SP values. Relatively recently, quantum cascade laser absorption spectroscopy (QCLAS) was introduced, which allows us to measure these values in near real-time with the analytical precision similar to those obtained by GC-IRMS (Mohn et al., 2012; Köster et al., 2013).

Because the SP values of N_2O produced via nitrification and those produced via bacterial denitrification are different, the N_2O SP has been widely used for N_2O source partitioning (Baggs, 2008; Ostrom and Ostrom, 2017; Yu et al., 2020) (Fig. 2). Although microbial N_2O reduction can increase the $\delta^{15}N^{bulk}$ of the unreacted N_2O (i.e., enrichment of ^{15}N) as well as the SP values of N_2O, it is possible to take account

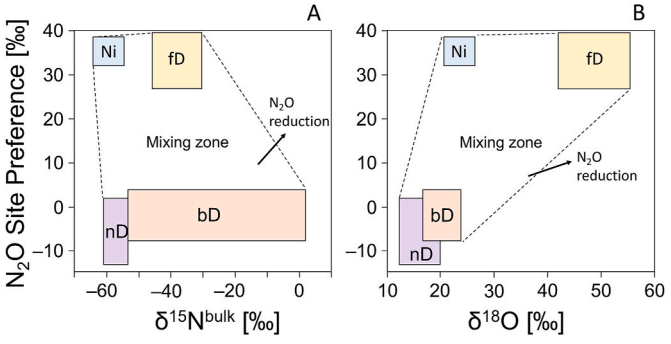

Fig. 2. Dual isotope plots showing the relationships between the site preference and (A) $\delta^{15}N^{bulk}$ and (B) $\delta^{18}O$ values of N_2O produced via various biological reactions. Legends: bD, bacterial denitrification; fD, fungal denitrification; nD, nitrifier denitrification; and Ni, nitrification (NH_2OH oxidation). Microbial N_2O reduction can increase the site preference, $\delta^{15}N^{bulk}$, and $\delta^{18}O$ values of N_2O as indicated by arrows. Values for chemo-denitrification are not shown. Modified from Yu et al. (2019).

this process when determining the relative contribution of nitrification and bacterial denitrification to N_2O production (Ishii et al., 2014; Wu et al., 2019; Yu et al., 2020).

The N_2O SP can be also used to distinguish N_2O produced via bacterial denitrification and that produced via fungal denitrification. The reported SP values for N_2O produced via fungal denitrification ranged between 16‰ and 37‰ (Sutka et al., 2008; Rohe et al., 2014; Yang et al., 2014; Maeda et al., 2015; Chen et al., 2016a). Some of these SP values may contain those of abiotically produced N_2O or via co-denitrification; therefore, care should be taken when using these values as the reference for fungal denitrification. These SP values overlap with the ranges of SP values for nitrification (13–37‰), and therefore, it is difficult to distinguish these processes based on the SP values alone. Combined analysis of $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP of N_2O would allow us to identify potential sources of N_2O (Rohe et al., 2020a), although the N_2O reduction process may complicate the calculation (Fig. 2). Analysis of $\delta^{15}N^{bulk}$ and $\delta^{18}O$ in the substrates of N_2O production reactions (e.g., $\delta^{15}N_{NO2}$, $\delta^{15}N_{NH4}$, $\delta^{18}O_{NO2}$, $\delta^{18}O_{H2O}$, $\delta^{18}O_{O2}$) can further increase the accuracy of the source partitioning (Rohe et al., 2017; Yu et al., 2020). Based on this approach, N_2O produced via fungal denitrification and/or nitrification accounted for 34–42% of total N_2O emissions in a grassland soil (Ibraim et al., 2019). Similarly, fungal denitrification and/or nitrification were identified as the dominant contributor to the N_2O emission from N-fertilized agricultural soils (Lin et al., 2020).

Analysis of "anomalous ^{17}O", which is expressed as $\Delta^{17}O$ where $\Delta^{17}O = [(1 + \delta^{17}O/1000)/(1 + \delta^{18}O/1000)^{0.525} - 1] \times 1000$ (Miller, 2002; Lewicka-Szczebak et al., 2016) in N_2O and NO_2^-, could allow us to discriminate N_2O produced via fungal denitrification from those produced via nitrification (Wankel et al., 2017). Based on this analysis, they found that fungal denitrification and/or chemodenitrification play a large role in N_2O production in coastal sediments.

It is still challenging to discriminate N_2O produced via fungal denitrification and those via chemodenitrification, in part because of the large variations (−4–37‰) seen in the SP values for the N_2O produced via chemodenitrification (Heil et al., 2014; Wei et al., 2017, 2019; Otte et al., 2019; Yu et al., 2020). Various SP values were obtained probably because of the different pH and redox conditions used, as well as the substrates and pathways for the abiotic N_2O-producing reactions (Yu et al., 2020). Further research is necessary to clarify the conditions and rates of specific abiotic N_2O-producing reactions (including co-denitrification) and to obtain the SP values for N_2O produced by each of these reactions. It is also important to refine the SP values for the N_2O produced via fungal denitrification by excluding those produced via co-denitrification.

4.2.2. Tracing the fate of ^{15}N-labeled substrates amended to soils

In addition to the analysis of the natural abundance of the nitrogen and oxygen isotopes, stable isotopes can be used to trace the fate of isotopically labeled substrates added to soils. This technique can be used to assess the potential contribution of various processes to N_2O and N_2 productions. For example, Laughlin et al. (2009) assessed the bacterial and fungal contribution to the N_2O production in a grassland soil by incubating the soil with ^{15}N-labeled NH_4^+ and NO_3^- as well as bacterial and fungal inhibitors (Laughlin et al., 2009). Similar approaches were used to measure the N_2 production rates of bacterial and fungal denitrification, fungal co-denitrification, and anammox in agricultural soils (Long et al., 2013; Abbas et al., 2019). Although $^{29}N_2$ can be produced by both fungal co-denitrification and anammox processes, their relative contribution to the $^{29}N_2$ production could be assessed, at least in part, by using bacterial and fungal inhibitors (Long et al., 2013).

4.3. Isolation and characterization of denitrifying and NO_3^-/NO_2^- reducing fungi

4.3.1. Media and conditions for fungal isolation

Culture-dependent analyses to study denitrifying and NO_3^-/NO_2^- reducing fungi begins with the isolation of fungi from environmental samples. Soil has been primarily used as the source to isolate fungi (Mothapo et al., 2015), although some other sources (e.g., woodchips collected from a denitrifying bioreactor) were also used (Aldossari and Ishii, 2021). Various denitrifying and NO_3^-/NO_2^- reducing fungi, mostly belonging to the phyla *Ascomycota* and *Basidiomycota*, have been isolated from these environments (reviewed in Mothapo et al., 2015). The most frequently isolated denitrifying fungi belong to the genus *Fusarium*. However, the results of fungal isolation may also depend on the media used.

There is no clear consensus for the media used to isolate denitrifying fungi. Various culture media have been used to isolate denitrifying fungi, including potato dextrose agar (PDA), Rose Bengal agar (RBA), glycerol peptone agar, and Czapek-Dox media with various modifications (e.g., Mothapo et al., 2013; Wei et al., 2014; Higgins et al., 2016; Novinscak et al., 2016; Aldossari and Ishii, 2021). These media can be supplemented with NO_3^- or NO_2^- and antibiotics (e.g., streptomycin) to promote denitrification and inhibit bacterial growth, respectively. Multiple media are often simultaneously used to obtain diverse fungi. Media to use also depend on the type of materials from which fungi are to be isolated. For example, to isolate denitrifying fungi from marine sediment, Cathrine and Raghukumar (2009) used malt extract agar, corn meal agar, malt extract broth, and Czapek-Dox agar, all of which were prepared in 1/5 strength with synthetic seawater with the addition of 10 mM sodium nitrate.

Similarly, various incubation conditions have been also used to isolate denitrifying fungi. Incubation temperatures range from 5 to 30 °C. Media are usually incubated under anoxic or microaerobic conditions, which can be created by replacing the air-phase with anoxic gas (e.g., N_2, Ar, He) or anoxic gas supplemented with a small amount (1–3% in headspace) of O_2, respectively. Anoxic or microaerobic conditions can be also produced by using O_2-consuming substrates or catalysts (e.g., AnaeroPak). Some studies also use oxygenic preincubation prior to incubating the media under anoxic or microaerobic conditions (e.g., Maeda et al., 2015). As mentioned above, a small amount of oxygen is required for fungal denitrification to occur.

4.3.2. Analysis of N gaseous products

Once fungal strains are obtained, their ability to reduce NO_3^- or NO_2^- to gaseous products should be examined and confirmed. Since the end product of fungal denitrification is in most cases N_2O, it can be measured using gas chromatography (GC) with an electron capture detector (ECD). When ^{15}N-labeled substrates are used, the production of N_2O and N_2 can be detected by GC-IRMS or GC coupled with mass spectrometry (GC-MS). Unlike the case of bacterial denitrification (Tiedje, 1994; Mahne and Tiedje, 1995), there are no standard methods for fungal denitrification confirmation. The culture conditions (e.g., medium, temperature, pH, incubation period, and O_2 concentration) vary between studies. Some media (e.g., glycerol peptone broth) contain organic N (Table S1), which could react with NO_2^- or NO to produce N_2O or N_2 (co-denitrification or BioNitrosation) (Aldossari and Ishii, 2021). This is problematic when identifying fungal denitrifiers *sensu stricto*. To discriminate fungal denitrification from co-denitrification (BioNitrosation), ^{15}N tracer experiments could be useful, in which the production of $^{46}N_2O$ or $^{30}N_2$ indicates the occurrence of denitrification whereas the production of $^{45}N_2O$ or $^{29}N_2$ indicates the occurrence of co-denitrification (Aldossari and Ishii, 2021). This approach, however, cannot exclude the possibility of N_2O or N_2 production via chemodenitrification. Therefore, proper negative controls (no biomass control, dead cell control, etc.) are necessary. Future research needs include the development of a standardized method for fungal denitrification confirmation ideally with organic-N free medium (e.g., modified Czapek-Dox broth; Table S1) with reduced amount of Fe^{2+}. It is ideal to measure the concentrations of key intermediates such as NO_2^- and NO, in addition to the substrate NO_3^- and the products (N_2O, N_2, NH_4^+, and CO_2) of fungal denitrification and other N reduction processes.

4.3.3. Presence of key denitrification genes

Detection of fungal *nirK* and *p450nor* in the isolated fungal strains can be done using PCR. Multiple PCR assays have been developed to amplify fungal *nirK* and *p450nor* from fungal strains and soils (Long et al., 2014; Maeda et al., 2015; Wei et al., 2015; Chen et al., 2016b; Higgins et al., 2016; Novinscak et al., 2016; Chen and Shi, 2017; Rohe et al., 2020b) (Table 1 and Fig. 3). Although these primers were designed to broadly cover diverse target gene sequences, fungal *nirK* or *p450nor* were often undetected by PCR in many N_2O-producing fungi. For example, Novinsack et al. (2016) reported that 75% of the N_2O-producing fungi were negative for *p450nor* by PCR. Similarly, Chen et al. (2016b) reported that 36% of their fungal strains were PCR-negative for *nirK*. Moreover, Aldossari and Ishii (2021) identified fungal strains capable of reducing ^{15}N-labeled nitrate to $^{30}N_2$ gas, although nitrous oxide reductase gene *nosZ* was not detected by PCR. Fungal *nirK* and *p450nor* as well as *nosZ* are highly diverse, and therefore, it might be difficult to design universal primers that can amplify these genes in all denitrifiers (Ma et al., 2019). Therefore, denitrification capability should not be ruled out by negative PCR results due to the PCR bias. Whole genome sequencing would be more useful in detecting denitrification genes because it does not have such biases (see section 4.3.4). It is important to note, however, that some of the fungal strains tested in the previous studies may not be denitrifiers *sensu stricto* (i.e., microbial dissimilatory reduction of nitrate/nitrite to gaseous end products for respiration purpose). Some of them may just reduce NO_3^- to NO_2^- or NO, which were then reduced to N_2O or N_2 via nitrosation (co-denitrification). Recently, Keuschnig et al. (2020) claimed that denitrification *sensu stricto* is only confirmed in the genus *Fusarium*. Future research is necessary to establish a standard protocol to confirm fungal denitrification by combining PCR, whole genome sequencing, and N_2O measurements with appropriate controls (see section 5 for more detail).

4.3.4. Genome sequencing

Whole genome sequencing is useful to assess the potential metabolisms of microorganisms. As discussed above, PCR alone is not sufficient to detect denitrification genes such as *nirK* and *p450nor*. By sequencing and analyzing the whole genomes of denitrifying fungi (including those reducing nitrate/nitrite and producing N_2O), it would be possible to identify *nirK* and *p450nor* as well as other genes responsible for denitrification. However, the number of fungal genomes that are experimentally verified to perform denitrification or produce N_2O is limited.

Recently, Higgins et al. (2018) analyzed the occurrences of denitrification-related genes (*narG*, *napA*, *norB*, *nirK*, *nosZ*, and *p450nor*) as well as the genes for NO-detoxifying flavohemoglobins (*fhb*) on the fungal genomes in the public databases. The genomes included in their analysis (712 genomes) are not necessarily of denitrifying fungi; however, they found wide-spread occurrence (23%, 167 out of 712 genomes) of *p450nor* in diverse fungi. Nitrate reductase gene (*napA* and *narG*) and nitrite reductase gene (*nirK*) were also found but at lesser extents (75, 3, and 82 genomes, respectively). These genes do not necessarily co-occur in the genomes. Among 167 *p450nor*-containing genomes, 30 (18%) and 48 (29%) genomes also harbor *napA* and *nirK*, respectively, and only 18 genomes (11%) harbored all gene set required for the reduction of nitrate to N_2O (*napA*, *nirK*, and *p450nor*). Phylogenetic analysis suggest the close relationship between fungal P450 Nor and actinobacterial CYP105 family P450s that involve in secondary metabolism (Higgins et al., 2018). In addition, secondary metabolism-related genes were identified around *p450nor* in many fungal genomes. Furthermore, *p450nor* and the genes for NO-detoxifying flavohemoglobins co-occur in many fungal genomes. Based on these findings, Higgins et al. (2018) proposed that some of the P450 Nor homologs might be involved in secondary metabolism instead of respiratory NO reduction or NO detoxification. Further biochemical experiments are necessary to clarify the role of P450 Nor in fungal denitrification or secondary metabolism, especially in non-*Fusarium* fungi.

Based on our analysis of Higgins et al. (2018) data, 76 out of 82 *nirK*-positive genomes (93%) harbor the genes encoding NO-removing reactions (*p450nor* or *fhb*) (Table S2). Because the product of nitrite reduction by NirK (= nitric oxide) can be toxic to cells, these fungi most likely need to remove NO by either using Fhb or P450 Nor. Presence of GSNO reductase was not analyzed by Higgins et al. (2018), although some fungi are known to possess this NO detoxification system (Cánovas et al., 2016). It is still unclear if Fhb and GSNO reductase are involved in denitrification, in addition to or as a substitute for p450 Nor, although Kim et al. (2010) reported high expression of Fhb under denitrifying conditions in *Fusarium lichenicola*. Further genome/transcriptome/proteome as well as biochemical analyses, especially of denitrifying fungi, are necessary to identify the key genes/proteins and pathways associated with fungal denitrification and N_2O production.

4.4. Culture-independent molecular analyses to study denitrifying fungi in environments

4.4.1. PCR-amplicon sequencing

PCR targeting fungal *nirK* and *p450nor* can be directly applied to soil

Table 1
List of PCR primers available to study fungal *nirK* and *p450nor*.

	Forward Primer		Reverse Primer		Amplicon Size (bp)	Coverage (%)[a]	Reference
	Name	Sequence (5'→ 3')	Name	Sequence (5'→ 3')			
Fungal *nirK*	nirKfF	TACGGGCTCATGtaygtnsarcc	nirKfR	AGGAATCCCACAscnccyttntc	480	88.1	Wei et al., 2015
	fnirK2F	GTYCAYATYGCYAACGGSATGTACGG	fnirK1R	GCRTGRTCNACMAGNGTRCGTCCC	468	32.7	Long et al., 2015
	EunirK-F1	GGBAAYCCICAYAAYATCGA	EunirK-R2	GGICCIGCRTTSCCRAAGAA	446	86.2	Maeda et al., 2015
	nirK1F	GTCCCHGGMCCHTTCAT	nirK1R	GGYTCRTGGTARAACTCGC	337	59.1	Novinscak et al., 2016
	FnirK_F1	GTCCCHGGMCCHTTCAT	FnirK_R1	CRTGGTARAACTCGCTYTGC	331	59.8[b]	Chen et al. (2016)
	FnirK_F1	GTCCCHGGMCCHTTCAT	FnirK_R2	TVCCGATDAYRTGGAAYGARC	554	40.9[b]	Chen et al. (2016)
	FnirK_F2	CATYGCCAAYGGYATGTA	FnirK_R2	TVCCGATDAYRTGGAAYGARC	314	62.9[b]	Chen et al. (2016)
	FnirK_F3	GCARAGCGAGTTYTACCAYG	FnirK_R2	TVCCGATDAYRTGGAAYGARC	233	54.7	Chen et al. (2016)
p450nor	p450nor394F	SCIACITTYGTIGAYATGGA	p450nor809R	ATCATGTTIACBAIIGTIGCIT	415	79.4	Higgins et al., 2016
	p450nor394F	SCIACITTYGTIGAYATGGA	p450nor1008R	GMSGCRATKATNCCYTC	614	85.3	Higgins et al., 2016
	P450nor1F	CCSACDTTYGTYGAYATGGA	P450nor1R	GTBGCRTTVCCNGCVAC	660	85.3	Novinscak et al., 2016
	Fnor1F	CCVACITTYGTBGAYATGGA	Fnor1R	TBACBAYRGTIGCRTTICC	~650	91.2[b]	Chen and Shi, 2017
	Fnor2F	TTYGTBGAYATGGAYSCICC	Fnor2R	TCATGTTBACCATRGTIGCRT	~650	79.4[b]	Chen and Shi, 2017
	P3.04fw	GCCACCATGGTVAAYATGAT	p5.02rev	GTGGTAGCGGCANAGYTCYTC	155	72.1[b]	Rohe et al. (2020)

[a] Coverages of the primers in the target sequences reported in Ma et al. (2019).
[b] Coverages were analyzed in this study by using Geneious Prime ver. 2021.0.3 (http://www.geneious.com) and the fungal *nirK* and *p450nor* databases generated by Ma et al. (2019) (http://hdl.handle.net/2142/101877) according to the criteria used by Ma et al. (2019).

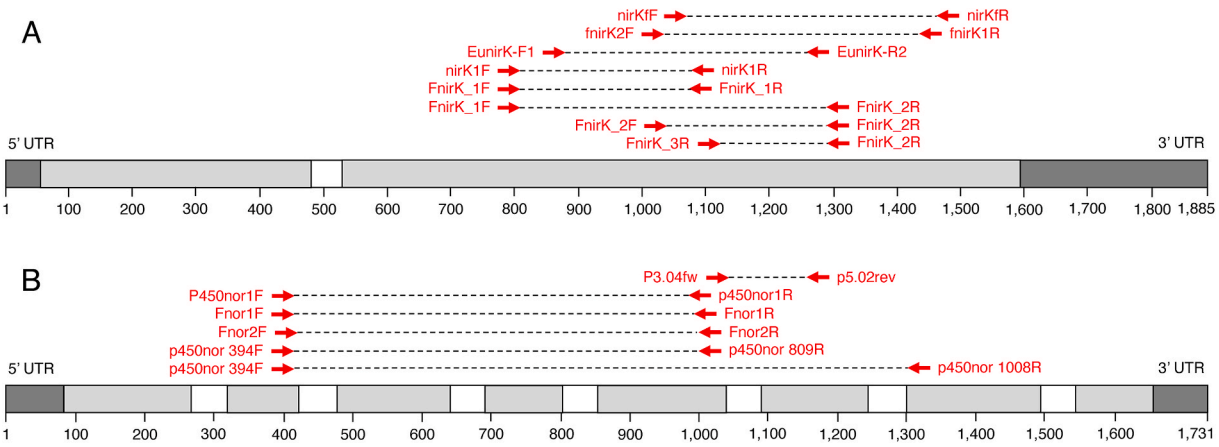

Fig. 3. Primer annealing sites for (A) fungal *nirK* and (B) *p450nor* PCR assays. The *nirK* and *p450nor* sequences from *Fusarium oxysporum* (GenBank accession numbers EF600898 and D14517, respectively) are used to map the primer sequences. Primers are shown as red arrows. Dashed lines indicate the expected PCR products with the primers. Grey and white regions indicate exons and introns, respectively. Dark grey regions indicate untranslated regions (UTRs). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and other environmental samples without fungal isolation. By sequencing the PCR amplicons, we can assess the diversity of *nirK* and *p450nor* sequences. While there may be PCR biases (i.e., not all fungal denitrifiers can be amplified), the PCR-amplicon sequencing approach is useful because it can provide diversity and relative abundance of the target sequences in high-throughput (Xu et al., 2019; Huang et al., 2021). For example, Xu et al. (2019) identified great *nirK* diversity in arable soils by sequencing the *nirK* PCR products with the Illumina MiSeq platform. Based on their analysis, *nirK* sequences closely related to those from the orders *Hypocreales*, *Sordariales*, *Eurotiales*, and *Mucorales* were frequently identified, although *nirK* sequences with no close relatives to the database sequences were also frequently detected.

Most primers shown in Table 1 have been used to directly amplify fungal *nirK* and *p450nor* from soil and other environmental samples. When assessing diversity by PCR-based approaches, it is important to evaluate the coverage of PCR primers (i.e., the proportion of target gene sequences that can be amplified by PCR with the primers). PCR with high-coverage primers can produce low false negatives. Based on the *in silico* analysis done by Ma et al. (2019), nirKfF/nirKfR (Wei et al., 2015) and EunirK-F1/EunirK-R2 (Maeda et al., 2015) primers had the highest coverage (>86%) among the fungal *nirK*-targeting primers. For *p450nor*-targeting primers, p450nor394F/p450nor1008R (Higgins et al., 2016), p450nor1F/p450nor1R (Novinscak et al., 2016), and Fnor1F/Fnor1R (Chen and Shi, 2017) had the highest coverage (>85%). Different groups of the target gene sequences can be also preferentially amplified by using different primers (e.g., Chen and Shi, 2017), and therefore, we need to be careful when comparing the results obtained with different primers.

For some assays, non-target amplicons (i.e., false positives) can be also produced when target genes (*nirK* or *p450nor*) are directly amplified from complex environmental samples (e.g., Higgins et al., 2016; Novinscak et al., 2016). In such cases, it may be necessary to excise bands of target amplicons from a gel after electrophoresis to purify the target amplicons (Higgins et al., 2016). Nested PCR approach can also increase the yield of target amplicons (Higgins et al., 2016), and therefore, it may help reduce the production of non-target amplicons. For the case of PCR targeting fungal *nirK*, previously developed primers often detect bacterial *nirK* most likely because of the high sequence similarities between fungal and bacterial *nirK*. The degree of cross-reaction with bacterial *nirK* varies by primers. Based on the comparative experiments done by Chen et al. (2016b), PCR assay with EunirK-F1/EunirK-R2 primers (Maeda et al., 2015) amplified more bacterial *nirK* than those with FnirK_F3/FnirK_R2 (Chen et al., 2016b) and nirKfF/nirKfR primers (Wei et al., 2015) from the same soil samples.

Based on previous studies, nirKfF/nirKfR primers (Wei et al., 2015) and p450nor394F/p450nor1008R (Higgins et al., 2016) or p450nor1F/p450nor1R (Novinscak et al., 2016) are most promising among the currently available primers to assess diversities of fungal *nirK* and *p450nor*, respectively, from soil and other environmental samples. Future research is necessary to further improve primer coverage and specificity.

4.4.2. Quantitative PCR

Quantitative PCR is useful to assess the quantity of denitrifying fungi in environments. The nirKfF/nirKfR primers (Wei et al., 2015) have been used for qPCR to measure the abundance of fungal *nirK* in the soils (Xu et al., 2019). They reported a significant positive correlation between fungal *nirK* abundance and fungal N_2O emissions (measured by SIRIN method) from arable soils, suggesting the usefulness of *nirK*-qPCR to assess fungal N_2O emissions. The qPCR assay for *p450nor* was recently developed (Rohe et al., 2020b). The levels of *p450nor* transcription as measured by reverse transcription qPCR with P3.04fw/P5.02rev primers correlated with the production of N_2O with high SP values (Rohe et al., 2020b). Although their *p450nor*-qPCR assay has not been tested with soil and other environmental samples, this assay would be useful to assess the production of N_2O of fungal origin.

Sensitivity, specificity, and accuracy are the common issues for qPCR (Bustin et al., 2009). Most of the previously reported primers for fungal *nirK* and *p450nor* (Table 1) are designed for amplicon sequencing purposes (except for Rohe et al., 2020b), and therefore, are not always ideal for qPCR purposes. In general, TaqMan probe-based assays have higher sensitivity (i.e., can detect target genes present at lower concentrations) than intercalating dye-based assays (Ishii, 2020). However, all assays designed so far use intercalating dye (e.g., SYBR green). Amplification of non-target fragments is problematic for qPCR, especially for intercalating dye-based assays. One approach to increase the specificity of intercalating dye-based assays is to read fluorescent signals at elevated temperatures (e.g., 80–85 °C), at which short non-target amplicons are denatured but target amplicons are not (e.g., Henry et al., 2004). Amplification efficiency is an important parameter to assess the accuracy of quantification. Amplification efficiency of 90–110% is generally considered adequate for qPCR (Bustin et al., 2009). Amplification efficiency is usually higher in small (<200 bp) than large amplicons. However, amplicon sizes of most of the previous assays are greater than 400 bp, which could lower the amplification efficiency of these assays. The presence of PCR inhibitors such as humus in the template DNA can also influence the amplification efficiency.

Digital PCR (dPCR) is another way of quantifying target genes,

although this technology has not been used to quantify fungal *nirK* and *p450nor* as far as we know. In dPCR, the number of PCR-positives are counted out of thousands of nano-liter reaction chambers or droplets (Quan et al., 2018). In general, dPCR is considered more sensitive than qPCR. In addition, amplification efficiency does not influence the quantitative results of dPCR much (Ishii, 2020). Therefore, dPCR would be more suitable to quantify fungal *nirK* and *p450nor* in environments than qPCR. This should be examined in the future.

4.4.3. Metagenomics

Shotgun metagenomics (or simply metagenomics) is an approach to sequence DNA fragments directly obtained from environmental samples without target gene amplification by PCR (e.g., Nadeau et al., 2019; Roco et al., 2019). Because metagenomics is free of PCR biases, it has the potential to detect a wide variety of fungal denitrification genes. A previous attempt was not successful in detecting *p450nor* in agricultural soil metagenomes, most likely due to insufficient sequence depths (a few hundred million reads) (Higgins et al., 2016). By contrast, fungal *nirK* and *p450nor* were detected in forest soils by metagenomics with smaller library sizes (30–40 million reads) (Jang et al., 2021), suggesting that the number of reads necessary to detect these genes depend on samples.

5. Future research needs

As discussed in this review article, recent research has greatly advanced our knowledge in fungal denitrification and NO_3^-/NO_2^- reduction processes. It has been reconfirmed that at least some fungi, especially those belonging to the genus *Fusarium*, can perform denitrification *sensu stricto* (Keuschnig et al., 2020). The molecular mechanism of fungal denitrification has been also clarified (Shoun et al., 2012; McQuarters et al., 2014). However, there are still many unknowns regarding fungal denitrification, especially about their ecology.

First of all, we need to re-define fungal denitrification. In some papers, fungi that can reduce NO_3^- or NO_2^- to N_2O are called "denitrifying fungi"; however, this is not accurate because fungal N_2O production is not necessarily related to their denitrification activity *sensu stricto*: N_2O can be produced by partially abiotic processes in some fungi as discussed in this article. It is more accurate to refer them as nitrate- or nitrite-reducing fungi, depending on the substrates used. In bacterial denitrification, strains that can reduce >80% of NO_3^- in the medium to N_2O + N_2 are considered denitrifiers (Mahne and Tiedje, 1995). Less stringent criteria have been also used for oligotrophic denitrifiers (Ishii et al., 2011; Tago et al., 2011). It would be useful to have similar criteria to identify fungal denitrifiers. Because N_2O production activities can vary by culture media and incubation conditions, we would need to establish a standard protocol for fungal denitrification confirmation, which remains as a future task. Since denitrification is a respiratory process, microbial growth should be measured during incubation and used as a parameter to define fungal denitrifiers. In addition, appropriate controls (e.g., no biomass control, dead cell control) should be included to detect abiotic N_2O and N_2 productions.

It is probably more useful to combine molecular and genomic tools with N_2O measurement to identify fungal denitrifiers. We propose to define fungi having both *nirK* and *p450nor* as putative fungal denitrifiers. Strains having both genes (*F. oxysporum* and *F. lichenicola*) were confirmed as bona fide denitrifiers, although those having only one of these genes were not (Keuschnig et al., 2020). The roles of these gene products (NirK and P450 Nor, respectively) in fungal denitrification have been clarified in *Fusarium* species (Shoun et al., 2012). It remains unclear, however, if these proteins are involved in denitrification in non-*Fusarium* species. Both fungal *nirK* and *p450nor* have been identified in various non-*Fusarium* genomes (Higgins et al., 2018) (Table S2). While these NirK and P450 Nor may be involved in fungal denitrification, they may play different roles, including the synthesis and detoxification of a signaling molecule NO (Cánovas et al., 2016) and secondary metabolism (Higgins et al., 2018). Therefore, the denitrification ability of these strains needs to be carefully examined in the future to verify if *nirK* and *p450nor* can be used as the marker genes for fungal denitrification.

Many fungi possess only one of the two genes (fungal *nirK* and *p450nor*) on their genomes. Having NirK or P450 Nor alone may be still beneficial for fungi because NirK and P450 Nor play an important role in the synthesis of ATP and the recycling of NAD(H), respectively (Shoun et al., 2012). Presence of *nirK* on fungal genome is of particular interest because NirK is shown to be involved in respiration. Many fungi have an NO detoxifying flavohemoglobins (Fhb) and GSNO reductase, and therefore, the product of nitrite reduction by NirK (= nitric oxide) can be removed without the need of P450 Nor. Indeed, Fhb is highly expressed together with NirK and P450 Nor under denitrifying conditions in *Fusarium lichenicola* (Kim et al., 2010), suggesting the potential involvement of Fhb in fungal denitrification. The end product of the NO detoxification by Fhb is NO_3^-; therefore, Fhb may recycle the denitrification substrate similar to the bacterial NO dioxygenase gene identified near the denitrification gene clusters in *Azospirillum* (Jang et al., 2019b). The denitrification ability of fungi that have *nirK* and Fhb genes but lack *p450nor* on their genome (see Table S2 for the list of candidate strains) should be examined in the future to clarify the role of Fhb in denitrification.

PCR is frequently used to detect fungal *nirK* and *p450nor*. Although multiple PCR primers have been developed and widely used, there is room for improvement to further increase specificity and coverage. Most studies used DNA as the template for PCR; however, RNA-based study is also important to analyze active transcription of fungal *nirK* and *p450nor* in environmental conditions. For this purpose, it is important to make sure if primers anneal to the exon region of the target gene (all primers shown in Table 1 do) (Fig. 3).

Amplification of fungal *nirK* and *p450nor* directly from environmental samples can allow us to assess the diversity and abundance of potentially denitrifying fungi without fungal isolation. Fungal *nirK* and *p450nor* sequencing data, however, should be carefully interpreted by considering the limitation of the approach (e.g., primer biases). In addition, the fungal *nirK* and *p450nor* sequences detected are not necessarily involved in denitrification as we discussed in this article. To make the amplicon sequencing data more meaningful, we would need better-curated sequence databases, which contain the fungal *nirK* and *p450nor* sequences from various bona fide fungal denitrifiers. For this purpose, we need to have the genomes of diverse fungal strains that can perform denitrification *sensu stricto*.

Environmental relevance of fungal denitrification and NO_3^-/NO_2^- reduction processes can be assessed by various methods, including the SIRIN method, stable isotope analyses, and culture-dependent and -independent molecular and genomic approaches. As we discussed, these methods have their unique strengths and weaknesses in detecting fungal denitrification signatures. Therefore, it is important to use multiple techniques to complement their strengths. Further research is also needed to improve the specificity and sensitivity of these methods. In particular, discrimination of N_2O originated from fungal denitrification, co-denitrification, and chemodenitrification is much needed.

6. Concluding remarks

Importance of fungal denitrification and NO_3^-/NO_2^- reduction processes is now widely recognized for the emission of N_2O. Fungal denitrification and NO_3^-/NO_2^- reduction processes can also have a strong potential for N bioremediation. To mitigate N_2O emission and control N bioremediation processes, it is necessary to understand the ecology of (denitrifying) fungi. There are still many unknowns; however, this means we have great opportunities to explore in the future. We now have various tools to investigate fungal denitrification and NO_3^-/NO_2^- reduction processes, and these methods keep improving. We expect to see more exciting findings regarding fungal denitrification in the near future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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