

2021

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### Recommended Citation

Ray, Nicholas E.; Hancock, Boze; Brush, Mark J.; Colden, Allison; (...); Kellogg, M. Lisa; and et al, A review of how we assess denitrification in oyster habitats and proposed guidelines for future studies (2021). *Limnology and Oceanography Methods*, 19(10), 714-731.  
doi: [10.1002/lom3.10456](https://doi.org/10.1002/lom3.10456)





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## A review of how we assess denitrification in oyster habitats and proposed guidelines for future studies

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### Abstract

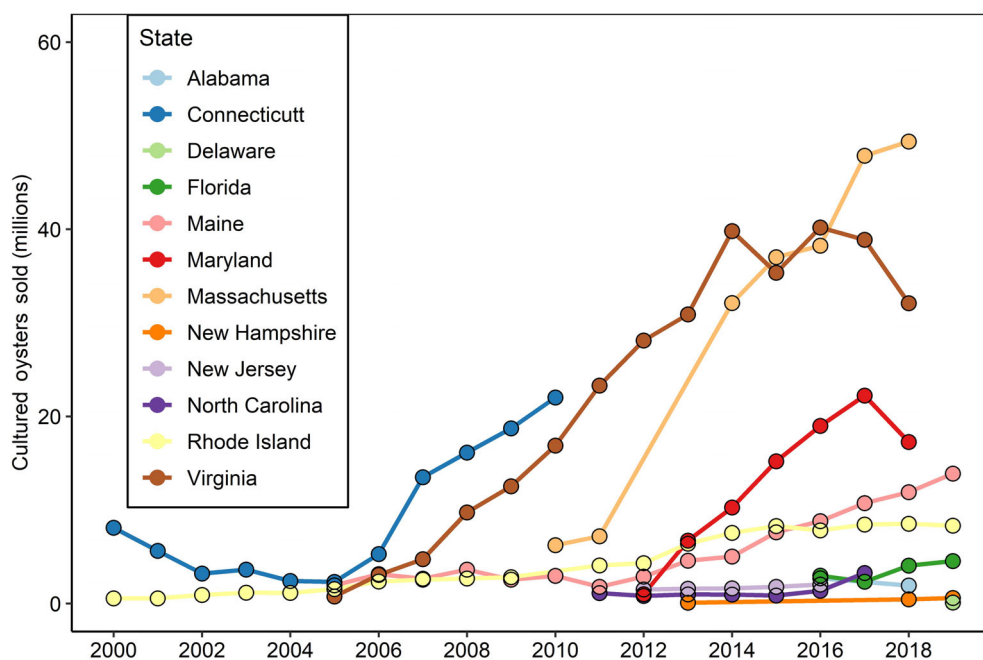
Excess nitrogen (N) loading and resulting eutrophication plague coastal ecosystems globally. Much work is being done to remove N before it enters coastal receiving waters, yet these efforts are not enough. Novel techniques to remove N from within the coastal ecosystem are now being explored. One of these techniques involves using oysters and their habitats to remove N via denitrification. There is substantial interest in incorporating oyster-mediated enhancement of benthic denitrification into N management plans and trading schemes. Measuring denitrification, however, is expensive and time consuming. For large-scale adoption of oyster-mediated denitrification into nutrient management plans, we need an accurate model that can be applied across ecosystems. Despite significant effort to measure and report rates of denitrification in oyster habitats, we are unable to create such a model, due to methodological differences between studies, incomplete data reporting, and inconsistent measurements of environmental variables that may be used to predict denitrification. To make a model that can predict denitrification in oyster habitats a reality, a common sampling and reporting scheme is needed across studies. Here, we provide relevant background on how oysters may stimulate denitrification, and the importance of oyster-mediated denitrification in remediating excess N loading to coastal systems. We then summarize methods commonly used to measure denitrification in oyster habitats, discuss the importance of various environmental variables that may be useful for predicting denitrification, and present a set of guidelines for measuring denitrification in oyster habitats, allowing development of models to support incorporation of oyster-mediated denitrification into future policy decisions.

The past 20 yr have seen rapid development of the oyster aquaculture industry (Fig. 1) and substantial oyster reef restoration (Berssoza Hernández et al. 2018; Duarte et al. 2020). In

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this same time period, there has been a large research effort to quantify whether—and by how much—oysters enhance benthic denitrification (the conversion of biologically available dissolved nitrogen [N] to inert di-nitrogen [N<sub>2</sub>] gas). Early suggestions of the potential for enhanced denitrification in oyster habitats piqued the interest of ecologists, coastal managers, and oyster farmers who imagined a new N mitigation tool that could be included in nutrient management plans (Newell et al. 2002, 2005; Porter et al. 2004; Rose et al. 2021). Nearly



**Fig. 1.** Annual sales of oysters (*Crassostrea virginica*) raised in aquaculture from Atlantic and Gulf Coasts of the United States. Data were aggregated from publicly available reports and Sea Grant news articles. States where no data were available, and those that include oysters harvested from the wild and grown out on a lease were excluded. Data sources and assumptions available in the Figshare Repository here: <http://10.6084/m9.figshare.12933677>.

two decades of work (Table 1; Fig. 2) has led to two important realizations: denitrification in oyster habitats is generally higher than in bare sediments and can vary tremendously over space and time (Ray and Fulweiler 2021) and, although the enhancement of denitrification associated with oyster aquaculture can sometimes be similar to that associated with reefs, it is inappropriate to extrapolate rates measured on reefs to aquaculture, or vice versa. Despite this significant progress and related successes toward including oyster-mediated denitrification in predictive ecosystem models, we are not yet able to accurately predict rates of N removal via denitrification processes in oyster habitats writ large—that is, we are still measuring rates and collecting environmental data in an effort to build broadly applicable predictive relationships. Before we can easily and widely include oyster-mediated denitrification in N management plans and credit programs, we must better understand the ecological drivers and predictive variables of denitrification in these habitats.

Our inability to predict denitrification in oyster habitats stems, in large part, from the fact that denitrification is notoriously difficult to measure and predict in general (Groffman et al. 2006; Fennel et al. 2009). In oyster habitats, this problem is compounded by the variety of methods used (Table 1), different environmental predictor variables measured, and inconsistent data reporting across studies. In this review, we provide relevant background on changes in eastern oyster (*Crassostrea virginica*) populations and describe how these oysters can enhance benthic denitrification. Further, we provide guidelines

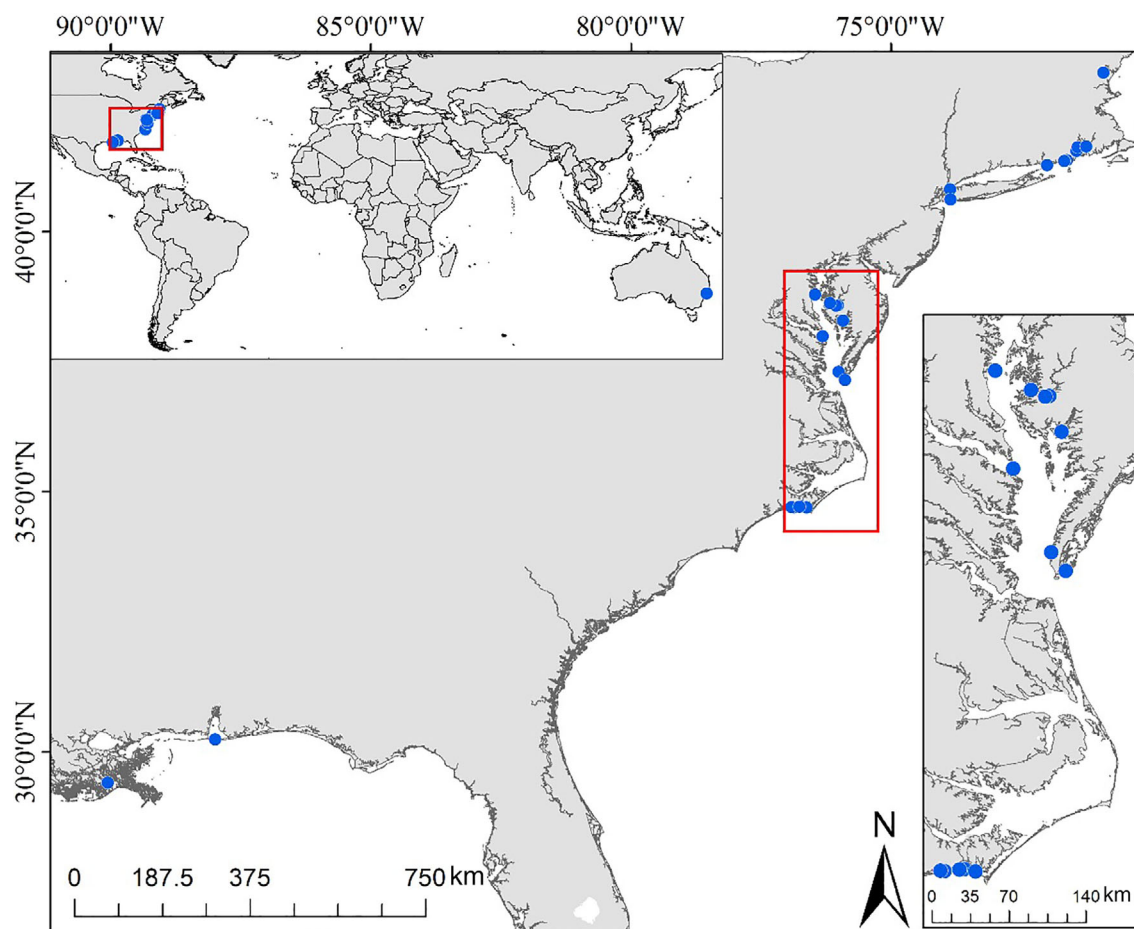
for how to best measure denitrification and relevant environmental parameters to facilitate the development of system-level nitrogen removal for use in nutrient management. We focus on *C. virginica* because most of the research on denitrification via oysters has been done on this species, however our suggestions can be applied to other reef-forming species and other bivalves raised in aquaculture. We summarize measurement considerations and methods previously used for quantifying denitrification in oyster habitats and discuss the importance of measuring and reporting common environmental variables that might help us predict denitrification. Finally, we propose a tiered set of guidelines for measuring and reporting rates of denitrification in oyster habitats to help guide future studies. We hope this review provides a useful reference for those designing and implementing studies that measure denitrification in oyster habitats and facilitates improved and widespread data collection and distribution for more rapid development of predictive models that can be used in management planning and N credit trading programs.

### Changing oyster populations

Over 85% of the historic extent of oyster reefs has been lost globally (Beck et al. 2011). Following a peak in harvest in the late 1800s and early 1900s, continued fishing combined with disease and environmental changes have reduced the aerial extent of oyster reefs to < 10% of their historic levels in most fisheries and < 1% in many systems (Beck et al. 2011; Zu Ermgassen et al. 2012; Gillies et al. 2018). The loss of reefs to

**Table 1.** Published studies that report rates of benthic denitrification in oyster habitats. All studies investigated *Crassostrea virginica* on the Atlantic and Gulf Coasts of the United States, except for Eriker et al. (2017), which was conducted using *Saccostrea glomerata* on the southeast coast of Australia. See Fig. 2 for study locations.

Study	Habitat		Incubation method				Denitrification method			In situ or ex situ?				
	Reef	Aquaculture	Other	Batch	Flow-through	Slurry	Other	N <sub>2</sub> /Ar	IPT	Acetylene block	Molecular	In situ	Ex situ (benthic)	Ex situ (whole reef)
Porter et al. (2004)														
Holyoke (2008)														
Piehler and Smyth (2011)														
Higgins et al. (2013)														
Kellogg et al. (2013)														
Smyth et al. (2013a)														
Smyth et al. (2013b)														
Hoellein and Zarnoch (2014)														
Hassett (2015)														
Hoellein et al. (2015)														
Mortazavi et al. (2015)														
Smyth et al. (2015)														
Testa et al. (2015)														
Humphries et al. (2016)														
Smyth et al. (2016)														
Arfken et al. (2017)														
Eriker et al. (2017)														
Vieillard (2017)														
Jackson et al. (2018)														
Lunstrum et al. (2018)														
Onorevole et al. (2018)														
Smyth et al. (2018)														
Jackson (2019) ch. 3														
Jackson (2019) ch. 4														
Westbrook et al. (2019)														
Ray et al. (2020)														
Ray and Fulweiler (2020)														
Ayvazian et al. In review														
Kellogg et al. In review														



**Fig. 2.** Map of location of studies that have measured sediment denitrification in oyster habitats. See Table 1 for a list of studies and citations.

support wild harvest has promoted the development of a robust and expanding aquaculture sector for the eastern oyster (Fig. 1).

In 2016, oyster aquaculture made up 31% of global mollusk culture production, and like other bivalve aquaculture, oyster aquaculture production has rapidly increased, with 26% growth in production between 2010 and 2016 (FAO 2018). Production of oysters in the United States has increased even more rapidly, with 37% growth between 2011 and 2016 (FAO 2018; Fig. 1). Oyster aquaculture uses either “extensive” method, where oyster shell or spat are planted directly on the substratum and later harvested using the same techniques utilized in wild oyster fisheries, or “intensive” method, where bags, cages, or trays are used to hold oysters in the water column (Forrest et al. 2009). A major difference between these approaches relevant to measuring and/or prediction denitrification rates is the spatial separation of oysters from benthic habitats in intensive culture systems.

In addition to potential food production, recognition of the value of ecosystem services provided by oyster reefs has led to a rapid increase in reef restoration (Grabowski et al. 2012; Berzoza Hernández et al. 2018; Gillies et al. 2018). Generally, oyster reef restoration involves placing oyster shell or other

materials on the seabed to serve as substrate on top of which oysters settle naturally or are planted as part of restoration activities (Fitzsimons et al. 2019). Native oyster shell is often used as the substrate, but in areas where the availability of shell is limited other materials have been used to supplement reef restoration activities including stone, mixed shell (e.g., whelk, clam, or scallop), crushed concrete, and engineered structures (e.g., Reef Balls, oyster “castles”). Often the goal of these activities is not only to increase the areal extent of reefs and biomass of oysters, but also to enhance ecosystem services once provided by healthy oyster reefs (Coen and Luckenbach 2000; Grabowski and Peterson 2007; zu Ermgassen et al. 2016). Since 2000, nearly 1500 oyster reef restoration projects have been reported globally (Duarte et al. 2020), with many other restoration projects not reported.

#### Oysters, nitrogen cycling, and denitrification

Oysters are filter-feeders that can selectively ingest or reject suspended particulates as they feed. Waste products from ingested particles are excreted as feces, and rejected particulates are wrapped in mucus and ejected as pseudofeces (Cranford et al. 2011). Both feces and pseudofeces (collectively





sediment denitrification rates are correlated with water column chlorophyll *a* (Fennel et al. 2009; Fulweiler and Heiss 2014), sediment oxygen demand (Seitzinger and Giblin 1996), sediment OM content (Caffrey et al. 1993), and  $\text{NO}_3^-$  availability (Seitzinger and Nixon 1985). These same system characteristics are likely important in driving benthic denitrification in oyster habitats, although we do not yet have the data to statistically demonstrate these relationships in oyster habitats across time and space.

Over the past decade, a growing body of research documents the wide variety of oyster reef and oyster aquaculture components that contribute to the enhanced  $\text{N}_2$  flux observed from these systems, including the sediments within the footprint of the reef (Piehler and Smyth 2011), the oyster reef structure (Jackson et al. 2018) which contains oysters that can harbor denitrifying bacteria on their gut, gill, and shells (Smyth et al. 2013a; Arfken et al. 2017; Ray et al. 2019), and the associated macrofaunal community (Kellogg et al. 2013). The shells of oysters, living or dead, can serve as habitat for nitrifying bacteria (Arfken et al. 2017), as can the shells of other organisms living on the reef (Welsh and Castadelli 2004). The increase in surface area for these bacteria and the abundant supply of precursors for nitrification likely explain the net positive fluxes of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in measured oyster reef fluxes when oysters and the associated community are included in incubation chambers compared to those that only included oyster reef sediments (Kellogg et al. 2013; Jackson et al. 2018). The oyster digestive system provides habitat for denitrifying microbes (King et al. 2012; Arfken et al. 2017), and denitrification and net denitrification associated with oysters themselves proceeds at significant rates (Smyth et al. 2013a; Caffrey et al. 2016; Arfken et al. 2017; Jackson et al. 2018; Ray et al. 2019).

In habitats with sufficient light available for photosynthesis, the hard substrate provided by oyster habitats provide attachment sites for benthic microalgae and macroalgae. By competing for DIN required for denitrification, these algae have the potential to decrease rates of denitrification (Gonzalez et al. 2013; Bourke et al. 2014).

Many of the species found in high abundance and/or biomass in oyster habitats are also filter feeders. In Chesapeake Bay, hooked mussel (*Ishadium recurvum*) biomass can exceed oyster biomass on restored oyster reefs (Gedan et al. 2014; Lipcius and Burke 2018). The sea squirt *Molgula manhattensis* can also exceed oyster biomass on reefs and is one of the most problematic fouling organisms for oyster aquaculturists (Carman et al. 2010). In addition to increasing filter feeder biomass and the associated mass of biodeposits, this increased diversity of filter feeders will increase the range of particle sizes that can be filtered in oyster habitats (Gedan et al. 2014). It is also possible that greater macrofaunal abundance will reduce total OM loading to benthic habitats by increasing the total demand for food. The relationship between non-oyster macrofauna and net denitrification in oyster habitats is still unclear and is an open research question.

Especially for oyster aquaculture, the location of the oysters within the water column and any structures they are associated with will play a role in determining filtration capacity, biodeposition, loading of OM to benthic habitats, and ultimately rates of net denitrification and N removal. Because oysters grown in extensive aquaculture settings grow in conditions very similar to natural and restored oyster reefs (e.g., grown directly on substratum with no cages or other gear, little handling/disturbance prior to harvest), many of the same factors that influence oyster reef net denitrification rates will influence net denitrification rates on extensive aquaculture farms. On restored oyster reefs and extensive aquaculture, the height and topography can determine the degree to which oyster biodeposits are retained within the vicinity of the reef (Lenihan 1999; Colden et al. 2016). In contrast, intensive aquaculture practices place oysters in bags or cages that can be placed in any location within the water column ranging from the substratum to just beneath the sediment surface. Mesh cages or bags that slow the water as it passes through reduce the flux of seston to the oysters inside. In addition to reducing the supply of seston, aquaculture gear that is held off the bottom physically separates oysters from benthic habitats. The farther the gear is held from the benthos, the greater the chance that biodeposits will be transported outside of the main aquaculture site (Testa et al. 2015), particularly in areas with high flow rates. Thus, even though oyster biomass or density may be similar, net denitrification rates may not be between one aquaculture site that differ in the time of gear used, the location of the oysters within the water column, or local hydrodynamic regime.

### Linking observations and modeling

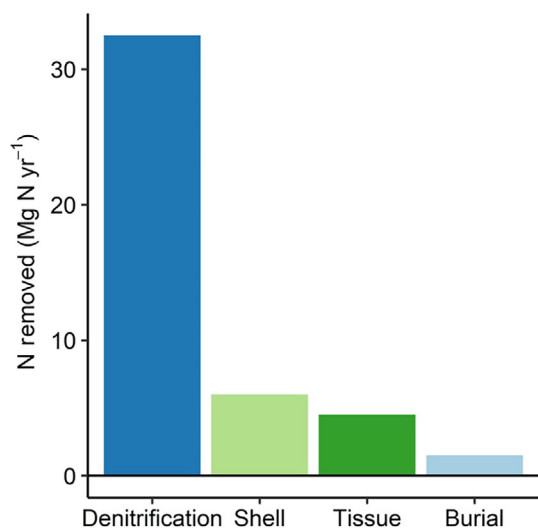
Numerical simulation models provide important tools for scaling site-specific measurements of denitrification to the ecosystem level, computing bivalve N and particulate removal and recycling, and placing N removals into context with external loads. Models thus have the potential to be useful tools for computing the effectiveness of shellfish restoration and aquaculture as best management practices (BMPs) for nutrient mitigation strategies, including in the context of nutrient trading. Bivalve—and specifically oyster—modeling has been an active area of research over the last three decades, with most efforts focused on modeling filtration and growth in the context of understanding population dynamics (Hofman et al. 1992; Powell et al. 1992), estimating ecosystem carrying capacity for aquaculture (Filgueira and Grant 2009; Filgueira et al. 2010; Ibarra et al. 2014), and simulating enhanced ecosystem service provision such as filtration, water quality improvements, and increased fish production associated with bivalve restoration (Cerco and Noel 2007; Fulford et al. 2007; Ehrich and Harris 2015; zu Ermgassen et al. 2016). Applications to compute nutrient removal have occurred more recently (i.e., last decade), with most efforts focused on sequestration in tissue



and shell in aquaculture settings (Ferreira et al. 2007, 2008; Rose et al. 2014).

A few efforts, however, have focused on modeling N removal via net denitrification in aquaculture or reef settings. Testa et al. (2015) combined measured rates of biodeposition with a mechanistic benthic-flux model to compute net denitrification and N burial under a floating oyster farm, and found that approximately 90% of biodeposits were removed from the local system by currents rather than denitrified on site. Cerco (2015) applied the Chesapeake Bay Program Eutrophication Model to compute nutrient removal by oyster reefs ( $2.8 \times 10^5 \text{ m}^2$  area) in the Great Wicomico River subestuary of Chesapeake Bay, and found that oysters removed over 30% of the watershed N loading (oysters removed  $6.2 \text{ t N yr}^{-1}$  of an estimated N load of  $18.6 \text{ t N yr}^{-1}$ ). Kellogg et al. (2018) modeled the tributary-wide oyster restoration in the Harris Creek subestuary of Chesapeake Bay, and found that oysters are now removing over 200% of watershed N inputs ( $\sim 21.4 \times 10^6 \text{ g N yr}^{-1}$ ), and 4.6% of total N inputs to the creek ( $\sim 967 \times 10^6 \text{ g N yr}^{-1}$ ); oyster-mediated net denitrification was the dominant loss term, accounting for 73% of total N removal (Fig. 4).

These last two studies highlight the importance of oyster-mediated denitrification as an N removal mechanism, and the importance of including the process in consideration of oysters as a BMP. As with all models, however, efforts to simulate oyster-mediated N removals will only be as good as the data available to constrain them, and the limited availability of benthic flux data has often been a limiting factor in verification of models of coastal systems. In Harris Creek, Kellogg et al. (2018) explored two approaches to constrain predicted rates of denitrification using the available observational data.



**Fig. 4.** Modeled annual nitrogen removal by oysters via different pathways in the Harris Creek subestuary of the Chesapeake Bay. Modified from Kellogg et al. (2018).

The initial model used data from nearby sites to compute net denitrification as a function of oyster biomass and temperature; however, the lack of data to constrain the temperature function introduced a high degree of uncertainty in modeled estimates. Following 3 yr in which extensive net denitrification measurements were collected in Harris Creek, this function was replaced with a site-specific, empirical relationship between net denitrification and other measured variables (i.e., biomass of oysters and associated fauna). While these functions rooted model predictions directly in the observations, the variability in observed net denitrification rates continued to introduce a high degree of uncertainty in model predictions. Given this continued uncertainty, and the desire to find more generally applicable formulations for use in models across multiple systems, there is a critical need for continued collection of net denitrification data together with associated predictor variables remains.

#### Denitrification as an ecosystem service

The prevalence of N-driven coastal eutrophication (Malone and Newton 2020) has resulted in public investment in numerous land-based BMPs that decrease N inputs (Lintern et al. 2020). However, for mitigation of N inputs *within* tidal waters, tidal wetland restoration, macrophyte transplant and restoration, oyster reef restoration, and oyster aquaculture are among the only opportunities for N removal. While N-trading programs remain limited, recent trades for N assimilated in oyster tissue have been valued between US\$50 and \$400 lb N yr<sup>-1</sup> (Rose et al. 2021). If these same rates were applied to N removal by oyster-mediated net denitrification in Harris Creek, the total value of N removal would range from 3.5 to 28.7 million US dollars per year from N removal alone. The value of oyster reefs and aquaculture is even higher when considering other services they provide, such as enhancement of commercial fisheries and reduction of storm surge. Coastal N trading programs are currently limited (Rose et al. 2021), but high-quality measurements of net denitrification rates are fundamental for the inclusion of oyster-mediated net denitrification and other in situ processes in future plans and for attribution of environmental and economic value. Prior to certification of enhanced denitrification associated with oysters as a BMP, sufficient data using scientifically defensible assessment approaches are critical.

#### Review of methods used to measure denitrification

Many methods have been used to measure rates of benthic denitrification and net denitrification. These methods have been described and compared thoroughly elsewhere (Seitzinger et al. 1993; Cornwell et al. 1999; Groffman et al. 2006), so here we will briefly describe those that have been used to estimate rates of denitrification and net denitrification in oyster habitats. We do not discuss estimates of net denitrification using stoichiometric or mass balance

approaches as these are imprecise and have large error terms associated with their estimates (Cornwell et al. 1999; Groffman et al. 2006). Instead, we focus on direct measurements of denitrification and net denitrification. Each of these methods requires several considerations, and all have benefits and drawbacks. Some are more appropriate and useful for developing predictive models of N removal by denitrification in oyster habitats than others.

### General considerations for sample collection

Most commonly used denitrification measurement techniques require enclosing a sample of the benthic habitat and water in a chamber and collecting samples for analysis of  $N_2$  concentration at regular intervals for a given period of time. These are often referred to as incubations. Over the course of an incubation the change in  $N_2$  concentrations over time is determined using a regression approach (for batch, or static cores; list of studies using this technique in Table 1), or by the difference in  $N_2$  concentrations of the inflow and outflow samples (for flow-through incubations; list of studies using this technique in Table 1). Regardless of incubation approach, the concentration change is prorated for the volume and cross-sectional area of the core and generally reported in  $\mu\text{mol } N_2\text{-N m}^{-2} \text{ h}^{-1}$ .

When choosing an incubation chamber, it is important to consider size (including surface area to water column volume ratio) and how closely it will mimic the natural environment. Chamber size affects both the simplicity of doing incubations (e.g., smaller devices are easier to operate) and the ability to include a representative part of the ecosystem. With small cores, multiple simultaneous incubations allow assessment of more sites for a given amount of effort. In more complex environments such as oyster reefs, small cores generally cannot include important biotic components, and may miss “hot-spots” of denitrification (Groffman et al. 2009). Large devices can be used to conduct in situ or ex situ incubations and have the advantage of capturing a more realistic benthic community. However, their large size incurs additional cost and logistical challenges. In regard to measurements of net denitrification in oyster habitats, smaller chambers are more frequently used for laboratory incubations of sediment, with cross-sectional area of 0.003–0.008  $\text{m}^2$  and 1–2 L of overlying water common (Smyth et al. 2013b, 2015, 2018; Ray and Fulweiler 2020). Chambers with greater cross sectional area 0.11–0.13  $\text{m}^2$  cross sectional area and volume (35–50 L) are often used in ex situ and in situ incubations, and can more easily contain sections of reef (Kellogg et al. 2013; Humphries et al. 2016).

Replicating in situ flow can be particularly challenging as we often do not know the physical conditions of a given system, and most systems are constantly changing due to winds and tides. Further, to capture gas fluxes, as when measuring denitrification, you must use a gas-tight chamber which by its very design shuts the system off from the environment. To

address this, the overlying water in both small and large chambers is mixed using stir bars or impellers, and/or internal or external water is pumped through the system. Stirring approximates flow in the environment by thoroughly mixing the water column, preventing stratification, and establishing an appropriate benthic boundary thickness (Boynton et al. 1981; Glud et al. 2007). Different speeds of rotation can result in different rates of biogeochemical exchange (Coley 2003), and thus the stirring rate is an important parameter to report. The ideal stirring rate might vary between studies, and site-specific conditions should be considered while also ensuring the stirring rate is high enough to ensure mixing, without being so high as to artificially alter benthic biogeochemical processes. Many studies report a stirring rate of  $\sim 40$  rpm and thus we recommend this rate as a starting point with the actual rate adjusted as needed based on sampling site and sample characteristics.

Dissolved oxygen ( $O_2$ ) concentrations in the incubation chamber are an important consideration, and care should be taken to ensure cores do not become hypoxic ( $O_2$  concentration  $\leq 2.0$   $\text{mg } O_2 \text{ L}^{-1}$ ) or anoxic. In static incubations, the timing of sample collection is often spaced to allow for a total drop in  $O_2$  concentration of at least 2.0  $\text{mg } O_2 \text{ L}^{-1}$  without allowing the water overlying the sample in the core to become hypoxic. In flow-through incubation chambers, the flow rate of water through the incubation chamber can be modulated to maintain continuously oxygenated overlying water.

It is also important to maintain the vertical architecture of the benthic sample to quantify a realistic denitrification rate. Slurry methods involve the collection of benthic samples which are then mixed and moved to containers, often before the addition of an isotope label (see section 2.3 below). This approach allows for a large number of samples to be analyzed and can describe the potential denitrification of benthic habitats as well as changes to rates of different pathways, but does not accurately describe denitrification, or net N removal in an unaltered ecosystem. Slurries should be avoided when attempting to quantify net denitrification in oyster habitats, although they may provide important information on potential rates of individual N pathways (Gilbert et al. 1997; Hoellein and Zarnoch 2014).

Finally, for assessments of net denitrification in habitats with significant quantities of micro or macroalgae, the presence of light and resultant photosynthetic processes can alter benthic fluxes. In this case, differences between light and dark fluxes may be substantial. For example, in a laboratory biodeposition study, sediments exposed to sufficient light supported a benthic microalgal/cyanobacterial community that consumed inorganic nitrogen released from the organic material and even fixed nitrogen (Newell et al. 2002). On the other hand, some studies have reported no change in oyster habitat denitrification between light and dark (Holyoke 2008; Sisson et al. 2011).

### When should oysters be included in chambers?

It is vital that samples collected for incubation and denitrification measurements account for the complex biological, physical, and chemical interactions that occur in oyster habitats. In the absence of a method for measuring net denitrification in situ, care should be taken to ensure that the portion of the oyster habitat that is encapsulated and removed from the system is as representative as possible of the oyster habitat of interest. For oyster reefs, studies of net denitrification have shown that the presence of oyster clumps and oyster shells in samples significantly enhances denitrification rates (Kellogg et al. 2013; Jackson et al. 2018). Thus, the most accurate net denitrification estimates will likely be derived from samples that incorporate oysters, oyster reef sediments, and associated macrofauna. As noted above, the size and type of incubation chamber used should take into consideration of the type and complexity of oyster habitat, with more complex habitats necessitating the use of larger chambers to capture the many components of the habitat that may contribute to net  $N_2$  production. In extensive oyster aquaculture, oysters are in direct contact with sediments, similar to natural and restored reef sites. In these cases, measurement of net denitrification can be achieved using the same techniques as those used for oyster reefs and oysters should be included within incubation chambers.

In oyster aquaculture settings where oysters are physically separated from benthic habitats (i.e., intensive aquaculture), although potentially desirable, it is rarely practical to include both the underlying habitat and aquaculture gear in the incubation. Because net denitrification associated with the oysters themselves has been documented (Ray et al. 2019), not including oysters in incubations may underestimate N removal at the oyster farm scale. Net denitrification measurements at aquaculture sites can be complicated by the size and variety of the aquaculture gear commonly used, the potential for biodeposits to be exported from the aquaculture site (as in floating aquaculture), and farm management practices. To date, no methods have been developed to measure net denitrification rates associated with whole cages of oysters, though this may be estimated using rates of net denitrification for individual oysters, which appears to be similar across systems (Smyth et al. 2013a; Caffrey et al. 2016; Arfken et al. 2017; Ray et al. 2019). Instead, measurements have focused on sediments adjacent to bottom gear or under/adjacent to gear held above the bottom and thus capture only the benthic component of the system. In cases where gear is suspended off the bottom, the further the gear is from the sediment surface and the greater the current speeds, the more likely it becomes that oyster biodeposits will be exported out of the aquaculture site. For example, Testa et al. (2015) modeled significant export of biodeposits from an oyster farm. Methods have yet to be developed to allow direct assessment of the fate of biodeposits exported from an aquaculture farm and all estimates have relied heavily on modeling. Farm practices such as harvest and

removal of fouling organisms can also be expected to alter deposition of OM. Similarly, some farms have considerable human activity, likely changing the properties benthic habitats and their rates of net denitrification (Lunstrum et al. 2018).

### Analytical methods

Rates of benthic denitrification and net denitrification are measured as the change in analyte concentration over time for a known area of substratum. Several approaches have been used in oyster habitats (Table 1): measurement of the change in  $N_2/Ar$  and calculation of change in  $N_2$  concentration ( $N_2/Ar$  method; Kana et al. 1994), the addition of an isotope label ( $^{15}N$ ) and measurement of the labeled product (IPT method; Nielsen 1992), and inhibition of  $N_2O$  reduction using acetylene ( $C_2H_2$ ) inhibition and subsequent measurement of  $N_2O$  (acetylene inhibition technique; Sørensen 1978). Microbial community analysis may also provide some insight to denitrification processes in oyster habitats.

The  $N_2/Ar$  method relies upon a mass spectrometer equipped with a semipermeable borosilicate inlet (i.e., a membrane inlet mass spectrometer, or MIMS) through which dissolved gases can pass, allowing the operator to collect water samples and analyze these at a high precision (0.05%) with no headspace equilibration step. Ratios of dissolved  $N_2$  and Ar are quantified, and then  $N_2$  concentrations are back calculated using the theoretical concentration of Ar at the sample temperature and salinity compared to the measured concentrations, under the assumption that as a biologically inert gas, measured Ar concentrations should not vary from their theoretical value at the given experimental conditions. The  $N_2/Ar$  method also considers net  $N_2$  fluxes in unamended water, providing a rate of benthic  $N_2$  exchange most representative of natural conditions. There are two primary concerns when using the  $N_2/Ar$  method. First, any bubble formation in the incubation chamber may lead to erroneous values. For example, oxygen bubbles formed during photosynthesis can lead to a false nitrogen fixation signal. For this reason, if light/dark measurements are of interest, we recommend conducting the dark incubation first and then the light (Eyre et al. 2013). If bubble formation occurs during a light incubation, we recommend not using the  $N_2$  data associated with it. Second, as dissolved oxygen concentrations decrease in the incubation chamber, the potential production of nitrosonium ( $NO^+$ ) during MIMS analysis may result in the appearance of higher  $N_2$  production, and thus a higher net denitrification value (Eyre et al. 2002). However, this concern has largely been laid to rest, as even when it does occur the change is within the precision of the instrument (Kana et al. 2004). Further, if you are concerned, you can run a simple test on the MIMS to determine if  $NO^+$  is being produced, and/or add an in-line furnace to remove oxygen during sample analysis (Kana et al. 2004). Despite these limitations, we think the  $N_2/Ar$  method is the most promising approach for quantifying N removal from oyster habitats via stimulated net denitrification because it is a

direct measurement that does not require any amendments, or assumptions that can be easily violated. It also has the added benefit of possible simultaneous measurement of  $O_2$  fluxes if  $O_2$  is not removed as part of the analysis (Eyre et al. 2002; Lunstrum and Aoki 2016).

The IPT method requires labeling the DIN pool in water overlying benthic sample with  $^{15}NO_3^-$  or  $^{15}NH_4^+$ , then follows the movement of the tracer to the  $N_2$  pool. While IPT (and more recent modified versions of the IPT) can provide useful information about mechanistic processes contributing to net denitrification, it may be difficult to properly meet all of the methodological assumptions (Robertson et al. 2019) when conducting isotope tracer measurements in oyster habitats. For example, the method is sensitive to the amount of label added and as a result the DIN concentrations may not be environmentally relevant in systems with low background DIN. In systems with low background DIN, the addition of  $^{15}NO_3^-$  or  $^{15}NH_4^+$  N provides a measure of denitrification that may not be reflective of the actual rate. IPT is also sensitive to the activity of other processes. Rates of denitrification calculated using IPT are typically lower than measures of net denitrification measured using the  $N_2/Ar$  method from the same system (Eyre et al. 2002). For example, Higgins et al. (2013) reported lower mean rates of denitrification, anammox, and  $N_2$  production in sediments beneath oyster aquaculture relative to bare sediment when using isotope tracer methods, but higher net  $N_2$  fluxes beneath oysters when using the  $N_2/Ar$  approach. Hoellein et al. (2015) measured greater net  $N_2$  flux than the sum of denitrification measured following  $^{15}NO_3^-$  and  $^{15}NH_4^+$  addition in both sediments adjacent to oyster reefs and those from nearby bare sediments. In the third study we could locate that used both the  $N_2/Ar$  and IPT method, Hassett (2015) found no difference in net  $N_2$  flux between sediments in restored oyster reefs relative to bare sediments, but slightly higher rates of denitrification in oyster habitats using IPT. A recent meta-analysis demonstrated a stronger effect of oysters on net denitrification when the  $N_2/Ar$  method was used compared to the effect of oysters on benthic denitrification measured via IPT (Ray and Fulweiler 2021). The difference in reported rates between these two approaches may be directly related to violations of IPT assumptions. For example, oyster reefs and aquaculture provide habitat for other macrofauna and burrowing organisms, a challenge when applying the IPT. The benefits and drawbacks of IPT relative to  $N_2/Ar$  have been thoroughly documented elsewhere (Eyre et al. 2002; Kana et al. 2004; Groffman et al. 2006; Robertson et al. 2019), but in the context of quantifying N removal via net denitrification processes in oyster habitats for use in N management plans and trading schemes,  $N_2/Ar$  is more accurate and simpler to use. Isotope tracer methods can be used in addition to  $N_2/Ar$  measurements to quantify rates of individual N-cycling processes and the relative contribution of those processes to the net  $N_2$  flux, helping to identify underlying biogeochemical mechanisms.

The acetylene inhibition technique requires the addition of  $C_2H_2$  to incubation chambers, inhibiting the  $N_2O$  reductase enzyme and preventing the final step of denitrification ( $N_2O \rightarrow N_2$ ; Fig. 3), and allowing for estimation of denitrification by measuring the change in concentration of the  $N_2O$  pool via gas chromatography. The technique has several drawbacks that result in an underestimation of denitrification: (1)  $C_2H_2$  inhibition may not be complete, particularly at low  $[NO_3^-]$ , (2)  $C_2H_2$  may not penetrate benthic samples efficiently, further reducing full inhibition of  $N_2O$  reductase, and (3) nitrification may also be blocked by  $C_2H_2$  through inhibition of  $NH_4^+$  mono-oxygenases, stopping coupled nitrification : denitrification. Further, the addition of  $C_2H_2$  appears to immediately alter the active microbial community (Fulweiler et al. 2015). In the context of net N removal, the acetylene inhibition technique does not consider nitrogen fixation or anammox, as neither of these processes that contribute to the net  $N_2$  flux contain an  $N_2O$  intermediate. The acetylene inhibition technique should not be used when quantifying rates of denitrification in oyster habitats.

The composition of microbial communities and expression of their N metabolism genes can complement measurements of denitrification in oyster ecosystems (Lindemann et al. 2016; Damashek and Francis 2018). In particular, the genes *nirS* (nitrite reductase), *norB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase) are responsible for denitrification and have been measured in parallel with  $^{15}N$  or  $N_2 : Ar$  rates (Braker and Tiedje 2003; Halm et al. 2009). DNA and RNA-based methods determine the abundance and expression of these genes, respectively. Recent developments have made it much cheaper and easier to conduct molecular analyses, and while these data provide important information, it is difficult to link microbial community with actual biogeochemical flux rates, and quantification of nitrogen genes is not a direct prediction of gas production rates (Bowen et al. 2014). Until relationships between microbial community and microbial gene expression are better understood, molecular techniques alone cannot be used to quantify or predict rates of net denitrification in oyster habitats.

### Predictor variables to model denitrification

Denitrification is challenging and expensive to measure. Often, it is easier and more cost effective to measure environmental variables that may in turn provide a useful method for accurately predicting rates of net denitrification in oyster habitats. In the following section, we describe the most relevant variables, and how they can influence net denitrification in oyster habitats.

### Site history and management

While it is expected that oyster habitats will have higher rates of net denitrification than areas without oysters, not all oyster habitats are the same. For example, a study of intertidal

oyster reefs in North Carolina found that reefs located adjacent to salt marshes or seagrasses do not enhance net denitrification relative to bare sediments, while oyster reefs located on mudflats, away from adjacent habitats, can have elevated rates of net denitrification (Smyth et al. 2015). This difference may be the result of the oyster habitat being functionally redundant when positioned in an area where there is already a high supply of OM (Westbrook et al. 2019), or where carbon does not limit denitrification (Hoellein and Zarnoch 2014).

The age of the oyster habitat may also affect net denitrification. As the oyster habitat ages, changes in sediment properties like bulk density, grain size, and benthic nutrient pools can occur, leading to differences in net denitrification (Chambers et al. 2018; Onorevole et al. 2018). The relationship between restoration, or aquaculture age, and net denitrification is not universal and other studies have found no relationship (Ahn and Peralta 2012; Ray et al. 2020). One contributing factor may be oyster density. The expectation is that as the reef ages, oyster density will increase (if not harvested). An increase in the number of oysters would lead to higher rates of net denitrification because there is more OM, higher surface area, and more complex structure while also increasing N availability. However, this relationship continues to be only marginally significant and is likely dependent on other aspects of the site (Smyth et al. 2015; Jackson et al. 2018; Onorevole et al. 2018). Total OM loading and net denitrification may decrease if the oyster density becomes great enough that food availability becomes limiting, or sulfides buildup and stop nitrification (Newell 2004).

Oysters can live in both subtidal and intertidal conditions. Continuously submerged environments have different oxygen conditions and light levels than intertidal environments, which can impact N cycling processes (Joye and Paerl 1993; Piehler and Smyth 2011). In addition to position in the tidal frame, the orientation of the oyster habitat within the estuary should be considered when measuring net denitrification. It is common to find oyster reefs parallel or perpendicular to the shoreline, but oyster reefs can also form as round mounds, and the orientation of the reef likely influences locations of high benthic net denitrification due to interactions between the water column and benthic habitats that modify local flow regimes (Lenihan 1999; Colden et al. 2016). Oyster farmers may also orient racks and bags in a specific way to maximize particulate food supply to oysters.

### Oyster size, density, and distribution

The primary feature of an oyster habitat is the population of oysters. Measured net denitrification rates should only be extrapolated to oyster habitats with similar characteristics to that of the sample used for net denitrification measurements. Data collected should allow estimation of oyster biomass per unit area. The most common method of assessing oyster biomass at the habitat scale is to measure the length and biomass of a subsample of oysters to develop a length to biomass

relationship (Higgins et al. 2011). Biomass for the remainder of the oyster population is then estimated based on oyster length. The biomass of oysters per unit area is important because, although filtration capacity per unit area is expected to initially increase linearly with increasing oyster biomass per unit area, the slope of the relationship may be expected to decrease as filtration capacity starts to exceed seston supply.

In addition to limiting extrapolations of measured data to habitats with similar oyster biomass, extrapolations should also be limited to oyster habitats with similar distribution of biomass and, in the case of aquaculture, similar gear type. On natural reefs, oysters are not evenly distributed and habitats with a similar mean biomass per unit area may have very different oyster distributions. Because of this, seston depletion may be an issue in habitats with high-density patches of oysters but not in a habitat with a more evenly distributed population. In addition to altering the filtration capacity, the distribution of oysters on the substratum will interact with the local hydrodynamic regime to determine whether biodeposits are retained within the oyster habitat or exported from the system. Dense patches of structural elements like oysters commonly create flow patterns that slow current speeds adjacent to the substratum.

### Water quality parameters and location within parameter gradients

The effects of oysters on net denitrification will likely vary with water chemistry. Oysters supply OM to benthic habitats, excrete  $\text{NH}_4^+$ , and consume oxygen, but do not influence salinity, or temperature—important regulators of denitrification through either direct control (Seitzinger et al. 2006), or in part due to the effects that these variables have on food supply, oyster growth, filtration, biodeposition, or nitrification occurring on the oyster shell (Carmichael et al. 2012; Kellogg et al. 2013; Smyth et al. 2013a). However, the relationships between temperature and salinity on net denitrification in oyster habitats are equivocal.

Estuaries—where salinity requirements are suitable for oysters—are generally characterized as having increasing salinity and decreasing  $\text{NO}_3^-$  nearer to the ocean. The supply of  $\text{NO}_3^-$  is controlled by watershed inputs and nitrification. In areas with a well-mixed water column, short residence time, and oxic benthic habitats, nitrification is the likely source of  $\text{NO}_3^-$ . Under this scenario where denitrification is coupled to nitrification, variation in oxygen would lead to difference in net denitrification from oyster habitat (Newell et al. 2002). In higher salinity areas, where denitrification is coupled to nitrification, there is evidence that oyster biodeposits prime benthic habitats for enhanced denitrification when  $\text{NO}_3^-$  is available (Smyth et al. 2015).  $\text{NO}_3^-$  can also stimulate denitrification associated with the shell and living oyster, although the effect is greater when live oysters are present (Caffrey et al. 2016). At OM-rich sites where  $\text{NO}_3^-$  loading is high, oysters may not have the same effect and may not promote enhanced net

denitrification relative to reference sites (Higgins et al. 2013; Westbrook et al. 2019) because denitrification in oyster habitats is not regulated solely by water column  $\text{NO}_3^-$  but also by C (Hoellein and Zarnoch 2014). Thus, the location of the oyster habitat within the estuary likely influences its impact on net denitrification in general and the ability of the oysters to enhance net denitrification in particular.

### Benthic habitat characteristics

Several benthic habitat physical and chemical characteristics may be useful when attempting to predict rates of net denitrification. Physically, rates of DIN exchange between oxic and anoxic zones within benthic habitats regulate rates of denitrification by controlling rates of nitrification, and transport of dissolved  $\text{NO}_3^-$  from the water column. Factors that enhance diffusion between oxic and anoxic zones can facilitate higher rates of coupled nitrification–denitrification, such as greater pore space volume (Cook et al. 2006). The presence of oyster shell must also be taken into consideration, as large shell pieces, live oysters, and sections of reef conglomerate can impede diffusion.

The C : N ratio of benthic habitats provides a rough predictor of whether N may be removed through denitrification (low C : N) or recycled via DNRA (high C : N; Burgin and Hamilton 2007; Smyth et al. 2013b; Hardison et al. 2015; Lunstrum et al. 2018). Similarly, the total amount of OM in the benthic habitats controls the availability of material needed for heterotrophic denitrification, as well as remineralization of  $\text{NH}_4^+$  and subsequent nitrification. There is evidence that net denitrification in some oyster habitats can be predicted based on benthic OM concentrations (Hoellein and Zarnoch 2014; Smyth et al. 2016, 2018), though areas with very high OM loading may experience buildup of hydrogen sulfide ( $\text{H}_2\text{S}$ ), an inhibitor of both nitrification and denitrification (Joye and Hollibough 1995).

### Other fluxes

The generation of  $\text{N}_2$  efflux data from benthic habitats is best put in both a scientific and quality control context by the simultaneous assessment of other relevant biogeochemical flux parameters. There is an expectation that high rates of oyster community respiration are required for high rates of denitrification; the remineralization of labile OM and release of inorganic N may be stoichiometric to the fluxes of dissolved inorganic C or  $\text{O}_2$ . For example, benthic  $\text{O}_2$  demand may serve as a proxy for denitrification, and several studies have reported a significant relationship between  $\text{O}_2$  consumption and  $\text{N}_2$  release from sediments in oyster habitats (Higgins et al. 2013; Smyth et al. 2013b, 2016; Humphries et al. 2016; Lunstrum et al. 2018), though the relationship is less clear in other cases (Kellogg et al. 2013; Hoellein et al. 2015; Ray et al. 2020). This relationship may not always exist for a variety of reasons. For example, buildup of  $\text{H}_2\text{S}$  can inhibit of nitrification and denitrification, or low availability of  $\text{NO}_3^-$  in the water column

may lead to alternative redox pathways dominating. Nevertheless,  $\text{O}_2$  consumption is a key, and relatively easy, variable to measure and we propose that with more data we may see a pattern emerge linking  $\text{O}_2$  consumption and denitrification in specific types of oyster habitats.

Recent advances in measuring oyster reef oxygen fluxes using underwater eddy covariance appear to hold promise for accurately measuring oyster habitat fluxes in the field (Volaric et al. 2018, 2020). This method involves in situ measurement without disturbance of the site, and provides instantaneous measurements over time, possibly incorporating both hotspots and hot moments of net denitrification (Groffman et al. 2009). Comparison of oxygen fluxes using eddy correlation and tray incubations of intact reef section have yielded similar estimates, suggesting that incubation of reef sections produces reasonable approximations of actual biogeochemical fluxes (Kellogg et al. In review). Directly measuring  $\text{N}_2$  fluxes using eddy covariance is difficult and has not yet been done in oyster habitats. However, if robust relationships between benthic  $\text{O}_2$  consumption and  $\text{N}_2$  production in oyster habitats exist across space and time, eddy covariance measurements of  $\text{O}_2$  flux may be a useful technique for rapid quantification of denitrification in oyster habitats. Continued development of underwater mass spectrometers and reduced cost of aquatic eddy covariance instruments will likely make this method useful in the future.

### Guidelines for measuring denitrification in oyster habitats

To improve interstudy comparisons and modeling efforts, and to inform future net denitrification measurements and data reporting, we developed a set of guidelines for variables to measure and report alongside rates of denitrification (Table 2). We split this set of variables into three tiers based on their importance. Tier 1 is the minimum set of measurements required for producing useful net denitrification data that should be taken and reported in all net denitrification studies. Tier 2 includes additional or more detailed measurements and observations that help contextualize tier 1 measurements. Tier 3 measurements provide additional information needed for improved mechanistic understanding of underlying processes regulating net denitrification and will help move the field forward.

These guidelines were developed during day-long discussion between the authors at the “Synthesizing the Nitrogen Removal Capacity of Oyster Aquaculture via Denitrification” Workshop hosted at the Frederick S. Pardee Center for the Study of the Longer-Range Future at Boston University in September 2019. Further discussion took place over the subsequent months until manuscript submission. We assigned variables to be measured to different tiers based on their potential for influencing rates of net denitrification, and the relative ease and affordability required for measurement.

**Table 2.** Guidelines for variables to measure and report when quantifying benthic denitrification in oyster habitats. These measurements are divided into three tiers: required (tier 1), recommended (tier 2), and desired (tier 3). The sampling scheme/plan is in bold. We recommend following the protocols described in *Protocol handbook for NICE – nitrogen cycling in estuaries* (Dalsgaard et al. 2000).

	<b>Tier 1</b>	<b>Tier 2</b>	<b>Tier 3</b>
Site description	<b>Visually identify upon sample collection</b> Length of time oysters present at site Oyster size distribution Oyster density (ind. m <sup>-2</sup> ) Diploid vs. triploid (aquaculture) Type of aquaculture practice/reef habitat Frequency of husbandry activity (aquaculture) Description of benthic community Tidal range Depth Orientation of reef to flow Adjacent habitat	<b>Collect once during core/incubation chamber collection</b> Oyster biomass (g dry tissue weight m <sup>-2</sup> ) Handheld flow-meter (m s <sup>-1</sup> )	<b>Measure immediately following incubation</b> Biomass of benthic organisms (g m <sup>-2</sup> ) Species ID/count of benthic organisms <b>Continuous measurement via data logger</b> Shear stress Acoustic Doppler of current speed and direction
Water quality	<b>Collect duplicate samples of each</b> Temperature Salinity Dissolved NO <sub>3</sub> <sup>-</sup> concentration (μmol L <sup>-1</sup> ) Dissolved NH <sub>4</sub> <sup>+</sup> concentration (μmol L <sup>-1</sup> ) Dissolved O <sub>2</sub> concentration (μmol L <sup>-1</sup> ) Chlorophyll <i>a</i> concentration (μg L <sup>-1</sup> )	<b>Water column profile</b> Dissolved NO <sub>3</sub> <sup>-</sup> concentration (μmol L <sup>-1</sup> ) Dissolved NH <sub>4</sub> <sup>+</sup> concentration (μmol L <sup>-1</sup> ) Dissolved PO <sub>4</sub> <sup>3-</sup> concentration (μmol L <sup>-1</sup> ) Secchi/turbidity/TSS Light penetration to benthic habitat (P.A.R.)	<b>Continuous measurement via data logger</b> Water column pH Water column O <sub>2</sub> concentration (μmol L <sup>-1</sup> ) Chlorophyll <i>a</i> fluorescence
Benthic habitat description	<b>Visually identify upon sample collection</b> Distance from aquaculture to substratum Macroalgae % coverage Shell presence/absence Shell % coverage	<b>Measure for each core or chamber after incubation</b> Grain size Benthic chlorophyll <i>a</i> (μg m <sup>-2</sup> ) % organic matter	<b>Measure for each core or chamber after incubation</b> Macroalgae biomass Porosity (0–1 cm; %) Density (0–1 cm; g cm <sup>-3</sup> ) C:N (0–1 cm; mol/mol) Apparent redox depth Pore-water H <sub>2</sub> S (4 cm; μmol L <sup>-1</sup> ) Pore-water nutrients (4 cm; μmol L <sup>-1</sup> )
Other fluxes (report all as: μmol m <sup>-2</sup> h <sup>-1</sup> )	<b>Collect at beginning and end of denitrification incubation (flux calculated as [end]-[start])</b> Benthic O <sub>2</sub> demand NH <sub>4</sub> <sup>+</sup> flux NO <sub>x</sub> flux	<b>Collect throughout incubation (flux calculated by regression)</b> PO <sub>4</sub> <sup>3-</sup> flux Benthic O <sub>2</sub> demand NH <sub>4</sub> <sup>+</sup> flux NO <sub>x</sub> flux	<b>Collect throughout incubation (flux calculated by regression)</b> Dissolved inorganic carbon flux Nitrous oxide flux Additional incubations using isotope tracers to identify mechanisms of net flux

Reporting these variables along with rates of net denitrification will generate a large dataset of potential predictor variables that can be used to develop accurate predictive models of N removal via net denitrification in oyster habitats at a specific point in time based on site-specific characteristics that are cheaper and easier to quantify than net denitrification.

In addition to collecting data according to these guidelines, accurate reporting and dissemination of this data is critical for it to be useful in model development. We strongly recommend that all future studies of net denitrification in oyster habitats publish the full dataset generated during the study period, with sufficient metadata for ease of interpretation by future users (Gil et al. 2016).



Interest in improving our ability to predict N removal via oyster-mediated net denitrification is in part linked with nutrient management frameworks and regulations intended to maximize N removal in coastal ecosystems anthropogenically enriched in N. The application of net denitrification in oyster habitats to these nutrient removal efforts is currently limited by availability of data across broad geographic areas, in varied oyster habitats, and through time. If data collection improves and modeling studies can reliably predict N removal, aquaculturists and restoration practitioners can leverage this ecosystem service to spur further investment in oyster recovery. Implementing the data collection and reporting recommendations we make here will provide the information necessary to move this effort forward.

In addition to the provided guidelines that will facilitate development of models for predicting net denitrification in oyster habitats across time and space, the three most important points of this paper are summarized below:

- Studies seeking to quantify rates of net denitrification in oyster habitats in the context of N removal should use the N<sub>2</sub>/Ar method to analyze samples collected from batch or flow-through incubation chambers.
- When possible, oysters and associated reef fauna should be included in the chamber when measuring net denitrification from oyster reefs and in aquaculture settings where oysters are in contact with the substratum. A thorough description of the oyster habitat, and what parts of the habitat are included in incubation chambers is necessary.
- Reporting of environmental variables associated with individual flux measurements—not just mean and error/deviation—is critical for the development of predictive models. The full dataset of the fluxes and ancillary variables measured should be published on a free to access website (e.g., Dryad or Figshare), with easily interpreted metadata. If raw data from previous studies are readily accessible, those datasets should also be published.

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### Acknowledgments

This manuscript was initially developed during a two-day workshop, “Synthesizing the Nitrogen Removal Capacity of Oyster Aquaculture via Denitrification.” The workshop was funded through a fellowship to R.W.F. from the Frederick S. Pardee Center for the Study of the Longer Range Future at Boston University. R.W.F. was also supported by a grant through RI Sea Grant. M.J.B. was supported by NOAA grant no. NA18NOS4780177. We want to thank the Pardee Center for their support. We give special thanks to Cynthia Barakatt, John Prandato, and Tony Janetos. This paper is contribution #6037 of the University of Maryland Center for Environmental Science and contribution number 4036 from Virginia Institute of Marine Science.

Submitted 07 October 2020

Revised 28 April 2021

Accepted 23 July 2021

Associate editor: Michael Beman