

## Application of the isotope pairing technique in sediments: Use, challenges, and new directions

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### Abstract

Determining accurate rates of benthic nitrogen (N) removal and retention pathways from diverse environments is critical to our understanding of process distribution and constructing reliable N budgets and models. The whole-core <sup>15</sup>N isotope pairing technique (IPT) is one of the most widely used methods to determine rates of benthic nitrate-reducing processes and has provided valuable information on processes and factors controlling N removal and retention in aquatic systems. While the whole core IPT has been employed in a range of environments, a number of methodological and environmental factors may lead to the generation of inaccurate data and are important to acknowledge for those applying the method. In this review, we summarize the current state of the whole core IPT and highlight some of the important steps and considerations when employing the technique. We discuss environmental parameters which can pose issues to the application of the IPT and may lead to experimental artifacts, several of which are of particular importance in environments heavily impacted by eutrophication. Finally, we highlight the advances in the use of the whole-core IPT in combination with other methods, discuss new potential areas of consideration and encourage careful and considered use of the whole-core IPT. With the recognition of potential issues and proper use, the whole-core IPT will undoubtedly continue to develop, improve our understanding of benthic N cycling and allow more reliable budgets and predictions to be made.

The global nitrogen (N) cycle has been heavily perturbed by human activities. Globally, 160 Tg yr<sup>-1</sup> are estimated to be introduced to the environment through agriculture and combustion of fossil fuels (Gruber and Galloway 2008) and is now within the same order of magnitude as natural terrestrial (110 Tg yr<sup>-1</sup>) and oceanic (140 Tg yr<sup>-1</sup>) inputs from N fixation (Gruber and Galloway 2008; Jickells et al. 2017). Fluvial transport via rivers and groundwater is the major route of N addition to the marine environment (Jickells et al. 2017) with

increased inputs leading to eutrophication and subsequent detrimental ecosystem effects such as hypoxia (< 2 mg L<sup>-1</sup> O<sub>2</sub>) and anoxia (0 mg L<sup>-1</sup> O<sub>2</sub>; Diaz and Rosenberg 2008).

Sediments play a major role in the transformation of N in aquatic systems through three principle anaerobic nitrate (NO<sub>3</sub><sup>-</sup>) reducing processes. Excess NO<sub>3</sub><sup>-</sup> may be removed by the conversion of NO<sub>3</sub><sup>-</sup> to dinitrogen (N<sub>2</sub>) through the processes denitrification and/or anaerobic ammonium (NH<sub>4</sub><sup>+</sup>) oxidation (anammox). NO<sub>3</sub><sup>-</sup> may also be reduced through dissimilatory NO<sub>3</sub><sup>-</sup> reduction to NH<sub>4</sub><sup>+</sup> (DNRA) by which N is retained in the system in a bioavailable form. Extensive measurements from a diverse range of environments worldwide have resulted in the emergence of patterns in the distributions of these processes. Denitrification is often found to play a major role in N removal in coastal zones with well-oxygenated bottom water where organic matter availability and NO<sub>3</sub><sup>-</sup> concentrations are high (King and Nedwell 1985, 1987). While

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anammox rates in sediments are generally low, the highest contributions of anammox to  $\text{NO}_3^-$  reduction are typically found at deep sites with oxygenated overlying water and with lower organic carbon content (Thamdrup 2012; Na et al. 2017). DNRA is often observed to dominate over  $\text{N}_2$  removal processes under reducing conditions (Buresh and Patrick 1981; Christensen et al. 2000; An and Gardner 2002; Burgin and Hamilton 2007; Algar and Vallino 2014) such as those experienced when bottom waters become hypoxic or anoxic (Tiedje et al. 1982; Nizzoli et al. 2010; Jäntti and Hietanen 2012). Additionally, while denitrification, anammox, and DNRA are considered anaerobic, varying tolerances to low oxygen concentrations have been reported between these processes; e.g. (Robertson and Kuenen 1984; Kalvelage et al. 2011; Dalsgaard et al. 2014b; Marchant et al. 2017). Thus rapid oxygen fluctuations and increasingly regular occurrences of low-oxygen bottom waters worldwide may have significant implications for benthic N cycling in natural environments. It is therefore of critical importance to accurately quantify processes which retain or remove N from the system under a wide variety of environmental conditions.

The  $^{15}\text{N}$  stable isotope pairing technique (IPT) has been used to determine rates of benthic N loss and retention processes for over 25 yr (Nielsen 1992) and has been one of the most widely used methods of assessing in situ contributions of  $\text{NO}_3^-$ -reducing processes. The addition of labeled  $^{15}\text{N}$ -nitrate ( $^{15}\text{NO}_3^-$ ) to overlying water of sediment cores permits the production of  $^{15}\text{N}$ - $\text{N}_2$  and  $^{15}\text{NH}_4^+$  from benthic  $\text{NO}_3^-$ -reducing processes to be determined despite high background  $\text{N}_2$  levels. The calculation of rates using the IPT relies on the central assumption that denitrification is the only  $\text{N}_2$ -producing processes, and specific relationships between  $^{15}\text{N}$  and  $^{14}\text{N}$  distribution both in  $\text{NO}_3^-$  in the  $\text{NO}_3^-$ -reducing zone and in the resulting  $^{15}\text{N}$ - $\text{N}_2$  species (discussed in “Evolution of the whole-core IPT” section of this manuscript). Since its original development, the whole-core IPT, along with its assumptions and associated calculations have been revised on several occasions to account for the presence of anammox as an additional  $\text{N}_2$ -producing process (Risgaard-Petersen et al. 2003; Trimmer et al. 2006) and the co-occurrence of denitrification, anammox, and DNRA at some locations (Song et al. 2016; Salk et al. 2017). The application of the IPT across a wide range of environments has added substantially to our understanding of how the N cycle is controlled in aquatic environments.

The whole-core IPT is ideally suited to cohesive sediments with well-oxygenated water columns. In these sediments the diffusive boundary layer (DBL) is important in regulating solute exchange (Jørgensen and Boudreau 2001) and is easily artificially generated by mixing of overlying water. While these conditions are representative of a significant proportion of benthic environments, natural non-static conditions may deviate from these ideals and can cause issues with the application of the whole-core IPT. To provide brief examples, variability in sediment oxygen penetration over time (e.g., due to

diurnal changes in photosynthesis, advective flow) can complicate the calculation of  $^{15}\text{NO}_3^-$  equilibration time. Oxygen may be drawn down substantially in sealed cores during incubation at locations with low-oxygen bottom water concentrations or during long incubations. Microorganisms storing  $\text{NO}_3^-$  (e.g., large sulfur bacteria, foraminifera, diatoms) may release and respire stored  $^{14}\text{NO}_3^-$  during incubation (Preisler et al. 2007; Sokoll et al. 2012; Song et al. 2013), complicating the relationships between  $^{15}\text{N}$  and  $^{14}\text{N}$  important to the IPT assumptions. Ventilation of sediments by bioturbating organisms, advective flow or gas bubbles can impact nitrification and thus the calculation of nitrification-derived  $\text{NO}_3^-$ -reduction is complicated by non-homogenous  $^{14}\text{NO}_3^-$  and  $^{15}\text{NO}_3^-$  distributions. Failure to simulate advective transport in permeable sediments may lead to significant underestimation of true rates of  $\text{NO}_3^-$  reduction during diffusive core incubations (Gao et al. 2012).

Despite these complications, it is nevertheless important to quantify  $\text{NO}_3^-$ -reducing processes accurately in environments where non-ideal conditions occur to provide reliable N budgets and to improve our extrapolative and predictive abilities related to nutrient transport and retention. Furthermore, we must be able to understand how methodological limitations affect the quantitative outcome of process rates and how this could lead to incorrect conclusions regarding N budgets. The focus of this review is not on the intricacies of the IPT calculations, which have been presented and discussed in detail previously (e.g., Nielsen 1992; Risgaard-Petersen et al. 2003; Trimmer et al. 2006; Song et al. 2016) and here we concentrate on the importance of good methodology and replicating in situ conditions during incubations. We discuss the proper use of the whole-core IPT both generally and under conditions which pose the most severe complications to its use. We highlight the importance of the sensible use of the IPT and discuss potential issues which must be taken into account to enable an accurate determination of N cycling process rates in sediments. Furthermore, we discuss progress in ways to apply the whole-core IPT.

### Evolution of the whole-core IPT

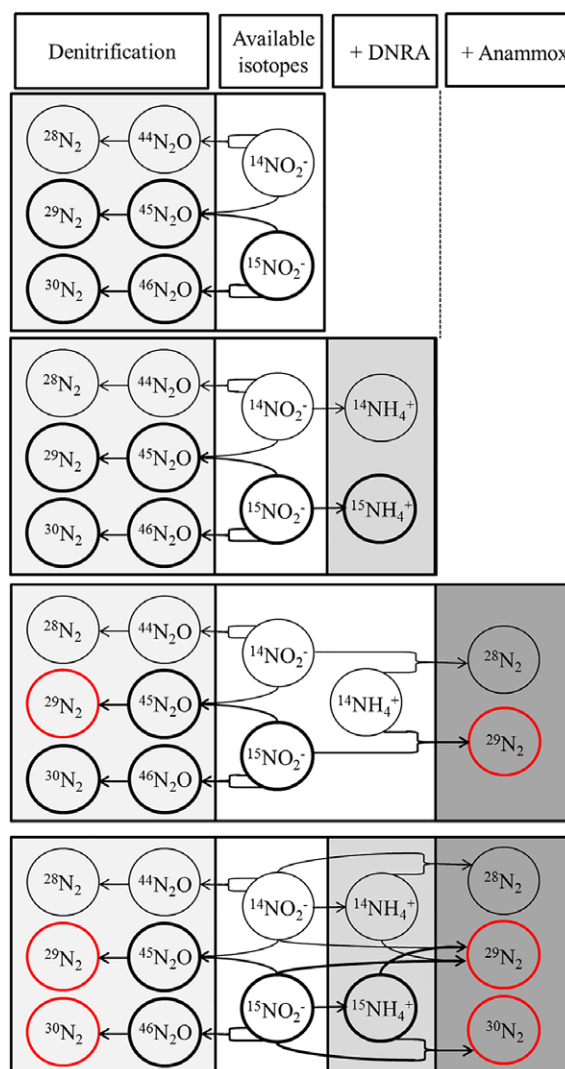
The IPT has gone through several iterations since its initial development due to the discovery of new processes and their potential co-occurrence (Risgaard-Petersen et al. 2003; Trimmer et al. 2006; Song et al. 2013, 2016; Salk et al. 2017). The basis of the IPT is that the addition of labeled  $^{15}\text{NO}_3^-$  to the overlying water and its subsequent diffusion into the  $\text{NO}_3^-$ -reducing zone can be used to trace the production of  $^{15}\text{N}$ - $\text{N}_2$  gas ( $^{30}\text{N}_2$ ,  $^{29}\text{N}_2$ ) despite a high atmospheric  $^{14}\text{N}$ - $\text{N}_2$  ( $^{28}\text{N}_2$ ) background as well as other  $^{15}\text{N}$ -labeled end products ( $^{45}\text{N}_2\text{O}$ ,  $^{46}\text{N}_2\text{O}$ ,  $^{15}\text{NH}_4^+$ ). This is achieved during a comparatively short incubation time relative to other methods directly measuring  $\text{N}_2$  accumulation (Groffman et al. 2006). It additionally permits the determination of the  $\text{NO}_3^-$  source—either the overlying water ( $D_w$ ) or sediment nitrification ( $D_N$ ). Here, we briefly highlight the events leading to the current state of the IPT.

The original IPT (Nielsen 1992) is built on three key assumptions which are discussed below:

1. The addition of  $^{15}\text{NO}_3^-$  does not affect the production of  $^{14}\text{N-N}_2$ , with the validity of this assumption relying upon denitrification as the sole process producing  $\text{N}_2$  from  $\text{NO}_3^-$  and the requirement that the process under investigation is  $\text{NO}_3^-$ -limited. This is easily tested as demonstrated by Eyre et al. (2002)
2. The ratio of  $^{15}\text{N-NO}_3^-$  and  $^{14}\text{N-NO}_3^-$  throughout the  $\text{NO}_3^-$ -reducing zone is constant after  $^{15}\text{NO}_3^-$  amendment.
3. If the above assumptions are met, the produced  $\text{N}_2$  species from  $^{15}\text{N-}$  and  $^{14}\text{NO}_3^-$  are binomially distributed.

If these assumptions are met, the resulting production of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  can be used to partition overall  $\text{N}_2$  production into  $D_W$  and  $D_N$  to determine the relative importance of the two  $\text{NO}_3^-$  sources. However, it seems that the application of the IPT was not without question, even during the early phases of its use. In response to Nielsen (1992), Middelburg et al. (1996a) asserted that the homogenous  $^{15}\text{N-NO}_3^-$  and  $^{14}\text{N-NO}_3^-$  labeling (assumption 2) is only achieved when nitrification and denitrification occur in spatially distinct zones (Middelburg et al. 1996a) and the potential for anoxic microsites in aerobic layers would invalidate this assumption. Middelburg et al. further suggested that dividing  $\text{NO}_3^-$  source into different components ( $D_N$ ,  $D_W$ ) is not necessary to understand benthic N cycling. The resulting dispute relating to IPT calculations and the value (or lack thereof) of determining  $D_W$  and  $D_N$  was discussed in two short communications (see Middelburg et al. 1996b; Nielsen et al. 1996). Today, determining the partitioning between water column-derived  $\text{NO}_3^-$  and  $\text{NO}_3^-$  produced from nitrification remains an important aspect in investigating how  $\text{NO}_3^-$  reducing processes and their  $\text{NO}_3^-$  sources are controlled in different environments. While discussions such as this have provided important insights from different research fields, they additionally highlight the need for the thorough understanding of the IPT—from the correct implementation to potential caveats of its use—which we discuss in the present review.

The first assumption was shown to be violated when the anammox process—previously only described in fluidized bed reactors (Mulder et al. 1995; Vann de Graaf et al. 1995)—was found to occur in natural sediments (Thamdrup and Dalsgaard 2002) and anoxic water columns (Dalsgaard et al. 2003; Kuypers et al. 2003). The coexistence of both anammox and denitrification violates the assumption that the production of  $^{14}\text{N-N}_2$  is independent from the concentration of  $^{15}\text{NO}_3^-$  added (Risgaard-Petersen et al. 2003) due to anammox combining added  $^{15}\text{NO}_3^-$  with unlabeled  $^{14}\text{NH}_4^+$  in sediments to produce extra  $^{29}\text{N}_2$ . As such a binomial distribution of  $^{14}\text{N}$  and  $^{15}\text{N}$  in the  $\text{N}_2$  pools cannot be assumed. Using the original IPT calculations, denitrification would be overestimated due to excess  $^{29}\text{N}_2$  production from anammox (Fig. 1; Risgaard-Petersen et al. 2003;



**Fig. 1.** Overview of potential complications associated with co-occurring  $\text{NO}_3^-$  and  $\text{NO}_2^-$  ( $\text{NO}_x^-$ )-reducing processes, (here shown as  $\text{NO}_2^-$  reduction). Bold black circles indicate isotopically labeled ( $^{15}\text{N}$ ) substrates and products. Red circles indicate an overlap of the end products of  $\text{NO}_x^-$  reduction leading to over- and under-estimations of process rates (see text). Based on Holtappels et al. (2011), reproduced with permission.

Trimmer et al. 2006). These problems led to a revision of the initial assumptions (revised-IPT: r-IPT; Risgaard-Petersen et al. 2003, 2004), permitting the potential contribution of anammox to  $\text{N}_2$  production to be calculated. This is achieved through time series experiments accompanied by sediment slurry incubations or through start-end whole-core incubations carried out at different  $^{15}\text{NO}_3^-$  concentrations (Risgaard-Petersen et al. 2003). These two incubation types are discussed in greater detail in “General use of whole-core IPT” section. For the purpose of this review, we herein refer to IPT (Nielsen 1992), r-IPT (Risgaard-Petersen et al. 2003), and other revised/updated calculations (e.g., Song et al. 2016; Salk et al. 2017) collectively as “IPT.”

The determination of DNRA through experimental  $^{15}\text{NO}_3^-$  addition has been carried out for many years (Koike and Hattori 1978; Sørensen 1978; Jørgensen 1989; Binnerup et al. 1992). However, the implications for the co-occurrence of denitrification and anammox with DNRA have only been discussed (Hietanen 2007; Jäntti et al. 2012) and measured (Dong et al. 2009; Trimmer and Nicholls 2009; De Brabandere et al. 2015; Song et al. 2016; Bonaglia et al. 2017; Salk et al. 2017) more recently. In the case of all three processes co-occurring, the application of the IPT becomes further complicated, with movement of  $^{15}\text{N}$  from the  $\text{NO}_3^-$  to  $\text{NH}_4^+$  pools through DNRA leading to additional  $^{30}\text{N}_2$  production from anammox (Fig. 1). In this situation, anammox and DNRA are underestimated and denitrification may be overestimated (Song et al. 2013, 2016; Salk et al. 2017) using earlier versions of the whole-core IPT (Nielsen 1992; Risgaard-Petersen et al. 2003). Calculations have therefore been further revised for slurries (Song et al. 2013) and whole-core experiments (Song et al. 2016; Salk et al. 2017). In addition to  $\text{N}_2$ , the isotopic composition of  $\text{NH}_4^+$  (Song et al. 2013, 2016; Salk et al. 2017) and of  $\text{N}_2\text{O}$  (Salk et al. 2017) are measured throughout incubations to enable differentiation between coupled anammox-DNRA and canonical anammox.

In many benthic environments, particularly those with low bottom water  $\text{NO}_3^-$ , denitrification rates have been shown to be coupled to nitrification, which in turn is controlled by oxygen availability and transport limitation in the sediment due to the varying thickness of the oxic zone (Jensen et al. 1996; Gihring et al. 2010; Marchant et al. 2016). Direct methods for the determination of nitrification rates are compromised by the fact that  $^{15}\text{NH}_4^+$  additions increase the  $\text{NH}_4^+$  pool at low pore-water  $\text{NH}_4^+$  concentrations several-fold, thereby artificially enhancing rates of  $\text{NH}_4^+$ -limited nitrification (Jäntti et al. 2012). Alternatively, isotope dilution approaches with added  $^{15}\text{NO}_2^-$  have been used to quantify nitrification rates (e.g., Jäntti et al. 2012; Marchant et al. 2016). Other ways of applying the IPT have also been implemented (e.g., see Steingruber et al. 2001), such as stand-alone slurries or bag incubations (e.g., Engström et al. 2005; Brandsma et al. 2011) which provide potential rates of  $\text{NO}_3^-$ -reducing processes but cannot be reliably used to reflect in situ rates. Nevertheless, such incubations can provide important information on environmental parameters which may influence the individual N cycling processes through manipulation experiments. IPT experiments may also be carried out using continuous flow-through chambers where oxygen (and  $\text{NO}_3^-$ ) concentrations can be controlled by continual replacement and removal of overlying water (Risgaard-Petersen et al. 1994, 1998b; Risgaard-Petersen and Jensen 1997). These set ups have the benefit of sampling the same sediment area over more prolonged time periods (several days) at steady state (Steingruber et al. 2001) and do not have the problems associated with oxygen draw-down inside cores. However, the equipment required is more complex, expensive, and less easy to transport (e.g., for field work). While the use and effectiveness of alternative  $^{15}\text{N}$  substrates to

whole-core incubations and the different experimental set-ups can provide important information on benthic N cycling, for the purposes of this review, we focus on the more well-established protocol of  $^{15}\text{NO}_3^-$  addition in static whole-core studies to determine in situ rates of  $\text{NO}_3^-$ -reducing processes.

### General use of whole-core IPT

To apply the IPT correctly, several considerations must be taken into account to ensure the above assumptions can be met and to ensure reliable process rates are calculated. We briefly describe the use of the two main whole-core IPT experiments—time-series and concentration-series incubations—and highlight the importance of good practice in the use of these techniques. In most respects, both the time-series and concentration-series experiments are similar to that of Nielsen (1992). Addition of  $^{15}\text{NO}_3^-$  to the overlying water of intact cores is followed by an initial equilibration period which varies depending on sediment oxygen penetration depth and  $\text{NO}_3^-$  diffusivity (see Dalsgaard et al. 2000 for calculations). This equilibration period is critical to allow added  $^{15}\text{NO}_3^-$  to diffuse into the  $\text{NO}_3^-$ -reducing zone so that  $\text{NO}_3^-$  reduction can be determined at *quasi-steady* state. During this equilibration period, overlying water is maintained at the desired (in situ) oxygen concentration by bubbling with air or with gas mixtures before cores are sealed to begin the incubation. After the cores are sealed, oxygen concentrations inside the sealed cores are not permitted to drop below 20% of the starting concentration to avoid major alteration of biogeochemical processes (Dalsgaard et al. 2000). Over the course of the incubations, cores are sacrificed by gently slurrying sediment with the overlying water to homogenize end products of  $\text{NO}_3^-$  reduction. The sediment is allowed to settle for a few minutes before water samples for dissolved gas and nutrients are withdrawn from the slurried core.

IPT incubations using a concentration-series rely on the addition of multiple  $^{15}\text{NO}_3^-$  concentrations to at least two (but typically more) sets of intact sediment cores. Following an equilibration period, incubations of half the cores in each  $^{15}\text{NO}_3^-$  treatment are terminated and the remaining cores are terminated after several hours of incubation. Linearity of  $\text{N}_2$  production is assumed between the two time points following the pre-incubation period. As anammox does not produce  $^{30}\text{N}_2$  pairs, the contribution of anammox can be calculated from the difference between expected and measured distributions of labeled  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  species in different  $^{14}\text{NO}_3^- : ^{15}\text{NO}_3^-$  ratio incubations. In concentration-series incubations, the contribution of anammox to measured  $\text{N}_2$  production is indicated by a positive correlation between production of  $^{14}\text{N-N}_2$  and  $^{15}\text{NO}_3^-$  concentrations and calculations accounting for anammox should be used for rate calculations (e.g., Risgaard-Petersen et al. 2003). If  $^{14}\text{N-N}_2$  production does not show a positive correlation with the different added  $^{15}\text{NO}_3^-$  concentrations, denitrification is assumed to be the only  $\text{N}_2$  producing process and Nielsen's 1992 calculations can be used. Later studies developed the IPT further by using the  $^{15}\text{N}$  distribution in  $\text{N}_2\text{O}$  as well as

$N_2$  to determine anammox and denitrification rates (Trimmer et al. 2006; Salk et al. 2017). Thereby the distribution of  $^{14}N$  and  $^{15}N$  in  $N_2O$  (as  $^{44}N_2O$ ,  $^{45}N_2O$ , and  $^{46}N_2O$ ) and in  $N_2$  (as  $^{28}N_2$ ,  $^{29}N_2$ , and  $^{30}N_2$ ) is determined after chromatographic separation and reduction of  $N_2O$  to  $N_2$  over copper. As  $N_2O$  is produced from denitrification but not from anammox (Trimmer et al. 2006), the  $^{14}N$  and  $^{15}N$  in  $N_2O$  should thus be binomially distributed, reflecting the ratios of  $^{14}N$  and  $^{15}N$  in  $NO_3^-$  being reduced through the denitrification pathway.

In time-series incubations, one set of intact sediment cores is incubated at a single  $^{15}NO_3^-$  concentration and cores are sacrificed over several (at least three) time points (Dalsgaard et al. 2000). A method of choosing an appropriate  $^{15}NO_3^-$  concentration is provided in Dalsgaard et al. (2000), with a suggested final enrichment of at least 30 atom % in the  $NO_3^-$  pool and a final concentration of at least 20% of the initial oxygen concentration. Compared to the concentration-series incubation, time-series incubation has the benefit of explicitly demonstrating a linear production of  $N_2$  over several time points. The contribution of anammox to overall  $N_2$  production (*ra*) is determined by carrying out parallel anoxic sediment slurry incubations. In these experiments, labeled  $^{15}N$  substrates are added and process rates are typically slowed down by dilution with site water (although in some cases rates have been observed to increase; e.g., see Crowe et al. 2012). Slurry incubations only produce potential process rates, and conditions in slurries are markedly different from intact sediment due to the absence of geochemical zonation. Different isotopic treatments are added to three sets of slurries to enable the contribution of each process to be identified; A:  $^{15}NH_4^+ + ^{14}NO_3^-$ ; B:  $^{15}NH_4^+$ ; and C:  $^{15}NO_3^-$  (Thamdrup and Dalsgaard 2002). In this way, the presence of anammox is confirmed only if treatment A showed that N from  $NH_4^+$  and  $NO_3^-$  was being combined to  $^{29}N_2$  and if treatment B showed no  $^{29}N_2$  production due to anammox being  $NO_x^-$  limited. The contribution of anammox to production of  $^{29}N_2$  in treatment C can then be calculated and *ra* deduced. One important and potentially overlooked factor is the need to ensure removal of residual oxygen and  $NO_3^-$  from slurries before addition of labeled substrates. This ensures that no additional  $^{14}NO_3^-$  is produced through nitrification and that this  $^{14}NO_3^-$ —or background  $^{14}NO_3^-$  in sediment—does not combine with added  $^{15}NO_3^-$  and lead to erroneous  $^{29}N_2$  production, indicative of anammox activity (Fig. 1). To achieve this, slurries should be prepared in a way which minimizes oxygenation of the sediment (e.g., using an  $N_2$ -filled glove bag), and subjected to a pre-incubation (8–12 h in darkness) where the residual oxygen and  $NO_3^-$  are allowed to be consumed by sediment organisms prior to substrate addition. Failure to pre-incubate, or to not incubate for long enough periods, can lead to potentially incorrect *ra* values.

In the most recent iterations of the whole-core IPT, the contribution of three co-occurring  $NO_3^-$ -reducing processes (denitrification, anammox, DNRA) can be determined. These

modifications require additional samples to determine the fraction and progressive accumulation of  $^{15}NH_4^+$  in the total  $NH_4^+$  pool (through  $^{15}NO_3^-$  reduction via DNRA) within the  $NO_3^-$ -reducing zone of whole-core incubations and in parallel slurry incubations (Song et al. 2016; Salk et al. 2017). Increasing observations of co-occurring  $NO_3^-$ -reducing processes demonstrate further that we lack some understanding of the controlling factors of when these processes occur together. This implies that in sediments where the contribution of each process to  $NO_3^-$  reduction is unknown, these extra samples and measurements are required to conclusively rule out interferences with the IPT.

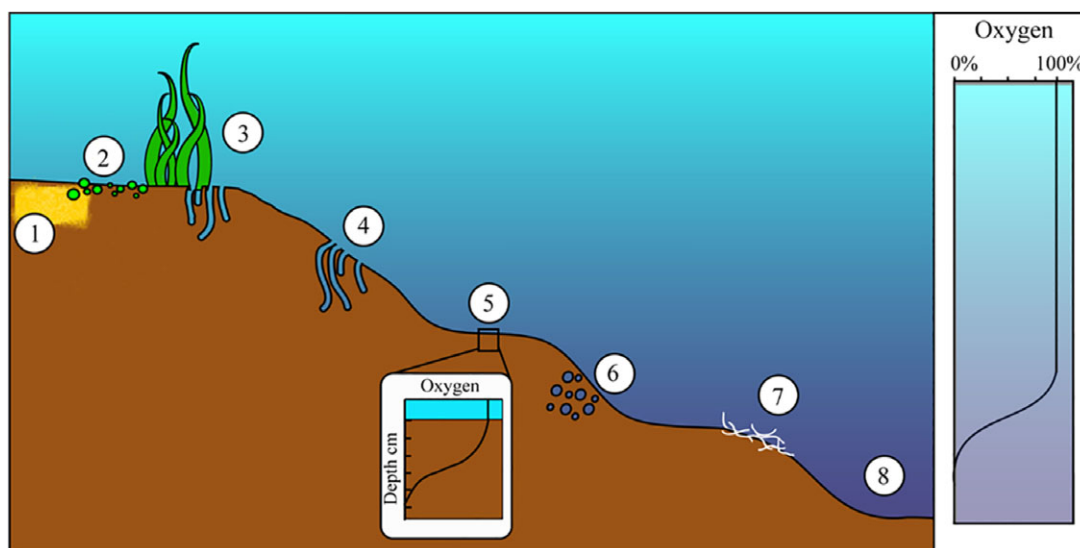
### Environmental challenges when applying the IPT

While the complications in using the IPT with co-occurring processes can be addressed by additional measurements and amendments to calculations, their application in natural environments can bring additional challenges. We provide an overview of various environmental factors with demonstrated effects on  $NO_3^-$ -reducing processes and discuss how these issues can complicate the application of the IPT (Fig. 2; Table 1). We furthermore suggest solutions of how these issues can be overcome to achieve the most reliable values of  $NO_3^-$ -reducing processes.

### Bioturbation

Burrowing and irrigating organisms enhance benthic fluxes of inorganic nutrients (Aller and Aller 1992; Griffiths et al. 2017) and effectively extend the depth to which oxygen and water column  $NO_3^-$  penetrate into sediment. Bioturbating organisms may stimulate nitrification and subsequently  $NO_3^-$ -reducing processes by expanding the volume of sediment in which these processes occur (Aller and Aller 1992; Pelegri et al. 1994; Pelegri and Blackburn 1995; Laverock et al. 2011; Bonaglia et al. 2013, 2014b; McTigue et al. 2016). In addition, they rework more labile organic matter from the sediment surface to deeper layers, stimulating remineralization by heterotrophic  $NO_3^-$  respiration (Kristensen 2000; Laverock et al. 2011). The impact of organisms on benthic denitrification has been clearly shown using the IPT in numerous studies (e.g., Risgaard-Petersen and Jensen 1997; Karlson et al. 2005; Bertics et al. 2010; Bonaglia et al. 2013, 2014b). More recently studies have also assessed the impact of bioturbation on DNRA (Bonaglia et al. 2013, 2014b; Nogaro and Burgin 2014; Murphy et al. 2016). However, to date anammox has not been shown to be a significant process in sediments in which the impacts of bioturbation have been investigated (Bonaglia et al. 2013, 2014b) and very few studies have been carried out on how bioturbation may impact benthic N-fixation (Bertics et al. 2010). As such the presence of benthic animals and their burrowing activities may produce as yet unknown antagonistic or synergistic effects on benthic N cycling processes (Magri et al. 2018).

The presence of burrowing fauna causes issues in the application of the whole-core IPT. The creation of multiple subsurface



**Fig. 2.** Schematic overview of environments which pose challenges to the use of the whole-core IPT. 1: Sandy sediments in intertidal zones dominated by advective pore-water flow. 2: Photic sediments (sands or muds) with microphytobenthos (MPB). 3: Photic sediments with rooted macrophytes and/or bioturbating organisms. 4: Deep, aphotic faunated sediments. 5: Low reactivity sediments with high oxygen penetration. 6: Gas-charged sediments which may be prone to ebullition during sampling. 7: Sediments with nitrate-storing microorganisms (e.g., *Beggiatoa* in this example). 8: Sediments with hypoxic/anoxic overlying water bodies and steep sediment oxygen gradients.

oxic/anoxic niches by organisms affects  $^{14}\text{NO}_3^-$  to  $^{15}\text{NO}_3^-$  ratios within sediments, making the calculation of the IPT problematic (Risgaard-Petersen and Jensen 1997; Bertics et al. 2010; Bonaglia et al. 2013). In this case, isolated pockets of  $^{14}\text{NO}_3^-$  produced by sub-surface nitrification will result in a heterogeneous distribution of  $^{15}\text{NO}_3^-$  and  $^{14}\text{NO}_3^-$ , violating the assumption 2. An additional issue to applying the IPT to faunated sediment is that the degree of oxygen penetration is unknown, making it impossible to reliably calculate an appropriate time for pre-incubation of  $^{15}\text{NO}_3^-$ . This issue could be checked by using time-series whole-core IPT experiments where sets of cores are terminated at several time points to follow  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production. Using the time-series approach does not change the fact that the time taken for  $^{15}\text{NO}_3^-$  to reach the zone of denitrification is unknown. However, using this approach it is possible to forgo the pre-incubation period and thus determine the point at which  $\text{N}_2$ -production is linear over time. Process rates would then be calculated only from where  $\text{N}_2$  production was demonstrated to be linear over time, solving the issue of not being able to calculate an exact pre-incubation time by leaving out the lag time due to long or variable diffusion paths. However, variability in faunal activity between cores results in different transport rates of  $^{15}\text{NO}_3^-$  within the sediment of different sediment cores, resulting in variable  $^{14}\text{NO}_3^- : ^{15}\text{NO}_3^-$  ratios in cores sacrificed at the same time point (this is generally not reported but commonly observed). In addition to faunal activity, heterogeneous mixing of  $^{15}\text{NO}_3^-$  may cause process rates calculated by IPT to be underestimated compared to  $\text{N}_2 : \text{Ar}$  methods (discussed later) due to differences in diffusion of  $^{15}\text{NO}_3^-$  across mucus linings of burrows relative to  $^{14}\text{NO}_3^-$  produced by nitrifiers already in burrow walls (Ferguson and Eyre 2007). Ferguson and Eyre

(2007) were also able to demonstrate that this underestimation was linearly related to infaunal biomass, which were removed at the study site by a trawling event which resuspended surface sediment.

Explicitly solving the issues related to bioturbation is not trivial. Further artifacts may occur from the typical experiments used to assess impacts of fauna on benthic N cycling. Early whole-core IPT studies on bioturbated sediments were carried out in the dark, on sediments which had been reconstructed following sieving, homogenization and addition of varying densities of single species (e.g., Pelegri et al. 1994; Tuominen et al. 1999). At locations where macrofaunal communities are dominated by a single species, results similar to those obtained in reconstructed cores may be expected. However, microbial communities (e.g., slow-growing nitrifiers) may need longer time periods (weeks as opposed to days) to become structured around burrows, within/around bio-deposits or in general within the multidimensional sediment matrix of a bioturbated sediment (Stocum and Plante 2006). As a result, the sediment microbial community in reconstructed cores may not behave as those in intact cores with established burrow microbiota due to the short pre-incubation period before the addition of  $^{15}\text{NO}_3^-$ . This may stimulate  $D_w$  as compared to  $D_N$  (i.e. if nitrifiers have not properly established), and underestimate denitrification efficiency due to large  $\text{NH}_4^+$  efflux, while the opposite may be true in the natural environment (Pelegri et al. 1994; Stief 2013). IPT-based investigations into the effect of macrofauna on randomly sampled intact sediment cores in which macrofaunal communities were assessed a posteriori are scarce. This is primarily due to the large sampling effort necessary to establish significant effects and to the sometimes contrasting effects of different

**Table 1.** Summary of the main methodological issues posed to the whole-core IPT by the discussed environments and potential solutions to overcome them in future studies.

Challenge	Main methodological issues	Potential solutions (where possible)
Bioturbation	<ul style="list-style-type: none"> <li>Heterogeneous distribution of <math>^{15}\text{NO}_3^-</math> and <math>^{14}\text{NO}_3^-</math> due to sub-surface nitrification (oxygen introduced although burrow irrigation)</li> <li>Unknown degree of oxygen penetration, unable to reliably calculate pre-incubation time</li> <li>Variability in faunal activity between cores causes differences in <math>^{15}\text{NO}_3^-</math> transport within sediments</li> <li>Reconstructed cores may alter sediment biogeochemistry and microbial communities</li> </ul>	<ul style="list-style-type: none"> <li>Further studies on mixed community assemblages, not just single species</li> <li>Use of in situ chambers</li> <li>Combination of IPT with <math>\text{N}_2:\text{Ar}</math> method (discussed in Eyre et al. 2002)</li> <li>Investigation of impacts of natural species assemblages and biodiversity assessment a posteriori (large sampling effort)</li> <li>Increase pre-incubation time (2–3 weeks) in reconstructed cores to allow microbial community to establish</li> </ul>
Phytoplankton	<ul style="list-style-type: none"> <li>Varying thickness of oxic layer in light/dark incubations</li> <li>Heterogeneous distribution of <math>^{15}\text{NO}_3^-</math> and <math>^{14}\text{NO}_3^-</math> due to sub-surface nitrification (oxygen release from root systems)</li> <li>Damage to plant root systems during core retrieval</li> <li>Uptake of <math>^{15}\text{NO}_3^-</math> by microphytobenthos (see below)</li> </ul>	<ul style="list-style-type: none"> <li>Assess intracellular <math>\text{NO}_3^-</math> pool in MPB</li> <li>Spatial distribution of <math>\text{NO}_3^-</math> storage (e.g., SIMS)</li> <li>Combination of IPT with push-pull methods or other perfusion/injection techniques</li> <li>Use of in situ chambers (e.g., Risgaard-Petersen et al. 1998a)</li> </ul>
Microbial nitrate storage	<ul style="list-style-type: none"> <li>Uptake of <math>^{15}\text{NO}_3^-</math></li> <li>Release of <math>^{14}\text{NO}_3^-</math> dilutes <math>^{15}\text{N}</math> fraction in <math>\text{NO}_3^-</math> pool</li> <li>Varying depth of <math>\text{NO}_3^-</math> transport into sediments</li> </ul>	<ul style="list-style-type: none"> <li>Additional experiments to determine <math>^{15}\text{NO}_3^-</math> storage and depth distribution</li> </ul>
Low oxygen concentrations	<ul style="list-style-type: none"> <li>Draw down of oxygen inside sealed cores and significant deviation of biogeochemistry from in situ</li> <li>Impaired nitrification (and nitrate supply) relative to in situ</li> </ul>	<ul style="list-style-type: none"> <li>Increase water volume over sediment in batch incubations</li> <li>Continuous oxygen monitoring throughout experiments (e.g., with optode system)</li> <li>Continuous oxygen regulation (e.g., in flow-through systems)</li> </ul>
Permeable sediment	<ul style="list-style-type: none"> <li>Inability to recreate advective flow regimes in individual cores</li> <li>Long diffusion distance and slow supply of <math>\text{NO}_3^-</math> to <math>\text{NO}_3^-</math>-reducing zone when cores from advective environments are incubated under diffusive conditions</li> </ul>	<ul style="list-style-type: none"> <li>Combination of IPT methods (e.g., flume experiments, percolation or flow-through techniques) with modeling techniques, especially in areas with high wave action (e.g., intertidal sands)</li> <li>Whole-core IPT may be applicable in sandy sediments which have insufficient permeability to facilitate significant pore water flow</li> </ul>
Gas ebullition	<ul style="list-style-type: none"> <li>Disruption of oxic layer and <math>\text{NO}_3^-</math>-reducing zone</li> <li>Homogenization of pore-water profiles</li> </ul>	<ul style="list-style-type: none"> <li>IPT in in situ advective chambers (e.g., Eyre et al. 2008, 2013)</li> <li>Shorter sediment core lengths (e.g., manual sampling)</li> <li>In situ measurements (e.g., benthic chambers)</li> </ul>

species within natural communities. Furthermore, as bioturbation experiments are typically carried out in the dark, they potentially exclude the complex relationships that establish between macrofauna and benthic primary producers which may also affect the application of the IPT e.g., as in Fig. 2 (discussed in the next section).

## Phytobenthos

### *Microphytobenthos*

Photosynthetic oxygen production by benthic microalgae (microphytobenthos, MPB) can increase the oxygen penetration by a factor of ~ 2 during the day (e.g., muddy locations 1–2 mm to > 4 mm; Risgaard-Petersen et al. 1994; Meyer et al. 2001; sandy sediments ~ 4 mm to > 8 mm; Revsbech et al. 1980). As a result, MPB may stimulate nitrification and  $D_N$  due to the increased volume of oxic sediment and subsequent increased  $\text{NO}_3^-$  availability (Risgaard-Petersen et al. 1994). The thicker oxic layer also increases the distance across which water column  $\text{NO}_3^-$  must diffuse to reach the zone of  $\text{NO}_3^-$  reduction and so  $D_W$  may be reduced (Risgaard-Petersen et al. 1994; Bartoli et al. 2003). In contrast, MPB also compete with nitrifying bacteria for uptake of  $\text{NH}_4^+$  (Risgaard-Petersen 2003; Risgaard-Petersen et al. 2004b, 2005). In this way, nitrification may become decoupled from denitrification in the presence of MPB and instead enhance internal recycling and retention, rather than removal of N (Risgaard-Petersen et al. 1994; Cabrita and Brotas 2000; Sundbäck and Miles 2000; Cook et al. 2004; Sundbäck et al. 2004, 2006). Endogenous  $^{14}\text{NO}_3^-$  and added  $^{15}\text{NO}_3^-$  may also be reduced through assimilatory pathways to  $\text{NH}_4^+$  even during darkness (Rysgaard et al. 1993; Cook et al. 2004) and has been identified as an important uptake pathway at some locations (Erler et al. 2013). While some studies have investigated explicit effects of MPB presence on anammox (Risgaard-Petersen et al. 2005) and DNRA (Dunn et al. 2012), much less is known about how these processes are affected in illuminated sediments with benthic microalgae.

The application of the whole-core IPT to sediments colonized by MPB is complicated by the varying thickness of the oxic layer between light and dark regimes as the time to reach a steady-state gradient of  $^{15}\text{NO}_3^-$  between overlying water and denitrifying zone will be longer in illuminated sediments. An additional issue is that at least some of the added  $^{15}\text{NO}_3^-$  is assimilated by MPB and may not reach the zone of  $\text{NO}_3^-$  reduction even if sufficient pre-incubation time is given. A further complicating factor is the ability of MPB (e.g., diatoms) to respire stored  $\text{NO}_3^-$  during temporary anoxia during night (see “Nitrate storage” section).  $\text{NO}_3^-$  respiration by MPB is therefore only captured during night time and the contribution of MPB to  $^{15}\text{NO}_3^-$  reduction will only be observed if the incubation covers both day and night periods. However, prolonged (24 h) incubation may lead to substantial oxygen consumption and possible anoxia inside cores (see “Variations in sediment oxygen penetration” section). Additionally, temporal uptake of water column  $^{15}\text{NO}_3^-$  and  $^{14}\text{NO}_3^-$  during the day and respiration

during night will likely result in non-homogenous distribution of  $^{14}\text{N}$  and  $^{15}\text{N}$  in  $^{15}\text{NO}_3^-$  and  $^{15}\text{N-N}_2$ .

Explicitly overcoming the collective impact of MPB (i.e., varying oxygen penetration, assimilation of  $\text{NO}_3^-$ , respiration of  $\text{NO}_3^-$ ) on the IPT is challenging. A comparison of IPT-generated rates with nutrient fluxes and N-assimilation from primary productivity would provide an overview of uptake and release of fixed N in the sediment. It may be beneficial therefore to carry out one incubation to measure net fluxes ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ ,  $\text{N}_2$ ) followed by IPT incubations on the same set of cores. Long (> 3 h) pre-incubation times may be useful to ensure the  $^{15}\text{NO}_3^-$  has can penetrate through the oxic layer to the zone of  $\text{NO}_3^-$  reduction; however, this also increases the time available to MPB to continuously take up  $\text{NO}_3^-$ . Additional experiments are required to specifically assess intracellular  $\text{NO}_3^-$  uptake, storage, and respiration during light and dark exposure such as spatially resolving isotope measurements by Secondary Ion Mass Spectrometry (SIMS) in combination with labeling experiments (e.g., Klawonn et al. 2016; Bergkvist et al. 2018).

### *Vascular plants*

In regions colonized by seagrasses and other vascular plants, diffusion of oxygen through root systems creates subsurface oxic niches (Maricle and Lee 2002; Jovanovic et al. 2015) where coupled nitrification–denitrification can occur (Caffrey and Kemp 1992; Risgaard-Petersen and Jensen 1997; Ottosen et al. 1999). Many studies investigating the impacts of rooted plants were carried out before the discovery of anammox and its co-occurrence with denitrification and DNRA. As such we still know relatively little about the quantitative impacts of plants on sedimentary DNRA and anammox.

In the same manner as in the presence of burrowing organisms, plants create multiple subsurface oxic niches causing heterogeneous  $^{14}\text{NO}_3^-$  to  $^{15}\text{NO}_3^-$  ratios within sediments and thus complicate the use of the IPT. This is due to both patchy production of new  $^{14}\text{NO}_3^-$  from nitrification and the fact that  $^{15}\text{NO}_3^-$  will not be able to diffuse deeply into the rhizosphere. Dealing with large plants growing in sediment also has practical issues, as damage to root systems or above-ground biomass during sampling is likely to alter plant’s photosynthetic or other metabolic capacities. In addition, the whole-core IPT is a destructive technique in that cores must be sacrificed for sample collection at each time point. Thus it is not possible to apply the IPT to the same set of cores, for example, during light–dark measurements and so true replication between treatments cannot be achieved due to sediment heterogeneity.

Previous studies employing IPT to sediments colonized by plants have utilized in situ incubation chambers (Risgaard-Petersen et al. 1998a) or perfusion techniques (Ottosen et al. 1999) to overcome such sampling artifacts and to discern contributions of discrete processes (e.g.,  $D_N$ ) at depth. In muddy sediments where perfusion is not possible,  $^{15}\text{NH}_4^+$  has also been injected into the rhizosphere of macrophytes to

determine subsurface  $D_N$  (Soana et al. 2015; Racchetti et al. 2017). However, as discussed in previous studies, the IPT is not the “perfect” assay with which to reliably quantify denitrification in these sediment systems (Nielsen 1992; Welsh et al. 2001) and studies may explicitly avoid the presence of macrophytes and their root systems to avoid complications to the IPT (e.g., Salk et al. 2017). In vegetated sediments, the potential for the combination of IPT with other methods may go some way toward overcoming issues associated with using IPT alone. For example, applying the IPT in push-pull methods (described further later in this article) may be an additional promising solution to investigate subsurface effects of the rooted plants on sediment N cycling (Koop-Jakobsen and Giblin 2009; Aoki and McGlathery 2017).

### Nitrate storage

The uptake and intracellular storage of  $\text{NO}_3^-$  has been observed in prokaryotic (Fossing et al. 1995; Schulz et al. 1999; Teske and Nelson 2006) and eukaryotic organisms (Piña-Ochoa et al. 2010; Kamp et al. 2011; Bernhard et al. 2012a). These intracellular pools can play an important role in transporting bioavailable N to sediments (Lomstein et al. 1990).  $\text{NO}_3^-$  may accumulate intracellularly to several hundred millimolar concentrations (Schulz et al. 1999; Glud et al. 2009; Xu et al. 2017). The stored  $\text{NO}_3^-$  may be respired by the organisms themselves as electron acceptor through DNRA (Otte et al. 1999; Teske and Nelson 2006; Kamp et al. 2011) or denitrification (Risgaard-Petersen et al. 2006; Høglund et al. 2008; Piña-Ochoa et al. 2010; Xu et al. 2017; but see also Bernhard et al. 2012b). As such, the presence of  $\text{NO}_3^-$ -storing organisms can have substantial effects on N biogeochemistry in sediments by directing the dominant  $\text{NO}_3^-$  reduction process (Sayama et al. 2005; Dale et al. 2016), providing a storage buffer to bridge periods when  $\text{NO}_3^-$  may be unavailable and when oxygen concentrations may fluctuate. In addition,  $\text{NO}_3^-$  can be transported deeper into sediments below surface diffusive layers (Prokopenko et al. 2011). Such  $\text{NO}_3^-$  would not equilibrate with the added  $^{15}\text{NO}_3^-$  from the water column over 4–8 h pre-incubation period. Any potential  $\text{N}_2$  production from deeply stored  $\text{NO}_3^-$  will not be accounted for by the IPT. Stored  $\text{NO}_3^-$  from the uppermost millimeters of sediment, however, can mix with added  $^{15}\text{NO}_3^-$  in the water column, generating additional  $^{29}\text{N}_2$  compared to systems without  $\text{NO}_3^-$ -transporting organisms. According to IPT calculations, the additional  $^{29}\text{N}_2$  would be falsely interpreted as nitrification–denitrification (i.e.,  $D_N$ ).

One of the most common organisms associated with  $\text{NO}_3^-$  storage are the large sulfur bacteria *Beggiatoa*, which reduce intracellular  $\text{NO}_3^-$  stores although DNRA (Preisler et al. 2007) or denitrification (Schutte et al. 2018, Zitzmann and Brüchert unpubl. data). Mats formed by these bacteria are a common feature of organic-rich coastal sediments exposed to low-oxygen conditions in the overlying water (Jørgensen and Revsbech 1983). The presence of these organisms is problematic

for applying the IPT because the added  $^{15}\text{NO}_3^-$  may not equilibrate completely with the large intracellular pools which will lead to an underestimation of the reduction of  $\text{NO}_3^-$  from these pools. Additionally, release of stored  $^{14}\text{NO}_3^-$  by mechanical rupturing during sample agitation (e.g., when conducting slurry incubations) may dilute the  $^{15}\text{N}$  fraction in the  $\text{NO}_3^-$  pool and affect the  $^{15}\text{N}$  fraction of  $\text{NH}_4^+$  or  $\text{N}_2$  produced; thereby leading to over- or under- underestimations of process rates (Sokoll et al. 2012; Song et al. 2013). Although these recent studies have shown that it is possible to account and correct for the presence of  $\text{NO}_3^-$ -storing organisms if biomass density, depth distribution, and intracellular  $\text{NO}_3^-$  concentrations are known (Sokoll et al. 2012; Song et al. 2013, Brüchert and Zitzmann unpubl. data), very few have yet considered this during application of the IPT.

For correct  $^{15}\text{N}$  experiments in the presence of *Beggiatoa* it is also important to determine their vertical distribution and intracellular  $\text{NO}_3^-$  content, since they also occur deeper in the sediment at higher bottom-water  $\text{O}_2$  concentrations and can be expected to migrate during the incubation and participate in the  $\text{NO}_3^-$  transformation processes (Jørgensen and Nelson 2004). Whole-core IPT incubations generally cannot provide information on the active contribution of  $\text{NO}_3^-$ -storing organisms and supplemental experiments with individual cells, chains, or bundles need to be performed (Otte et al. 1999; Preisler et al. 2007; Glud et al. 2009).

### Variations in sediment oxygen penetration

#### Low bottom-water oxygen concentrations

The occurrence and modern-day increases in the frequency and extent of low-oxygen water masses (Carstensen et al. 2014; Breitburg et al. 2018) are a major feature to consider when studying benthic N cycling. The determination of biogeochemical process rates under in situ bottom water oxygen conditions is of major importance for constructing models and for our capacity to predict future oxygen scenarios more accurately. Oxygen depletion of overlying water has profound effects on sediment N turnover and N-removal capacity (Seitzinger 1990; Rysgaard et al. 1994). The reduction in oxygen penetration with decreasing bottom-water oxygen concentration reduces the diffusion distance of  $\text{NO}_3^-$  to the  $\text{NO}_3^-$ -reducing zone and increases the importance of  $D_W$  (Rysgaard et al. 1994). However, with a reduced thickness of the oxic layer,  $\text{NO}_3^-$  supply may become impaired both due to the reduction of nitrification and the eventual consumption of  $\text{NO}_3^-$  in the water column if hypoxia or anoxia is prolonged. Additionally, DNRA is often observed to become dominant under low-oxygen conditions (e.g., Tiedje et al. 1982; Jäntti and Hietanen 2012, but see also Bonaglia et al. 2017) and shifts in dominant  $\text{NO}_3^-$ -reducing process may occur with variations in oxygen concentration—either in situ or during experiments. As such, changes in oxygen concentration during experimentation should be minimized to obtain representative in situ process rates.

Whole-core experiments have largely been developed for environments where sediments are exposed to well-oxygenated water columns. Accordingly, oxygen concentrations in the overlying water of whole-core experiments should be maintained at in situ bottom water oxygen concentrations before and during  $\text{NO}_3^-$  equilibration periods. Following equilibration, sediment cores are sealed with stoppers to allow end products of  $\text{NO}_3^-$  reduction to accumulate in individual cores over several hours. During IPT experiments (or other flux measurements) oxygen is not allowed to drop below 20% of the initial oxygen concentration (Dalsgaard et al. 2000), however this “rule of thumb” becomes problematic under hypoxic conditions. While oxygen consumption decreases with decreasing oxygen concentration (Hall et al. 1989; Rasmussen and Jørgensen 1992), at severely oxygen depleted sites oxygen may be entirely consumed between the time that cores were capped and the time the core is slurried at the end of the incubation period. The influence of prolonged and short-term hypoxia on N cycling in sediments has been investigated experimentally in laboratory (e.g., Hietanen and Lukkari 2007; Jäntti and Hietanen 2012; Roberts et al. 2012) and large-scale field experiments (De Brabandere et al. 2015) and have demonstrated varying effects. Laboratory experiments and large field experiments have shown varying effects of short-term hypoxia on N cycling in sediments. Jäntti and Hietanen (2012) and De Brabandere et al. (2015) showed a greater importance of DNRA under hypoxia with a concomitant reduction in denitrification, whereas Roberts et al. (2012) observed reduced DNRA relative to denitrification in cores exposed to long-term hypoxia. In studies in the North Sea, denitrification increased under short-term hypoxia while anammox remained constant (Neubacher et al. 2011) while both anammox and denitrification were shown to increase under sustained hypoxia (Neubacher et al. 2012). However, it is unknown how and if N cycle processes change in response to the rapid changes such as those experienced when oxygen is consumed in core experiments. It is also notable that, as with many environmental factors discussed in this review, there is a lack of information on how DNRA and anammox may change in relation to varying oxygen conditions.

The temporal variability of the in situ oxygen concentration in some areas requires that whole-core incubations are either conducted under several oxygen concentrations or as repeated measurements over the year. For example, in situ bottom water oxygen concentrations at stations in the Eastern Gotland Basin in the Baltic Sea fluctuated between  $160 \mu\text{mol L}^{-1}$  to less than  $10 \mu\text{mol L}^{-1}$  within just 24 h (Noffke et al. 2016). Observations from locations near Tvärminne in the north-western Gulf of Finland (K. Attard, unpubl. data) and Askö in the southern Stockholm archipelago (V. Brüchert, unpubl. data) have shown erratic fluctuations in oxygen between over  $200 \mu\text{mol L}^{-1}$  to below  $100 \mu\text{mol L}^{-1}$  during multi-week lander deployments. In the shallow (< 3 m) Curonian Lagoon (Lithuania) bottom water oxygen fluctuated between  $400 \mu\text{mol L}^{-1}$  and  $110 \mu\text{mol L}^{-1}$  over just 3 h during summer months (M. Zilius, unpubl.

data). The strong oxygen fluctuations violate the assumptions made in static enclosed sediment incubations but are potentially highly relevant for studying nutrient dynamics in regions such as the Baltic Sea. How rapidly sediment processes respond to fluctuations in oxygen availability is also dependent on sediment exchange rates since high organic inputs and oxygen uptake reduce the depth of oxygen penetration.

Fluctuations in oxygen penetration depth in turn alter the available volume for benthic nitrification (and thus  $\text{NO}_3^-$  supply), as well as the availability of reduced compounds which may affect  $\text{NO}_3^-$ -reducing processes such as sulfide ( $\text{H}_2\text{S}$ ; Brunet and Garcia-Gil 1996; Burgin and Hamilton 2008; Jones et al. 2017) or ferrous iron ( $\text{Fe}^{2+}$ ; Coby et al. 2011; Laufer et al. 2016a; Robertson et al. 2016). Further insights into the kinetics of sediment  $\text{NO}_3^-$ -reducing processes may provide some insights as to how oxygen fluctuations may alter the balance among denitrification, anammox, and DNRA. While recent studies have demonstrated geographical variations in the fine-scale oxygen control of  $\text{NO}_3^-$ -reducing processes in oxygen deficient water columns (Kalvelage et al. 2011; Babbitt et al. 2014; Dalsgaard et al. 2014a), far less is known about precise oxygen control of  $\text{NO}_3^-$  reduction in sediments.

Oxygen concentrations inside cores can be monitored by using flow-through system where an oxygen sensor on the inside of the core (Binnerup et al. 1992; Rysgaard et al. 1993, 1994; Risgaard-Petersen and Rysgaard 1995). In these experiments, samples for  $^{15}\text{N}$ -labeled compounds are collected from the out-flowing water. While these systems permit a greater degree of control, they are more expensive to construct and maintain and may lead to compromises in replication capacity. A possible methodological modification may be the use of longer cores, allowing a greater water volume with smaller area/volume ratio. This may slow down the decrease in oxygen concentration in sealed cores. However, a larger water volume decreases measurement precision as end products are diluted.

### **Deep oxygen penetration**

The majority of conditions discussed so far in this review are typical of coastal sediments which have oxygen-penetration depths within the millimeter-range (excluding permeable sediments, see “Permeable sediment” section). Here, the diffusion of added  $^{15}\text{NO}_3^-$  to  $\text{NO}_3^-$ -reducing layers will be achieved in a pre-incubation period in the range of  $\sim 0.5$ – $3$  h (Dalsgaard et al. 2000). However, at some stations where sediment reactivity is low, much greater oxygen penetration depths (in the centimeter-range, or more) are observed (Reimers 1987; Glud et al. 1994, 2013; Cai and Reimers 1995; Li et al. 2012).

The main challenge of investigating benthic N cycling in sediments with deeply penetrating oxygen is the application of the IPT within a reasonable time frame. Relative to more reactive sediments with shallow oxygen penetration, sites with several centimeters, pre-incubation with  $^{15}\text{NO}_3^-$  and following incubation periods for IPT incubations are much

longer. For example, Glud et al. (2009) used 5 h pre-incubation and 18 h incubation period where oxygen penetration was up to ~10 mm in deep Sagami Bay sediments. Thus for sediments with even deeper oxygen penetration (e.g., Reimers 1987), the application of the IPT becomes unfeasible due to impractically long pre-incubation times to confirm that  $^{15}\text{NO}_3^-$  has equilibrated with the  $^{15}\text{NO}_3^-$ -reducing zone, as well as extreme incubation times required to achieve measurable signals. In the marine environment, deeply oxygenated sediments are generally located at deep stations (Glud et al. 1994, 2013; Cai and Reimers 1995) and the time, space and associated boat time cost required to carry out IPT incubations aboard research vessels may not be realistic.

Retrieval of cores from deep sites causes significant deviation of porewater profiles (Hall et al. 2007) compared to those measured in situ on benthic landers (Glud et al. 1999). In deep ocean sediments with high oxygen penetration the use of in situ methods (e.g., benthic landers, discussed previously) for assessing deep ocean biogeochemical processes is extremely valuable, however few research groups have so far had the opportunity and technology to develop deep-ocean IPT approaches. In general, locations with deeply oxygenated sediments have significantly lower  $\text{NO}_3^-$ -reducing rates than those with shallower oxygen penetration and higher organic matter turnover (Li and Katsev 2014). However, these deeply oxygenated sediments cover a large area of the ocean floor and are thus hugely understudied compared to coastal sediments. However, some sites with deep oxygen penetration occur in relatively shallow (< 500 m) sediments where retrieval of cores leads to fewer recovery artifacts. For example in sediments of Lake Superior, oxygen penetration varies greatly between sites (Li et al. 2012) and benthic N cycling has been previously calculated through flux-based N budgets (Li and Katsev 2014). In this environment, there is the potential for injection of  $^{15}\text{NO}_3^-$  to greater depth at side ports in cores as demonstrated in Crowe et al. (2017) where cores are retrieved from shallower depths and are not subject to as many artifacts as with retrieval from very deep stations. However, the ability to carry out such experiments requires prior knowledge of benthic oxygen penetration. The IPT is most appropriate for locations with steep biogeochemical gradients and so a more appropriate solution for assessing benthic N cycling in deeply oxygenated, low-activity sediments would be analysis of pore-water gradients (i.e.,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , etc.) and reaction transport modeling.

### Permeable sediment

Sandy sediments cover ~50% of the inner continental shelf (< 65 m; Hall 2002), which is an important area for sedimentary N turnover and removal (Seitzinger 1988). A large proportion of these sands can be expected to be permeable, which facilitates advective pore-water flow as a transport mechanisms of dissolved and particulate substances into and through the sediment (Thibodeaux and Boyle 1987; Huettel

et al. 2003). Advective pore water flow has significant impacts on sediment biogeochemistry and the redox-sensitive N cycle due to increased solute fluxes (Huettel and Gust 1992a; Shum and Sundby 1996), particulate organic matter intrusion (Huettel et al. 1996; Rusch and Huettel 2000) and oxic sediment volume (Ziebis et al. 1996), stimulating mineralization (Forster et al. 1996; Boudreau et al. 2001). Advective pore water flow is mainly driven by horizontal pressure gradients at the permeable sediment surface (Santos et al. 2012) which establish when a boundary layer flow interacts with micro- and macro-scale sediment topography (e.g., waveforms, stones, bioroughness; Thibodeaux and Boyle 1987; Huettel and Gust 1992b), or under the influence of waves with wave crests and troughs creating high and low pressure areas at the sediment ("wave pumping"; Riedl et al. 1972).

In cohesive sediments, the DBL above the sediment is important in regulating solute exchange and has a strong influence on N cycling processes (Jørgensen and Boudreau 2001). In IPT incubations, the DBL is easily artificially generated by mixing of the water overlying sediments by a magnetic stirrer. However, the presence of advective pore-water flow poses unique problems to the use of the typical whole-core IPT, which aims to specifically reproduce the DBL conditions over cohesive sediments and thus does not realistically replicate the in situ pore water flow field of advective systems. Using diffusive methods (here IPT) in an advective environment may lead to  $^{15}\text{NO}_3^-$  not reaching the deep zone of  $\text{NO}_3^-$  reduction in the appropriate amount and within a reasonable timeframe, which is further complicated by the presence of three-dimensional redox zones (Huettel et al. 1998). However, sandy sediments with insufficient permeability to facilitate pore water flow, e.g., at locations exposed only to very slow bottom water currents may not experience advective flow (Forster et al. 2003) and the application of the conventional diffusive whole-core IPT is possible (Hellemann et al. 2017).

In general  $\text{NO}_3^-$  reduction process rates from permeable sands are relatively scarce compared to those from cohesive muddy sediments but several studies have made efforts to overcome specific issues related to the application of the IPT on permeable sediments. At stations with slow bottom-water currents, a preliminary assessment of permeability is desirable to evaluate whether the conventional diffusive whole-core IPT can be applied in sandy sediments. Sediment permeability can serve as a practical way to characterize a sediment regarding its ability to enable pore water flow; the threshold for the onset of advective pore water flow with significant effects on sediment biogeochemistry has been given at  $> 2.5 \times 10^{-12} \text{ m}^2$  for coastal sediments of the Baltic Sea (Forster et al. 2003), and  $> 1 \times 10^{-12} \text{ m}^2$  for generally more energetic environments (Huettel et al. 2003). Thus in some off-shore permeable sediments, the typical IPT incubations and assumptions may be applicable. However, the majority of studies applying the IPT to permeable sediments have been largely carried out in dynamic intertidal environments where advection has a large impact on sediment

processes. Studies investigating N cycling in intertidal sandy sediments have used flow-through sediment columns (Rao et al. 2007; Evrard et al. 2012), percolation techniques (Gao et al. 2010; Marchant et al. 2014, 2016), experimental chamber simulations (Cook et al. 2006), stirred reactors (Cook et al. 2017), push-pull methods (Erler et al. 2014), flume experiments (Kessler et al. 2012), in situ advective chambers (Eyre et al. 2013), and various modeling techniques (Cook et al. 2006; Kessler et al. 2012, 2014a; Neumann et al. 2017) to account for advective solute exchange. These studies have demonstrated the great importance of this sediment type for benthic N turnover and loss through  $N_2$  (Eyre et al. 2008, 2013; Gao et al. 2012; Marchant et al. 2016) and/or  $N_2O$  production (Marchant et al. 2018). In advection-dominated environments, direct comparisons of diffusive and advective techniques demonstrated that advective flow regimes resulted in  $N_2$  production 1–2 orders of magnitude greater than under diffusive conditions (e.g., Gihring et al. 2010; Gao et al. 2012). Thus in intertidal environments realistic estimation of N cycling processes in permeable sediments is only achievable when advective processes are simulated.

### Gas ebullition

The acquisition of “undisturbed” sediment cores for whole-core IPT experiments is important to ensure that redox zonation is preserved. Changes in hydrostatic pressure are an important factor governing ebullition (Heyer and Berger 2000; Joyce and Jewell 2003). The subsequent pressure decrease after collection of sediment cores can cause substantial gas release which irrigates sediment releasing  $NH_4^+$  and phosphate into overlying water, homogenizing pore water profiles (Dale et al. 2013), disturbing the redox zonation and compromising the use of the IPT. Such sediment cores may thus not be representative of in situ sediment due to the oxic layer and  $NO_3^-$  reducing zones having been disturbed to varying degrees. Complications from sampling sediments with high gas contents have led to issues while conducting ex situ experiments with IPT (Zilius et al. 2016, S. Hietanen, unpubl. data).

The impacts of gas ebullition on the IPT—or how it can affect benthic N cycling in general—are rarely reported or experiments may be discarded entirely, and as such direct references to this are lacking. However, it is likely that several co-occurring and contradictory effects would disrupt the reliable application of the IPT. The disturbance of redox zonation would lead to non-homogenous  $^{15}NO_3^-$  and  $^{14}NO_3^-$  distributions as well as transporting reduced substances ( $H_2S$ ,  $Fe^{2+}$ ) which may be chemically oxidized and result in a thinner remaining oxic layer. This would reduce the volume of sediment where nitrification could occur and thus the uniformity of  $D_N$  over time could not be assumed. Alterations in the oxygen penetration depth will also complicate the calculation of appropriate IPT incubation times. In contrast the void left by gas bubbles may be replaced by oxic water, leading to subsurface oxygenated regions where  $^{14}NO_3^-$  can be produced

through nitrification and causing further issues related to non-homogenous  $^{15}NO_3^- : ^{14}NO_3^-$  ratios.

The unpredictability of sporadic gas release certainly makes this issue one of the most difficult to explicitly solve with regards to the IPT but also to other biogeochemical assessments. Sediment disturbance by ebullition may be overcome in some cases by the use of shorter sediment lengths in cores (4–5 cm), thereby avoiding the deeper sediment horizon where methane accumulates. In many cases, it may not be possible to select a precise core length at deeper stations where sediment cores are taken on board research vessels. However, the use of divers or other manual sampling at shallow sites may provide a greater degree of success in retrieving intact cores and such cores may provide a better representation of in situ conditions compared to surface-based coring methods (Mogg et al. 2017).

## Discussion and outlook

### Method progression

The whole-core IPT has provided an important dataset to address the diversity and distribution of N cycling processes. However, the emerging potential shortcomings of the use of the IPT should be acknowledged. We must be able to understand how these limitations affect the quantitative outcome of process rates and how this could lead to inaccuracies regarding N budgets. It is also clear that we cannot achieve a complete picture of benthic N cycling until we have data from the challenging environments discussed above. The IPT calculations have gone through several revisions, and analytical advancements have been made in recent years. Increased detector sensitivity in mass spectrometers and improved chromatographic separation have thus furthered our ability to accurately determine rates of benthic N cycling processes. Here, we discuss methodological advancements and combinations as well as some further considerations of whole-core IPT incubations (see also Table 1).

### Benthic chamber incubations

Benthic chamber incubations combined with the IPT principles have been used in several studies at shallow coastal stations (Nielsen and Glud 1996; De Brabandere et al. 2015) as well as deeper sites in the Baltic Sea (Bonaglia et al. 2017; Hall et al. 2017). This equipment has the benefit of enabling undisturbed sediment to be investigated without recovery artifacts (Aller et al. 1998; Hall et al. 2007), with minimal disturbance to infaunal assemblages (Nielsen and Glud 1996) and under true in situ environmental conditions with regards to bottom water oxygen and  $NO_3^-$  concentrations, temperature, salinity, and pressure. Benthic chambers additionally encompass a relatively large surface area and thus can account for sediment heterogeneity. Individual cores spread over a large surface area also account for surface sediment variation, but the assumption that all cores are equally representative of the same area of sea floor as replicates in time series may not be accurate,

especially not at stations with patchy substrate or surface variation (e.g., *Beggiatoa* mats, bioturbating organisms). At stations where methane accumulation leads to substantial artifacts from pressure release during sampling, the use of in situ IPT to measure  $\text{NO}_3^-$ -reducing processes is clearly valuable.

While the advantages of in situ incubations are clear, these methods do not allow for the in situ determination of anammox and DNRA. This requires that the lander is designed to recover sediment reasonably intact to allow sub-coring in the flux chamber once the lander is on deck (to recover  $\text{N}_2$  which has accumulated in sediments) or additional ex situ measurements from whole-core or slurries to obtain supplementary information on processes (Steingruber et al. 2001; De Brabandere et al. 2015; Bonaglia et al. 2017; Hall et al. 2017). Additionally, if the oxygen penetration depth is not determined with in situ microelectrodes on the benthic lander, it is difficult to determine the appropriate length of time for  $^{15}\text{NO}_3^-$  equilibration and starting time of the experiment. However, this can be overcome by carrying out time-series sampling to confirm linear  $\text{N}_2$  production. As with the whole-core incubations, the use of benthic chambers also has issues in the case of permeable substrate in that the in situ bottom water flow field—and thus the corresponding pore water flow field—is often not maintained.

#### $\text{N}_2 : \text{Ar}$

Another common method used to assess  $\text{N}_2$  production from sediment is the  $\text{N}_2 : \text{Ar}$  ratio (Kana et al. 1994; Laursen and Seitzinger 2002; Benelli et al. 2018). Changes in  $\text{N}_2$  concentration over time are measured relative to the inert gas Ar, the concentration of which should be constant during the incubation. Thus the overall net  $\text{N}_2$  dynamics from production by denitrification or anammox and consumption by  $\text{N}_2$  fixation can be determined. In contrast to the IPT, the direct  $\text{N}_2$  flux measurement based on the  $\text{N}_2 : \text{Ar}$  method does not require the addition of a  $^{15}\text{N}$  tracer which alleviates problems associated with pre-incubation and diffusion distance. However,  $\text{N}_2 : \text{Ar}$  can only determine net effects of several coexisting processes and it is not possible to distinguish between anammox, denitrification or coupled anammox-DNRA.

The potential for  $\text{N}_2 : \text{Ar}$  to be used in combination with IPT may be of benefit when working in shallow environments where plants, meio- and macrofaunal activity creates heterogeneous sediments, and therefore IPT assumptions could be invalid or too complex to be determined with a single method (Magri et al. 2018). In previous studies,  $\text{N}_2 : \text{Ar}$  has been shown to overestimate  $\text{N}_2$  production in permeable sediments under advective pore-water flow (Cook et al. 2006), underestimated  $\text{N}_2$  production in sediments reworked by macrofauna (Ferguson and Eyre 2007) and in estuarine sediments with hypoxic bottom water (Crowe et al. 2012), and give comparable results (Eyre et al. 2002; Deek et al. 2013) relative to whole-core-IPT measurements. In some environments, applying both methods could be beneficial in studying benthic

nitrogen fixation, which is now thought to be significant in a number of coastal systems (Fulweiler et al. 2007; Andersson et al. 2014; McCarthy et al. 2015; Newell et al. 2016). While this is attractive, this method comes with additional issues and the potential for determining incorrect rates (see Eyre et al. 2002).

#### *Push-pull methods*

Sediments colonized by vascular plants or other sediments where patchy  $\text{NO}_3^-$  reduction may occur below the  $\text{NO}_3^-$ -diffusion zone pose another challenge to the whole-core IPT. A recent adaptation of the IPT is its combination with a push-pull technique, which was developed for use in vegetated salt marsh (Koop-Jakobsen and Giblin 2009) and subtidal seagrass sediments (Aoki and McGlathery 2017). An additional variation of the push-pull and IPT method has also been applied to advection-dominated carbonate sands in combination with flow-through reactors (Erler et al. 2014). In these experiments, pore-water is extracted from saturated sediments using a micro-piezometer, and amended with  $^{15}\text{NO}_3^-$ . The amended water is then returned (“pushed”) back into the sediment where it is allowed to incubate and samples are taken (“pulled”) at time points over several hours. In contrast to traditional core incubations, the push-pull technique using IPT may more accurately capture background heterogeneity and natural range of  $\text{NO}_3^-$  reduction processes. Its more recent application has also been developed to specifically be able to account for DNRA (Aoki and McGlathery 2017) which may be of major importance in coastal vegetated systems (Giblin et al. 2013). The push-pull method has the additional benefit that root systems are not disturbed or damaged by the removal of cores and that in situ light and flow regimes are maintained. There are also some practical limitations of applying the method (see discussion in Aoki and McGlathery 2017); however, the combination of the two techniques may provide more robust information on the contribution of subsurface  $\text{NO}_3^-$  reduction to N removal and recycling.

#### *Combining IPT with models*

The use of various modeling techniques in conjunction with whole-core IPT measurements have proved valuable in advective systems. Advective pore-water modeling allows flushing rates to be constrained in permeable sediments in which  $\text{NO}_3^-$  reduction rates are challenging to evaluate experimentally. N dynamics affected by advective pore-water flow can nowadays be described reasonably well computationally with surface-subsurface coupled models and fluid dynamics approaches, at least at small scales (Kessler et al. 2014a,b). Applying modeling techniques, possibly in combination with experimental methods which account for advective flow is likely to continue to improve our understanding of N cycling in these environments (Cardenas et al. 2008; Kessler et al. 2012).

Modeling approaches to scale up to larger areas must be based on reliable information provided from experimental measurements, ideally taking into account the potential

influences of benthic substrate (e.g., see Deutsch et al. 2010), fauna or vegetation and how this can influence solute transport and process rates (Lessin et al. 2018). Nielsen et al. (1995) demonstrated that denitrification measurements from whole-core IPT compared well with mass balance calculated by numerical modeling. In this case, testing these independent approaches found that they were appropriate for application in a shallow fjord environment with muddy sediments. However, extrapolation of IPT-based rates over larger areas requires so far unconstrained assumptions. Spatial homogeneity of a particular sediment type is only starting to be reasonably accounted for in seascape habitat maps. The discreet and largely *ex situ* experimentation with  $^{15}\text{N}$  only covers time intervals over a few hours in which a habitat may experience significant natural variability. Additionally, in compiling data from multiple methods to calculate N removal and retention (e.g., whole-core IPT, acetylene-block, flux-based mass balances) to scale up to larger areas, the potential shortcomings of each method should be considered (e.g., see Groffman et al. 2006). An understanding of the available empirical data and an interdisciplinary approach are certainly needed to produce meaningful models (Lessin et al. 2018).

#### Further considerations for the use of whole-core IPT

##### *Interaction with other biogeochemical cycles*

The N cycle is comprised of a wide range of redox reactions where inorganic redox partners have the potential to influence process rates. This is particularly true in cohesive sediments where reduced species from deeper layers can diffuse into the narrow zone of  $\text{NO}_3^-$  reduction. Several studies have shown that  $\text{Fe}^{2+}$  (Straub et al. 1996; Weber et al. 2006; Robertson et al. 2016),  $\text{H}_2\text{S}$  (Brunet and Garcia-Gil 1996; Burgin and Hamilton 2008), and methane ( $\text{CH}_4$ ) (Haroon et al. 2013; Norði and Thamdrup 2014) can potentially be used as electron donors for  $\text{NO}_3^-$  reduction and influence the fate of N in sediments.

The reduction of  $\text{NO}_3^-$  coupled to the oxidation of inorganic electron donors does not implicitly pose issues to the whole core IPT. However, the influence of alternative donors may be episodic in response to environmental fluctuations (e.g., hypoxia; Roberts et al. 2014; Robertson et al. 2016), causing temporal changes in the dominant  $\text{NO}_3^-$  reduction pathway. Organisms carrying out these reactions may be autotrophic (e.g., Burgin and Hamilton 2007; Laufer et al. 2016b; Robertson and Thamdrup 2017), using  $\text{CO}_2$  as a C-source, or mixotrophic, oxidizing an organic co-substrate in addition to the inorganic substrate as a C-source for growth (e.g., Straub and Buchholz-Cleven 1998; Muehe et al. 2009). Thus, the use of inorganic electron donors as opposed to organic carbon to reduce  $\text{NO}_3^-$  could potentially lead to uncertainties in C budgets if all  $\text{NO}_3^-$  reduction is assumed to be heterotrophic. Substantial metabolic flexibility in terms of C-source has recently been demonstrated in mixed microbial cultures of  $\text{NO}_3^-$ -reducing  $\text{Fe}^{2+}$ -oxidizing organisms (Tominski et al.

2018). This suggests that these communities have the capacity to respond to environmental fluctuations in C-source availability and that the contribution of autotrophic vs. mixotrophic groups to benthic  $\text{NO}_3^-$  reduction may vary throughout the year as C-inputs change.

The true *in situ* contribution of multiple electron donors can be difficult to assess and high-resolution pore water and complimentary solid-phase sediment profiles are required to assess possible interactions. Information on specific N cycling processes and the influence of varying substrates in sediment can be obtained from additional slurry incubations supplemented with alternative electron donors carried out in parallel with the three typically used isotopic treatments (discussed previously) alongside the whole-core IPT. The quantitative importance of these interactions to N cycling in sediments still remains largely unknown however they may play a significant role in determining the fate of  $\text{NO}_3^-$  in sediments.

##### *Seasonal succession*

The presence of meio- and macrofaunal species is typically observed under well-oxygenated conditions during winter and spring and mobile organisms may migrate to more well-oxygenated areas in response to oxygen deficiency (Josefson and Widbom 1988; Nilsson and Rosenberg 2000). Activity of MPB is additionally controlled seasonally by temperature and light availability (Sundbäck et al. 2000). As such these organisms may substantially influence N cycling only during certain times of the year. Due to the impact of fauna and MPB on benthic N cycling, seasonal whole-core IPT studies at such ephemerally faunated sites and sites with intermittent MPB activity are very important for constructing annual nutrient budgets.

The impacts of seasonality (e.g., Rysgaard et al. 1995; Sundbäck et al. 2000; Ferguson and Eyre 2007; Jäntti et al. 2011) and fauna (e.g., Kristensen 2000; Laverock et al. 2011) on N cycling have been well established, however much less is known about seasonal successions of microbial populations and their impact on N cycling processes. As discussed above, the presence of  $\text{NO}_3^-$ -storing organisms can pose a significant issue for the IPT. However the presence of these organisms may be transient, with seasonal studies noting the presence of *Beggiatoa* mats typically only during warmer months when the overlying water is hypoxic (Jäntti and Hietanen 2012; Bonaglia et al. 2014a; Sulu-Gambari et al. 2016; Lipsewiers et al. 2017). Accordingly, during certain months of the year it may be necessary to carry out additional  $\text{NO}_3^-$  storage experiments or calculations (e.g., Glud et al. 2009; Song et al. 2013).

In addition to *Beggiatoa*, succession of other microbes that influence the biogeochemistry of the surrounding sediment may alter N cycling. The recently described cable bacteria have been found in a large number of locations worldwide (Burdorf et al. 2016). These organisms form chains to bridge spatially separated electron donor (sulfide) and acceptor (oxygen or  $\text{NO}_3^-$ ) (Nielsen et al. 2010; Pfeiffer et al. 2012; Marzocchi

et al. 2014) and thereby transfer electrons over centimeter distances in sediments. This metabolism locally decreases pH and causes the dissolution of Fe sulfides, and subsequent liberation of  $\text{Fe}^{2+}$  (Risgaard-Petersen et al. 2012) which may act as a potential electron donor for  $\text{NO}_3^-$  reduction, apparently favoring DNRA over denitrification (Roberts et al. 2014; Robertson et al. 2016, Kessler et al. 2018). In this way, cable bacteria may indirectly control sediment N loss or retention processes.

Recent studies on the seasonal succession of cable bacteria and *Beggiatoa* in a seasonally hypoxic coastal basin (Seitaj et al. 2015; Sulu-Gambari et al. 2016) demonstrated a dominance of cable bacteria during well-oxygenated spring months. The presence of cable bacteria was greatly reduced during summer hypoxia and absent under anoxia. Following autumn reoxygenation of bottom waters, *Beggiatoa* mats were observed to have colonized surface sediments (Sulu-Gambari et al. 2016). Thus throughout the year, the succession of the microbial community from cable bacteria- to *Beggiatoa*-dominated sediments may also play an important role in regulating N cycling at sites susceptible to periodic hypoxia.

Despite their ubiquity, we know relatively little on the quantitative impact of cable bacteria and further studies are needed to assess their impact on N cycling. To apply the whole-core IPT at such locations and obtain a proper understanding of seasonal changes in N cycling may require extensive additional experiments throughout the year. Thus in parallel to whole-core IPT experiments, determining the influence of *Beggiatoa* through  $\text{NO}_3^-$ -storage experiments, microprofiling (of  $\text{O}_2$ , pH and  $\text{H}_2\text{S}$ ) in surface sediment and high-resolution pore-water and solid-phase profiling of N, Fe, and S compounds would be required. The potential impacts of  $\text{Fe}^{2+}$  on  $\text{NO}_3^-$  reduction through slurry experiments could also help to determine how processes and interactions may fluctuate throughout the year. Thus as a consequence of the discovery of novel organisms, the need for extra experimental efforts and method development are required to understand their true impact on N cycling.

### **Sediment $\text{N}_2$ fixation**

Recent studies have revealed that N-fixation—the reduction of atmospheric  $\text{N}_2$  to organic N—can play a larger role in coastal systems than previously thought (Fulweiler et al. 2007; Andersson et al. 2014; Newell et al. 2016). Cyanobacteria have long been recognized as making a major contribution to N-fixation in illuminated aquatic sediments, however it is becoming increasingly clear that other sediment microorganisms can make an important contribution to benthic  $\text{N}_2$ -fixation (Severin and Stal 2010; Andersson et al. 2014). A particularly interesting aspect of research is subsurface N fixation by sulfate-reducing bacteria (SRB) which has been described in association with seagrass roots (Welsh et al. 1996; Nielsen et al. 2001) and more recently found to be important in bioturbated (Bertics et al. 2010) and seasonally hypoxic sediments (Bertics et al. 2013).

Although the physiological and ecological purpose for subsurface  $\text{N}_2$  fixation is poorly understood, the presence of N-fixing organisms in sediments could potentially affect the results of conventional whole-core IPT experiments as  $^{15}\text{N}$ - $\text{N}_2$  formation rates by anammox and denitrification may be underestimated in the presence of simultaneous  $\text{N}_2$  consumption. However, assuming  $\text{N}_2$ -fixation will fix  $^{15}\text{N}$  and  $^{14}\text{N}$  according to the  $^{15}\text{N}/^{14}\text{N}$  ratio in  $\text{N}_2$ ,  $\text{N}_2$ -fixation will not change the overall  $^{15}\text{N}/^{14}\text{N}$  ratio of  $\text{N}_2$ , and thus only affects the IPT method by lowering the overall  $\text{N}_2$  concentration. In many sediments,  $\text{N}_2$  consumption is likely to be much lower compared to the overall concentration of  $\text{N}_2$  and may only be of limited importance in most sediments. Regardless of this, very little is known about the contribution of this process and the presence of  $\text{N}_2$ -fixing organisms at depth requires specific supplementary  $^{15}\text{N}$ - $\text{N}_2$  fixation experiments. Considering the ubiquity of SRB and of sediments colonized by faunal and macrophyte assemblages,  $\text{N}_2$ -fixation by SRB may make an as-yet unknown and significant contribution to sediment N budgets in some locations.

### **Conclusions: using the whole-core IPT today**

Our understanding of benthic N cycling has progressed rapidly over the last 25 yr in terms of new processes, their co-occurrence and methods with which we can resolve individual process rates. The aim of this review is to highlight that, as with any method, the application of the IPT should not be careless and experimental design and consideration of artifacts is critical to its appropriate use and data interpretation. In light of our modern understanding of the N cycle it is only appropriate that the use of traditional methods should correspondingly progress.

Given the challenges associated with the application of the whole-core IPT caused by co-occurring processes, and other environmental factors, we encourage those employing the IPT in future studies to be vigilant with experimental design and the measurement of rates of  $\text{NO}_3^-$ -reducing processes. We suggest that given the uncertainties associated with the distribution of sediments where multiple N cycling processes co-occur, it is important to assess the presence of anammox and DNRA when applying the whole-core IPT. The complex environments which arise when primary producers or macrofauna are present require additional information regarding in situ biodiversity, patchiness, and experimental type (intact vs. reconstructed cores, light–dark regimes). In situ oxygen concentrations should be reproduced and maintained during the holding and equilibration period of whole-core experiments. More needs to be known about the contribution of  $\text{NO}_3^-$ -storing and  $\text{N}_2$ -fixing organisms to overall N turnover and their potential interference with the IPT should also be taken into account when conducting these experiments. Further studies taking advective flow in permeable sediments into account are required to adequately assess their contribution to N cycling.

In reality, the biological, chemical, and physical issues highlighted here commonly occur together (e.g., Fig. 2). For

example, the mats of  $\text{NO}_3^-$ -storing *Beggiatoa* are well known to occur at high densities under low oxygen concentrations in overlying water. While the IPT has revealed that microphytobenthos may depress N-loss via denitrification under N-limiting conditions (Bartoli et al. 2003; Risgaard-Petersen 2003; Sundbäck et al. 2004), the co-occurrence of macrofauna (and their associated microbial assemblages) in the same cores may facilitate N-availability from deep or scarcely available sedimentary pools. Settled cells of  $\text{N}_2$ -fixing cyanobacteria can be abundant in the surface sediment layer in shallow systems (e.g., the Curonian lagoon); however, their potential contribution to benthic  $\text{N}_2$ -fixation, resuspension, and decay remains poorly understood. Going further, the potential of microphytobenthos (e.g., benthic diatoms, Kamp et al. 2011) to respire stored (or added)  $\text{NO}_3^-$  in anoxic sediment layers further complicates the biogeochemical scenario—a factor which may vary in relation to exposure to dark-light shifts in shallow illuminated sediments.

There is no doubt that the future application of the whole-core IPT will continue to advance to meet the needs of new developments in N cycling research. It is the responsibility of those using the IPT to ensure that preventable methodological caveats are avoided and that the data collected is meaningful in relation to the environments investigated. In this way, more valuable experimental data can be produced to support more accurate models and predictions of future nutrient scenarios as well as providing well-founded information for policy makers.

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None declared.

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