Flavobacteria buffer nitrous oxide emissions from partial denitrifiers in coastal sediments

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Abstract

Nearly one-fifth of global emissions of the potent greenhouse gas nitrous oxide (N_2O) originate from the ocean, particularly from nutrient-polluted coastal regions. Permeable (sandy) sediments, which cover half of the continental shelf worldwide, are potential sources of N_2O due to increasing nutrient inputs from urbanization and agriculture. Yet, the microbial processes determining N_2O emissions in these dynamic and unique ecosystems remain understudied. Here, we combined environmental measurements, bacterial cultivation, and genomic analyses to understand the microbes and processes controlling N_2O cycling in permeable sediments from Port Phillip Bay (Australia). We established a genomic resource comprising 249 metagenome-assembled genomes and 95 new isolate genomes. Genome-based metabolic reconstructions and culture-based gas measurements revealed diverse bacteria in these sediments produce N_2O through incomplete denitrification pathways. However, these bacteria co-occurred with highly abundant clade II N_2O-reducing bacteria from the *Flavobacteriaceae* family. Kinetic profiling revealed both clade II *nosZ* flavobacterial isolates and whole sand communities exhibit a low affinity for N_2O, contrary to previous reports that clade II N_2O reducers

generally have a high substrate affinity. This indicates adaptation to the high
residence times of N_2O within production and consumption zones in the sands.
Collectively, these N_2O reducers remove most N_2O produced in permeable
sediments, supporting lower-than-expected coastal emissions predicted by
biogeochemical models. We conclude that permeable sediments host specialised
microbial communities that mitigate N_2O emissions and buffer marine nitrogen
cycling amid rising nutrient pollution.

41 Introduction

Nitrous oxide (N_2O) is a long-lived atmospheric trace gas contributing to
stratospheric ozone depletion and climate change [1]. With a warming potential
approximately 300 times higher than that of carbon dioxide on a 100-year time scale,
N_2O is the third largest contributor (6.4%) to total greenhouse gas emissions after
CO_2 and CH_4 [2,3]. Up to 57% of the total N_2O emissions come from natural sources,
in which marine ecosystems contribute to nearly half of such emissions at 2-7 Tg N
year^{-1} [4]. Estuarine and coastal zones function as a natural biofilter, removing
excess nutrients at the interface between land and ocean [5]. However, increasing
anthropogenic activities, such as nutrient loading, aquaculture and fertiliser use, are
inputting nitrogen to these ecosystems, which in turn results in widespread
eutrophication and exacerbates N_2O emissions [4,6–9]. N_2O emissions from coastal
environments remain insufficiently understood and poorly quantified in global N_2O
budgets due to complex N_2O production pathways and high spatiotemporal variability
[7,10,11].

Permeable sediments (i.e. sands and gravels) dominate coastal regions, covering
nearly half of the continental shelf worldwide [12]. These sediments are subject to
highly turbulent hydrodynamic conditions that vary across space and time, as
opposed to fine-grained impermeable (cohesive) sediments [13–15]. Advective pore-
water flows drive continuous exchange of dissolved solids and gases, suspended
particles and microorganisms between the sediment and overlying water column,
making redox conditions in the sediments highly variable over short distances and
timescales [13–15]. With microbial abundance of ~10^9 cells cm^{-3} sediments [16],

sands affect N_2O emissions in coastal regions due to enhanced microbial nitrogen
cycling linked to anthropogenic nitrogen inputs. Nitrification and denitrification are the
two main microbial processes that control the N_2O budget in aquatic environments
[17,18]. In well-oxygenated waters, N_2O can be released by ammonia-oxidizing
bacteria (AOB) and archaea (AOA) as a byproduct of ammonia oxidation to nitrite
(NO_2^-) (i.e. the first step of nitrification) [9,18]. By contrast, N_2O is produced as an
intermediate during denitrification of nitrate (NO_3^-) or NO_2^- under oxygen-depleted
conditions [17,19]. Many denitrifiers lack the genetic capacity for N_2O reduction,
resulting in N_2O released as the final product instead of inert dinitrogen gas (N_2)
[20,21]. Furthermore, abiotic processes, such as (photo)-chemodenitrification, can
also contribute to the production of N_2O in marine waters and sediments [22,23].
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Despite several processes leading to N_2O production, the major biological sink of this
gas is its reduction to dinitrogen gas (N_2) by microorganisms expressing the
periplasmic, copper-containing enzyme N_2O reductase (NosZ) [24,25]. Phylogenetic
analyses resolve the enzyme into clade I (NosZI), clade II (NosZII) and the recently
defined clade III (NosZIII) [26–28]. NosZI is often associated with complete
denitrifiers. In contrast, NosZII is found in much more diverse microbial taxa that
typically lack key denitrification enzymes (i.e. NirK and NirS), suggesting N_2O serves
as an electron acceptor even for non-denitrifiers [27–29]; for example, some non-
denitrifying bacteria encoding the NosZII, including the acidophilic bacterium
Desulfosporosinus nitrosoreducens [30] and methanotrophs *Methylocella tundrae*
and *Methylacidiphilum caldifontis* [31], reduce N_2O to conserve energy through
anaerobic respiration, while others use it to dispose excess electrons during
transient anoxia (Park et al., 2017). Beyond these two clades, a recent study
discovered a widespread and active clade III lactonase-type NosZ that is divergent in
sequence but similar in structure to NosZI and NosZII [26]. These findings have
expanded our understanding on the diversity of N_2O reducers and provide
opportunities for mitigating N_2O emissions.

Given the global extent of permeable sediments, any imbalances between N_2O
production and consumption can greatly impact marine N_2O budgets. While there is
evidence that these sediments are a potential source of N_2O [32–36], recent
hydrodynamic-biogeochemical models suggest that their contribution to coastal N_2O

emissions are relatively minor [37], highlighting uncertainties in the N_2O budget of
these sediments. Furthermore, current understanding of the microbial basis of N_2O
cycling within permeable sediments is largely limited to community surveys [33], with
little functional and ecophysiological characterization. In this study, we addressed
these knowledge gaps by investigating the functional diversity and capacity of N_2O-
cycling microorganisms in sandy sediments from moderate to high nutrient-impacted
beaches in Port Phillip Bay, Australia. To do so, we integrated bacterial cultivation,
metagenomic analyses, and biogeochemical assays to provide a newly compiled
genome collection of microorganisms inhabiting permeable sediments and assess
their role in N_2O cycling. Altogether, we demonstrate that diverse N_2O producers and
consumers are present and active in coastal sediments, with their tight coupling
buffering the N_2O emissions from these sediments. These findings provide microbial
evidence supporting hydrodynamic models [37] that coastal N_2O emissions may be
overestimated and reveal new mediators of nitrogen and greenhouse gas cycling in
marine ecosystems.

Results

Microbial communities in permeable sediments rapidly consume high concentrations of N_2O

To determine whether N_2O production occurs *in situ,* we measured N_2O in pore
waters of subtidal sandy sediments at St. Kilda and Werribee beaches located in
Port Phillip Bay, a nitrogen-limited marine embayment in Victoria, Australia. Trace
amounts of N_2O (~ 0.0055 μM or 84.6% saturation) were detected in some samples;
however, N_2O was generally below the limit of detection (0.004 μM dissolved gas
concentration) in porewater across most sampling depths and times (**Fig. S1, Table
S9**). The absence of detectable *in situ* N_2O concentrations is surprising given the
denitrification activity previously reported at the same studied sites [38,39]. This may
reflect three possibilities (or a combination of): (1) denitrification steps are tightly
coupled, producing minimal N_2O and efficiently reducing it to N_2, (2) N_2O is only
sporadically produced when NO_3^- is transported into anoxic zones or (3) non-
denitrifying bacteria with clade II N_2O reductases rapidly consume N_2O produced by
other community members.

We performed a series of slurry experiments to determine whether microbial
communities within permeable sediments consume N_2O at a range of
concentrations. Under anoxic conditions, microbial communities in the slurries
rapidly consumed 0.8 μM and 108 μM N_2O with the average rates of 0.65 and 49.8
nmol h^{-1} g^{-1} wet sediment, respectively (**Figure 1A**). Additionally, Werribee slurries
showed significantly higher N_2O consumption rates (P = 0.015 - Student's t-test) than
St. Kilda slurries at both concentrations, potentially reflecting long-term enrichment of
denitrifying populations in these sediments at the vicinity of a wastewater treatment
plant. Kinetic experiments were used to determine whether beach sand microbial
communities are adapted to high or low N_2O concentrations. N_2O reduction by the
sedimentary communities followed Michaelis-Menten-type kinetics (R^2 = 0.99 for St
Kilda, 0.93 for Werribee) with apparent half-saturation constants [$K_{m(app)}$] of 43 and
55 μM N_2O respectively and maximum reduction rates [$V_{max\ (app)}$] of 19.6 and 46.3
nmol N_2O h^{-1} g^{-1} wet sediment (**Fig. 1B**). The [$K_{m(app)}$] values observed in this study
are unusually high for marine communities, for example being orders of magnitude
higher than those observed in anoxic estuarine water in Chesapeake Bay, US (0.647
μM N_2O) [25] or those in the oxic-anoxic interface from the eastern Pacific oxygen
minimum zone (0.334 μM N_2O) [40]. This suggests that microbial communities within
sands are exposed to much higher N_2O fluxes compared to the water column,
presumably due to nutrient enrichment in these ecosystems. Furthermore, the
[$V_{max(app)}$] for N_2O reduction in these sediments were one order of magnitude higher
than previously reported [$V_{max(app)}$] values for denitrification in permeable sediment in
Port Phillip Bay (0.5 – 5 nmol g^{-1} h^{-1}) [39], implying the capacity for N_2O consumption
exceeded N_2O production in these sediments. Altogether, our results demonstrate
that while permeable sediments could potentially produce elevated N_2O levels due to
nutrient enrichment, rapid microbial consumption helps prevent N_2O accumulation
and emissions. Thus, these sediments may function as hotspots of N_2O turnover,
acting as a dynamic sink despite high N_2O exposure.

Permeable sediments host diverse N_2O producers and consumers

We used genome-resolved metagenomics to better understand the microbial and enzymatic mediators of N_2O cycling in permeable sediments. We sequenced and performed *de novo* co-assembly and binning of metagenomes from the Werribee and St. Kilda beaches, yielding 53 medium-quality and 17 high-quality metagenome-assembled genomes (MAGs). We additionally reanalysed 179 MAGs we previously obtained from St. Kilda permeable sediments [38,41] (**Table S3**). Overall, the resultant 249 MAGs span 19 phyla and 74 orders including the most abundant taxa previously reported for permeable sediment (e.g. *Woeseiaceae*, *Rhodobacteraceae* and *Flavobacteriaceae*) (**Fig. 2B, Table S3**). We then annotated the short reads, derived MAGs and unbinned contigs to determine the functional capacities and diversity of the permeable sediment microbiome.

In line with previous studies [38,41], microbial communities within permeable sediments have high capacity for denitrification. Specifically, many community members are predicted to mediate NO_3^- reduction via Nar and Nap (St. Kilda: 44%; Werribee: 58%), NO_2^- reduction via NirS and NirK (St. Kilda: 27%; Werribee: 54%) and nitric oxide (NO) reduction to N_2O via Nor (St. Kilda: 22%; Werribee: 24%) (**Fig. 2A; Table S2**). Metabolic reconstruction showed that 76 MAGs (30%) encode partial denitrification pathways, i.e. MAGs missing one or more denitrifying reductases (**Table S3**). To minimize artefacts of missing genes due to fragmented genomes, we performed more focused analyses of 64 high-quality MAGs (>90% genome completeness) to more reliably infer partial denitrification pathways. Among these, 16 MAGs were found to possess one or more denitrying reductases but lack the N_2O reductases. MAGs associated with *Arenicellaceae* (Gammaproteobacteria)*, JABDKV01* (Gammaproteobacteria) and *Pyrinomonadaceae* (Acidobacteriota) families encoded Nor for N_2O production, suggesting these microorganisms are N_2O producers within the sand communities. Compared to denitrification, fewer community members mediate nitrification via Amo and Nxr (St. Kilda: 0.4%; Werribee: 8%) or dissimilatory nitrate reduction to ammonium (DNRA via Nrf – St. Kilda: 13%; Werribee: 9%) (**Fig. 2A; Table S2**).

Consistent with our activity measurements, analysis of metagenomic short reads
shows that many bacteria encode genes to reduce N_2O via NosZ (St. Kilda: 23.3%;
Werribee: 29.1%; **Fig. 2A**). In detail, bacteria encoding the NosZII are more
abundant than the NosZI (19.6% and 20.2% versus 3.5% and 8.7% of St. Kilda and
Werribee communities, respectively) while only a minor fraction of the community
(St. Kilda: 0.01%; Werribee: 0.8%) encoded the NosZIII (**Fig. 2A**). Consistently, at
the MAG level, only five MAGs associated with Pseudomonadota (n = 4) and
Verrucomicrobiota (n = 1) encode NosZI whereas 61 MAGs spanning 8 phyla
encode the NosZII (**Fig. 2B**). Of NosZII containing MAGs, 49 (80%) were
Bacteroidota and 57% of these were *Flavobacteriaceae* family. Furthermore, the
majority of NosZII harbouring MAGs (79%) contained only *nosZ* but not any other
denitrifying genes, suggesting these microbial groups only consume, but do not
produce N_2O.

To gain further insight into the phylogenetic diversity of the N_2O-reducers, we
constructed a phylogenetic tree of NosZ including 647 amino sequences retrieved
from 36 isolate genomes, 24 MAGs, 245 unbinned contigs, and 342 reference
sequences (**Fig. 3**). Consistent with previous findings [27,29,42,43], our phylogenetic
tree revealed that the NosZI is predominantly restricted to the Pseudomonadota,
whereas the NosZII showed broader taxonomic distribution across several phyla
(**Fig. 3**). Most NosZII sequences affiliate with Bacteroidota, specifically the family
Flavobacteriaceae (**Fig. 3**). NosZII sequences were also affiliated with other phyla,
including Gemmatimonadota, Acidobacteriota and Chloroflexota. While the NosZIII
was reported to be associated with diverse taxa [26], this gene was not detected in
the isolate genomes or assembled contigs in our dataset, suggesting low prevalence
of clade III N_2O reducers in the studied sandy sediments.

An isolate collection of marine microbes uncovers novel
mediators of N_2O cycling

To complement the metagenomic insights into the potential mediators of N_2O
cycling, we conducted an isolation campaign to obtain high-quality genomes, confirm
metabolic activities, and gain ecophysiological insights into key N_2O cycling

microorganisms. To the best of our knowledge, this cultivation effort generated the largest collection of permeable sediment isolates (n = 95) to date. These spanned four main phyla, including Bacillota (n = 6), Actinobacteriota (n = 4), Bacteroidota (n = 36), Alphaproteobacteria (n = 29) and Gammaproteobacteria (n = 20). These isolates represent a considerable fraction of the community's phylogenetic diversity observed from metagenome-resolved data, including dominant lineages. Based on GTDB, our isolate collection includes two novel bacterial genera and 57 novel bacterial species (**Fig. 2B, Table S4**).

Among these isolates, 12 *Alphaproteobacteria* (*Rhodobacteraceae*, n = 5) and 7 *Gammaproteobacteria* encode a complete set of denitrifying genes, including clade I *nosZ*, indicating capacity to completely reduce NO_3^- to N_2. Another 12 proteobacterial isolates encode denitrification genes except *nosZ*, suggesting they produce N_2O through partial denitrifications. The partial denitrification phenotype was experimentally validated in two isolates, an *Antarctobacter* sp. and *Sedimentitalea* sp. of the *Rhodobacteraceae* family (Alphaproteobacteria) (**Fig. 4**). The *Antarctobacter* sp. has the genomic capacity to mediate stepwise NO_3^- reduction to N_2O ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$) via Nar, NirK, and Nor, while *Sedimentitalea* sp. is predicted to only mediate NO_2^- reduction to N_2O ($NO_2^- \rightarrow NO \rightarrow N_2O$) via NirS and Nor (**Fig. 4B, C**). Consistently, the *Antarctobacter* sp. isolate completely reduced 917 ± 30 µM NO_3^- to N_2O after 24 hours of incubation with the expected 1:1 stoichiometry, after transiently producing small quantities of NO_2^-. As expected, the strain also produced N_2O when supplemented with NO_2^-; however, this was a less efficient process and only ~62% of the initial NO_2^- was reduced to N_2O after 24 hours of incubation, suggesting delayed NO reduction (**Fig. 4B**). Also consistent with the genomic predictions, the *Sedimentitalea* sp. isolate could not consume NO_3^- but did convert NO_2^- to N_2O after an initial lag (**Fig. 4C**). Given both isolates lacked the N_2O reductases, they were unable to perform the last step of denitrification and thereby emitted N_2O as their primary end product.

On the other hand, most isolates showed widespread capacity for N_2O reduction. Clade I *nosZ* genes were identified in the 12 Proteobacteria capable of complete denitrification. In contrast, clade II *nosZ* genes were identified in 20 *Flavobacteriaceae* and three *Cyclobacteriaceae* isolates (Bacteroidota). Among

these, four encoded the clade II *nosZ* without any denitrifying genes*,* while 16 co-
encoded *nosZ* with *nor* for NO reduction to N_2O. Notably, some *Flavobacteria*ceae
isolates (e.g. *Lutibacter* sp., *Algibacter* sp. and *Winogradskyella* sp.) also encoded
nrf for DNRA whereas others (e.g. *Arenibacter algicola* or *Xanthomarina* sp.) also
encoded the *nirK* genes for NO_2^- reduction. Whereas metagenomic predictions
indicate that the most dominant *Flavobacteriaceae* lineages within permeable
sediments are exclusive N_2O reducers, results from microbial isolates suggest that
Flavobacteriaceae members can variably mediate NO_2^- reduction, DNRA along with
N_2O reduction.

Further genomic analyses on these isolates revealed their broader metabolic breadth
(**Table S4**). Most of them encode multiple terminal oxidases for aerobic respiration
while some (e.g. *Lutibacter* sp. or *Oceanimonas* sp.) also encode group 3b or 3d
[NiFe]-hydrogenases, which contribute to fermentative H_2 production within these
sediments as previously reported [14]. This suggests that N_2O-cycling
microorganisms can be extremely versatile switching between aerobic respiration,
anaerobic respiration and fermentation depending on availability of electron
acceptors. Additionally, many isolates encode sulfide-quinone oxidoreductase (Sqr)
for sulfide oxidation, while others encoded carbon monoxide dehydrogenase for
trace gas oxidation or rhodopsin for phototrophy, suggesting permeable sediment
microbes can variably use organic, inorganic and solar energy sources. Altogether,
these culture-based insights support our previous genome-resolved metagenomic
predictions [38,41] that permeable sediment microorganisms are highly metabolically
flexible, possibly as a response to frequent environmental fluctuations.

283 Clade II *nosZ* encoding bacteria from permeable sediments
284 show low affinity towards N_2O

To further determine the physiological characteristics of N_2O reducers in permeable
sediments, we investigated N_2O consumption by four marine bacterial isolates
capable of aerobic organoheterotrophic growth (**Figure 5A**). Two strains, *Olleya*
marilimosa and *Winogradskyella* sp. (both *Flavobacteriaceae*), carry the clade II
nosZ gene but lack key denitrification genes (i.e. *nirS* or *nirK*). In contrast,

Marinobacter adherens (Oleiphilaceae) and *Oceanimonas* sp. (*Aeromonadaceae)*
carry denitrification genes and clade I *nosZ*. These four isolates grew aerobically in
Difco 2216 Marine Broth using yeast extract and peptone as the carbon and energy
sources (**Fig.5 A, D,G and J**) and quickly reduced all headspace N_2O under anoxic
conditions (**Fig. 4B, E, H and K**). Whole-cell kinetic measurements revealed that
N_2O reduction followed Michaelis-Menten-type kinetics in all isolates (**Fig.4C, F, I**
and L). The mean apparent half-saturation constants [$K_{m(app)}$] of clade I *nosZ* strains
M. adherens and *Oceanimonas* sp. were 94.4 µM (95% CI: 47 - 230) and 34.1 µM
(95% CI: 19 - 72). In contrast, [$K_{m(app)}$] values of clade II *nosZ* flavobacterial isolates
O. marilimosa and *Winogradskyella* sp. were considerably lower at 10.1 µM (95% CI:
3.9 – 25) and 11.3 µM (95% CI: 5.6 – 22.3), respectively.

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We subsequently compared the N_2O sink strength of our permeable sediment
isolates with previously characterized N_2O reducing bacteria from other ecosystems
(**Fig. 6**). Clade II *nosZ* flavobacterial isolates in this study had much higher [$K_{m(app)}$]
values than most clade II *nosZ* microorganisms reported to date such as
Anaeromyxobacter dehalogens (1.34 µM) [44] or *Dechloromonas aromatica* RCM
(0.324 µM) [45] (**Fig. 6A)**, indicating a relatively low affinity for N_2O. In addition to
substrate affinity, we also compared their 'catalytic efficiencies' (V_{max}/K_m) to
understand their ability to reduce N_2O under low N_2O concentrations (**Fig. 6B**). While
the maximum reduction rates (V_{max}) of our isolates (except the clade I NosZ
containing *M. adherens*) were comparable to the average of the other bacteria, their
'catalytic efficiencies' (V_{max}/K_m) was low due to their low apparent affinity towards
N_2O (**Figure 6B, Table S5**). These results suggest that, among known N_2O reducing
bacteria, our isolates have relatively weak N_2O sink strength under both high and low
substrate conditions regardless of their clade type.

Discussion

Through integrative approaches, we demonstrate that N_2O cycling in permeable
sediment is mediated by a delicate balance between microbial production and
consumption. Consistent with previous studies [33,38,41], our results show that

permeable sediments host diverse microorganisms with partial denitrification
pathways (i.e. lacking one or more genes required for complete denitrification). Some
of the most abundant members of the community (e.g. *Rhodobacteraceae* members)
possessed either nitrite reductase and/or nitric oxide reductase, but lacked NosZ,
thus producing N_2O as the terminal product of denitrification and contributing to N_2O
emissions. Furthermore, we experimentally confirmed this partial genotype in two
Rhodobacteraceae isolates (i.e. *Antarctobacter* sp. and *Sedimentitalea* sp.), which
produced N_2O from either NO_3^- or NO_2^- without further reducing it to N_2, validating
metagenomic and culture-based genomic predictions. At the ecosystem level,
denitrification is recognized as a collective effort performed by different community
members and partial denitrification can be more common across diverse terrestrial
and aquatic environments [21,51,52]. Partitioning of denitrification steps among
community members could alleviate competition between enzymes acting on the
same substrates and enhance community flexibility and resilience under
environmental disturbances [51]. Furthermore, specialising in a certain step allows
partial denitrifiers to minimise resource allocation to denitrification, enabling broader
metabolic flexibility to adapt to complex, dynamic and oligotrophic ecosystems [52].
Given that permeable sediments are highly dynamic with strong spatiotemporal
variation in electron donor and acceptor availability, partial denitrification is more
prevalent than complete denitrification in these systems. Although we did not directly
measure N_2O production rates in this study, the high $[K_{m(app)}]$ values for N_2O
reduction of St. Kilda and Werribee sediments suggest that the N_2O reducing
community in these sites is adapted to high N_2O concentrations potentially generated
by these partial denitrifiers.

Despite substantial capacity for N_2O production, we provide biogeochemical,
metagenomic and culture-based evidence that most N_2O is consumed by the
sediment community before being emitted into the atmosphere. A diverse consortium
of N_2O-reducing microorganisms carrying the clade I and II *nosZ* gene were
detected, which efficiently reduce N_2O to N_2 before being released into the
atmosphere. These microorganisms span several phyla including Bacteroidota,
Pseudomonadota, Campylobacterota, and Gemmatimonadota, with metabolically
versatile members of the *Flavobacteriaceae* (Bacteroidota) carrying clade II *nosZ*
genes particularly dominant. Our results indicate that most flavobacterial isolate

genomes and MAGs lack canonical genes required for dissimilatory denitrification,
suggesting that these bacteria are unlikely to produce N_2O via the conventional
denitrification pathway. Instead, by expressing the clade II *nosZ* gene, they function
as N_2O respirers that scavenge N_2O generated by other microorganisms. Moreover,
their N_2O reducing capacity, together with the ability to use diverse energy sources,
electron donors, and electron acceptors, reflects their metabolic flexibility that allows
them to thrive under frequent oxic-anoxic shifts of these sediments. In this study,
clade III *nosZ* was scarcely detected in the top sediment layers. Previous work has
suggested that clade III *nosZ* carrying bacteria, such as members of
Desulfobacterota or Nitrospinota phyla, are more likely to inhabit anoxic
environments [26], whereas the highly dynamic redox conditions of permeable
sediments likely limit their occurrence.

In rippled permeable sediments where solutes such as NO_3^- and N_2O are continually
exchanged via advective porewaters [9,53], N_2O-reducing microorganisms might be
expected to rapidly scavenge N_2O with great sink strength. This hypothesis also
stemmed from our observation of few detectable *in situ* N_2O concentrations,
suggesting that N_2O reducers efficiently consume N_2O as it is produced, preventing
its accumulation in the environment. However, contrary to our hypothesis, our
physiological work demonstrates that bacterial isolates from permeable sediments
exhibited relatively weak N_2O-reducing capacity, as reflected in both average
maximum reduction rates (V_{max}) and low catalytic efficiency (V_{max}/K_m) compared with
other known N_2O-reducing bacteria. This unexpected finding raises questions about
the mechanisms regulating N_2O cycling within these sediments. The relatively weak
sink strength of these isolates may likely reflect their adaptation to the physical
characteristics of these sediments. Reactive transport models have suggested that
the majority of denitrification in permeable sediments is driven by NO_3^- fed from
overlying water [54,55]. In these sediments, advective flow and transport dynamics
can restrict interactions between oxic and anoxic zones [54]. As a result, under calm
conditions without physical disturbances such as intense waves, once NO_3^- enters
the reduced reaction zone and is denitrified to N_2O, long residence time would allow
N_2O reducers to fully reduce N_2O to N_2 without the need to invest in high-affinity or
high-rate N_2O reductases. This aligns with previous model predictions showing
minimal N_2O efflux under calm wave conditions which are predominant at these

studied sites, correlating with high Damköhler number, a dimensionless ratio of
reaction to transport rates [37]. Under more hydrodynamically active conditions, more
oxygen is introduced into the sediment, limiting the opportunities for denitrification
and N_2O production to take place. It is only under specific intermediate conditions of
sediment flushing that significant amounts of N_2O escapes the sediment, making it a
rare occurrence (<3% of the time) [37].
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Altogether, our findings suggest a potential cross-feeding interaction between N_2O
producers and non-denitrifying N_2O reducers, which efficiently buffers the emissions
of this potent greenhouse gas in eutrophic permeable sediments. The efficiency of
N_2O removal by clade II N_2O-reducing bacteria may be attributed to the unique
physical characteristics of these sediments rather than their biokinetic parameters.
This suggests that physical characteristics of these sediments not only influence
dynamics of greenhouse gases, but also drive the adaptation and ecophysiological
strategies of resident microbial communities. Future work should investigate how
these microorganisms respond and mediate N_2O emissions under variable
hydrodynamic regimes.
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Materials and Methods

Sampling and *in situ* N_2O level measurements

Different sampling campaigns were conducted at St. Kilda Beach (37.863159°S,
144.971026°E) and Werribee Beach (37.58132°S, 144.42112°E) between 2022 and
2024 for different purposes: i) bacterial isolation (02/06/2022, 03/08/2022,
19/10/2022 and 11/01/2023), ii) slurry incubations (10/05/2024 for St. Kilda samples;
23/09/2024 and 20/08/2025 for Werribee samples) and iii) *in situ* N_2O concentrations
(24/05/2024, 05/11/2024 for Werribee and 15/05/2024, 01/10/2024 and 18/11/2024
for St. Kilda). Subtidal surface sediment (0-5 cm deep) was collected in sterile 50 mL
Falcon tubes and were immediately transported to the laboratory and stored at 4°C
overnight before experiments. The sediments for metagenomic sequencing were
also sub-sampled, flash-frozen in liquid nitrogen and stored at -20°C until further
analysis. Seawater samples were collected for slurry incubations and were filtered

(0.22 µm) to remove most pelagic species. *In situ* N_2O levels were measured from
pore-water samples collected with a piezometer connected with a rubber tube and
over-filled in 12 mL exetainers. Three replicates were collected, each spaced 2 m
apart. Samples were preserved by adding 50 µL saturated $HgCl_2$ and stored at room
temperature. For dissolved N_2O concentration measurement, a 4 mL helium
headspace was introduced into each 12.5 mL Exetainer vial, shaken and equilibrated
at room temperature before being analysed by gas chromatography described
below.
429

Slurry incubations

Sediment experiments were conducted in order to determine N_2O consumption
capability of the sediment community. Slurries were prepared with 5 g of wet
sediment and 50 mL filtered seawater in 120 mL serum vials before sealing with a
butyl rubber stopper (Sigma-Aldrich) and Wheaton closed-top seals (Sigma-Aldrich).
Serum vials were purged with helium (He) gas for 10 minutes with vigorous shaking
every five minutes to exclude O_2 and create anoxic conditions. The headspace of
vials was amended with N_2O (Air Liquide Australia Limited) to give initial mixing
ratios of approximately 10 (0.8 μM) or 1,000 ppmv (108 μM). Before measurement, 2
mL He was injected into the headspace, then 2 mL of sample was taken from the
headspace and injected into a He pre-purged 3 mL exetainer. Samples were
analysed using a VICI Trace Gas Analyser with a limit of detection of 0.05 ppmv.
442

Metagenomic sequencing and assembly

Environmental DNA was extracted from six sediment samples collected at St.Kilda
and Werribee Beach using the PowerSoil® DNA kit (QIAGEN) following the
manufacturer's instructions. Extractions included a sample-free negative control.
Samples were sequenced on an Illumina NovaSeqX Plus (2 × 150 bp) at Monash
Genomics & Bioinformatics Platform (Monash University). Sequencing yielded an
average of 44,021,402 read pairs per sample, with 1,052 read pairs sequenced in
the negative control. The metagenomes were processed using the Metaphor
workflow [56]. Specifically, the metagenomes were quality filtered with fastp [57] and
co-assembled using MEGAHIT v1.2.9 [58] with default settings. Contigs shorter than

1,000 bp were discarded. The assembled contigs were binned with Vamb v4.1.3 [59],
MetaBAT v2.12.1 [60] and CONCOCT v1.1.0 [61]. The three bin sets were
subsequently refined using DAS Tool v1.1.6 [62], combined with MAGs previously
obtained from St. Kilda permeable sediments [38,41] and dereplicated with dRep
v3.4.2 [63] using the parameters -comp 50 -con 10. The completeness,
contamination and heterogeneity of the bins were assessed using CheckM2 [64].
Quality thresholds for bins were set as per MIMAG standards [65], retaining only
medium (completeness >50%, contamination <10%) and high (completeness >90%,
contamination <5%) quality bins for further processing, which were termed
metagenome-assembled genomes (MAGs). MAG taxonomy was determined using
the Genome Taxonomy Database Release R226 [66] via GTDB-Tk v2.3.2 [67].
CoverM [68] with the option 'genome' was used to calculate the relative abundance
of each MAG in each sample.

Bacterial isolation and whole genome analysis

Sediment samples for bacterial isolation were collected at St. Kilda Beach in three
sampling campaigns (02/06/2022, 03/08/2022 and 11/01/2023) and at Werribee
Beach (19/10/2022). Approximately 1 g wet weight of the surface sediment (0-5 cm
deep) was dispersed in 9 mL of sterilised 1× phosphate-buffered saline (PBS),
vortexed vigorously for three minutes and sonicated for another three minutes. The
resulting slurry was serially diluted with 1× PBS and stored in cryovials at -80 ^{o}C with
25% glycerol for further isolation if needed. Aliquots of 100 µL undiluted and serially
diluted slurry were spread on Marine Agar 2216 (Difco), modified Marine Agar
2216E, and Marine R2A Agar plates (ingredients in **Table S1**) and incubated
aerobically at 30 ^{o}C for at least one week until most morphologically different
colonies appeared on the plates. A series of enrichments were also performed using
1 mM $NaNO_3$ or $NaNO_2$ as electron acceptors and either 1 mM sodium acetate, 1
mM glucose, 0.002 g/L starch or 0.5 g/L yeast extract as electron donors.
Approximately 5 g of sediments were suspended in 50 mL of a bicarbonate-buffered
minimal saltwater medium (MSW) (**Table S1**) in 120-mL serum bottles and purged
with N_2 for three minutes to create anoxic conditions. Enrichments were transferred
to fresh medium when NO_3^-/NO_2^- was all consumed, using 10% inoculum (v/v). After
the third transfer, the slurries were serially diluted, isolated by streaking on Marine
Agar 2216 or MSW agar and incubated at the same conditions as mentioned above.

Bacterial stocks were preserved at -80 ^{o}C in 25% glycerol for long-term storage. For
isolate identification, DNA was extracted by heating 30 µL of colony suspension at
95°C for 15 minutes and immediately frozen at -20°C and the 16S rRNA gene was
amplified using the universal eubacterial primers 27F (5'-AGA GTT TGA TCC TGG
CTC AG-3') and 806rB (5'-GGA CTA CNV GGG TWT CTA AT-3') or 1492R (5'-GGT
ACC TTG TTA CGA CTT-3'). Purified PCR products were Sanger sequenced
(Monash Genomics & Bioinformatics Platform, Victoria, Australia) and the obtained
sequences were compared with those available in the GenBank database using
BLAST on NCBI (National Center for Biotechnology Information) website.
495
A total of 95 bacterial isolates from permeable sediments collected in St. Kilda and
Werribee beaches were selected for whole genome sequencing. The genomic DNA
of isolates was extracted using the Genomic DNA Kit (Meridian Bioscience) following
the manufacturer's instructions. Samples were sequenced on Illumina NovaSeq
6000 platform at GENEWIZ (AZENTA Life Sciences). Raw sequences were quality
filtered with the BBTools suite v.38.81 (https://sourceforge.net/projects/bbmap/),
using BBDuk to remove the 151st base, trim adapters, filter PhiX reads, trim the 3'
end at a quality threshold of 15 and discard reads below 50 bp in length. Processed
short reads were assembled using Unicycler v.0.4.7 [69]. The assembled genome
quality was assessed with CheckM2 [64] and taxonomic classification of each isolate
was performed using GTDB-tk v2.1.1 [67].
507

Metabolic annotation

Open reading frames (ORFs) were predicted using Prodigal v2.6.3 [70] for isolate
whole genome and MAGs, then annotated using DIAMOND v2.0.15 [71] for
alignment against a custom set of 51 metabolic marker protein databases
(https://doi.org/10.26180/c.5230745) covering the major pathways for aerobic and
anaerobic respiration, energy conservation from organic and inorganic compounds,
carbon fixation, nitrogen fixation and phototrophy. Gene hits were filtered by a
minimum percentage identity score by protein: 80% (PsaA), 75% (HbsT), 70%
(PsbA, IsoA, AtpA, YgfK and ARO), 60% (CoxL, MmoX, AmoA, NxrA, RbcL, NuoF,
[FeFe]-hydrogenases and NiFe Group 4 hydrogenases) or 50% (all other genes). To
determine gene abundance in the community, read counts of each gene were

normalized to reads per kilobase million (RPKM) and then divided by the mean
abundance of 14 universal single-copy ribosomal marker genes (in RPKM, obtained
from the SingleM v0.13.2 package) [72].
522

Phylogenetic analysis

To understand the distribution and diversity of microorganisms in marine permeable
sediments involved in the nitrogen cycling and N_2O emission, a phylogenomic tree
was constructed from 95 bacterial isolate whole genomes and 97 MAGs. Genomes
were aligned based on the AR53/BAC120 marker set of GTDB-Tk (v2.4.0) using the
"align" option and the tree was generated by maximum likelihood estimation with the
ultrafast bootstrap value of 1,000 and model Q.pfam+I+G4 using IQ-TREE [73]. In
addition, we also analysed the diversity of clade I and clade II *nosZ* genes present in
permeable sediments. To do so, we retrieved the NosZ amino acid sequences
derived from unbinned and binned contigs and isolate genomes. Any sequences
shorter than 300 amino acids were excluded. We also included the NosZ amino acid
sequences from the Greening Lab database (https://doi.org/10.26180/c.5230745)
and clade III NosZ sequences from [26] as reference. Sequences were aligned using
MAFFT v7.526 [74] and trimmed with TrimAL 1.2rev59 [75] with default settings.
Maximum-likelihood trees were constructed with the ultrafast bootstrap value of
1,000 and model Q.pfam+I+G4 using IQ-TREE [73]. The tree was visualised and
midpoint-rooted using the Interactive Tree of Life (iTOL) [76].
540

Isolate physiological characterisation

The partial denitrification phenotype was tested in two bacterial isolates (i.e.
Antarctobacter sp. and *Sedimentitalea* sp.). The N_2O-reducing phenotype was tested
in two clade II *nosZ* organisms (i.e. *Olleya marilimosa* and *Winogradskyella* sp.) and
two clade I *nosZ* organisms (i.e., *Marinobacter adherens* and *Oceanimonas* sp.). All
cultures were grown overnight in Difco 2216 Marine Broth medium under oxic
conditions. Overnight cultures were transferred to 50 mL of fresh media so that the
starting optical density (OD_{600}) reached 0.03. Oxic treatment remained as
unamended air while anoxic treatments were purged with He to exclude O_2 as
described above. For partial denitrification phenotype analysis, cultures were

amended with either 1mM $NaNO_3$ or 0.1 mM $NaNO_2$ and the concentrations of NO_x

(NO_3^- + NO_2^-) and NO_2^- were determined spectrophotometrically on a Lachat

Quickchem 8000 following the procedures in Standard Methods for Water and

Wastewater [77]. Headspace gas (2 mL) was sampled into He purged 12-mL

exetainers to monitor N_2O production. For N_2O reduction phenotype analysis, the

headspace of anoxic vials were amended with N_2O as the main electron acceptor to

give initial mixing ratios of approximately 100 and 1,000 ppmv. Vials were placed on

an orbital shaker (150 rpm) at $30^{\circ}C$ for 24 hours. To monitor N_2O concentration

changes, 2 mL of headspace gas was sampled into He purged 3-mL exetainers.

560   Kinetic analysis

Rates of N_2O consumption by *O. marilimosa, Winogradskyella* sp., *M. adherens* and

Oceanimonas sp. and by whole sediment communities from the two studied sites

were measured. For axenic cultures, overnight-grown cells under oxic conditions

(OD_{600} of 3.0 to 4.0) were inoculated into 50 mL fresh Difco Marine Broth medium in

120-mL serum vials to reach an initial OD_{6oo} of 0.03 then vials were sealed with butyl

rubber stoppers. Similarly, slurry was prepared with 5 g of wet sediment and 50 mL

filtered seawater in 120-mL serum vials and sealed with butyl rubber stoppers. Vials

were purged with He gas to exclude O_2 as described above. Triplicate cultures or

slurries were incubated with different N_2O concentrations in the headspace ranging

from 5 to 1,000 ppmv. Headspace N_2O concentrations were quantified by gas

chromatography at different time intervals. The final N_2O concentration in the liquid

phase was corrected and calculated as previously described [78]. Reaction rates of

N_2O consumption were determined from the slopes of linear regression lines fitted to

N_2O concentration measurements over time. Michaelis-Menten curves and

parameters were estimated using the non-linear fit (Michaelis-Menten, least squares

regression) function in GraphPad Prism v10.3.1.

577   Data availability

All sequences generated from this work were deposited to the NCBI Sequence Read

Archive under the BioProject accession numbers PRJNA1347951 for metagenomes

and metagenome-assembled genomes and PRJNA1348900 for isolate whole

genomes. Bacterial and archaeal MAGs generated from the same sites in previous

studies [38,41] were downloaded from NCBI Sequence Read Archive with BioProject
accession number PRJNA623061 and PRJNA609151.

Conflict of interest
585 The authors declare no conflict of interest.

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Author contributions
C.G, P.L.M.C and W.W.W conceived and supervised the study. Experimental planning and
design were conducted by T.N.D, C.G., W.W.W. and P.L.M.C. T.N.D and L.J were
responsible for bacterial isolation and culturing. T.N.D, H.P., and W.W.W. were responsible
for environmental microcosms and monitoring. T.N.D. and T.F.H were responsible for
physiological and kinetic characterization of isolates. Chemical analyses were performed by
V.E. Metagenomic and whole genome analyses were performed by T.N.D, F.R., R.L and
P.M.L. Resources, supervision and funding were contributed by C.G., P.L.M.C and W.W.W.
The manuscript was written by T.N.D and F.R. with contributions from all authors.
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Supplementary materials

Figure S1 | *In situ* N_2O concentrations in permeable sediments
Table S1 | Composition of different media used for isolation of bacteria from
permeable sediments in St. Kilda Beach and Werribee.

Table S2 (xlsx). Sequencing statistics and abundance of metabolic marker genes in
the short read metagenomic data. This includes the calculated average copies per
organism for each marker gene, average copies per organism for nitrogen
metabolising genes and a list of short read hits and their corresponding match in the
database.
610

Table S3 (xlsx). Summary and annotation of dereplicated metagenome-assembled-genomes (MAGs) obtained from this study and previous [38,41] studies. This includes taxonomic information for each MAG, relative abundance of each MAG per sample calculated by CoverM, a summary of metabolic marker genes identified in each MAG, a list of clade II *nosZ* containing MAGs, a list of high-quality MAGs and a full list of metabolic marker genes identified in the MAGs with alignment information and protein sequences.

Table S4 (xlsx). Summary of bacterial isolates. This includes taxonomic information for each isolate and a summary of metabolic marker genes identified in each isolate genome.

Table S5. Comparison of biokinetic parameters for marine permeable sediment isolates and other N_2O-reducing bacteria.

Table S6 (xlsx). Sequences of metabolic genes in unbinned contigs derived from homology-based searches.

Table S7 (xlsx). Measurements of N_2O concentrations and kinetic analyses. This includes measurement of N_2O concentrations in slurries and four bacterial isolates.

Table S8 (xlsx). Measurements of NO_x and N_2O in two *Rhodobacteraceae* isolates (i.e. *Antarctobacter* sp. and *Sedimentitalea* sp.) with partial denitrification pathways.

Table S9 (xlsx). *In situ* N_2O concentrations in porewaters of St. Kilda and Werribee permeable sediments.

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Figure captions

Figure 1. N$_2$O consumption by permeable sediments. (**A**) Anoxic slurry incubation

of surface sediments (0-5 cm) from St. Kilda (13/05/2024) and Werribee

(23/09/2024) with different N$_2$O concentrations. (**B**) Kinetics of N$_2$O reduction by

surface sediments in St. Kilda (13/05/2024 samples) and Werribee (20/08/2025

samples). The curves were fitted and kinetic parameters were approximated based

on a Michaelis-Menten non-linear regression model.

Figure 2. Nitrogen-cycling metabolic capacity of microbial communities in

permeable sediments. (**A**) Relative abundance of nitrogen metabolizing genes in

St. Kilda and Werribee beaches based on gene-centric metagenomic analysis (**B**)

Maximum-likelihood phylogenomic tree showing nitrogen-cycling metabolic gene

inventories of 95 bacterial isolates and 245 bacterial MAGs generated from this

study and acquired from (Chen et al., 2022, 2021). The tree was built with 1,000

ultrafast bootstrap replicates using the LG+F+I+G4 model via IQ-TREE. Tree

branches were colour-indexed to show phylum-level taxonomy of bacterial isolates

and MAGs. Key metabolic genes for denitrification were highlighted using coloured

rings. Specific isolates were selected for further analysis, including culture-based

activity and kinetic measurements (asterisks).

Figure 3. Diversity of NosZ in permeable sediments. The maximum-likelihood

phylogenetic tree of NosZ amino acid sequences was generated using Q.pfam+I+G4

substitution model (via IQ-TREE) and was midpoint rooted. The NosZ amino acid

sequences derived from MAGs (n = 24) and isolate genomes (n = 36) are shown in

bold, whereas sequences from unbinned contigs (n = 245) are marked with an

asterisk (*). Additionally, 342 reference NosZ sequences were included. Taxonomic

assignments are indicated by branch colours on the tree.

Figure 4. Partial denitrification genotype and phenotype of selected bacterial

isolates. (A) Presence and absence of denitrifying genes in two isolates. (**B**) Partial

denitrification phenotypes in *Antarctobacter* sp. (**C**) Partial denitrification phenotypes

in *Sedimentitalea* sp. Both isolates were grown on Difco 2216 Marine Broth with

either 1 mM NaNO$_3$ for NO$_3^-$ treatment or 0.1 mM NaNO$_2$ for NO$_2^-$ treatment under

anoxic conditions. Data are presented as mean ± SD, n = 3 biologically independent

samples, with media-only vials (n = 2) as negative controls.

Figure 5. N_2O reduction genotype and phenotype of clade I and II N_2O reducing

isolates. (A) The presence and absence of *nosZ* genes and other denitrifying genes

in selected isolates based on whole genome sequencing and metabolic annotation.

(**B-D**) Aerobic growth and N_2O reducing activity of *O. marilimosa* and

Winogradskyella sp. (clade II *nosZ* isolates) and *M. adherens* and *Oceanimonas* sp.

(clade I *nosZ* isolates) respectively. Isolates were grown on Difco 2216 Marine Broth

aerobically or anaerobically with N_2O as the sole terminal electron acceptor. Growth

was determined by optical density measurements at 600 nm, followed by

measurements of N_2O concentration in the headspaces of the culture vials. Kinetics

of N_2O reduction of four isolates were calculated based on a Michaelis-Menten non-

linear regression model. All experiments were performed in triplicate. Data are

presented as mean ± standard deviation (SD).

Figure 6. Comparison of N_2O reduction biokinetics between different N_2O

reducing microorganisms. (A) Affinities for N_2O reported for N_2O-reducing isolates

and whole communities from this and previous studies [40,44–48] (**B**) N_2O sink

strength of various microorganisms from different ecosystems, determined by

plotting the 'catalytic efficiency' (V_{max}/K_m) against the maximum reduction rates

(V_{max}). To enable consistent comparison, we converted all V_{max} values to mmol N_2O

g^{-1} cell dry weight h^{-1}, assuming the total protein amounts account for 55% of the

total dry biomass [49,50]. We also converted all values to V_{max} at 20^oC assuming the

rates increase exponentially with temperature by the factor of 2.5 per 10^oC increase

in temperature (Q_{10} = 2.5), as previously described [46].

A

	St. Kilda				Werribee		
napA							
narG							
nirS							
nirK							
norB							
Clade I nosZ							
Clade II nosZ							
Clade III nosZ							
nrfA							
amoA							
nxrA							
hzsA							
nod							
nifH							

Average gene copies per organism

0.3
0.2
0.1

Nitrogen cycling metabolisms

- Nitrate reduction
- Nitrite reduction to nitric oxide
- Nitric oxide reduction
- Nitrous oxide reduction
- Nitrite reduction to ammonium
- Nitrification
- Anammox
- Nitric oxide disproportionation
- Dinitrogen fixation

B

0.1   MAGs   Isolates

Phylum

- Patescibacteria
- Fusobacteriota
- Bacillota
- Actinomycetota
- Planctomycetota
- Verrucomicrobiota
- Eisenbacteria
- Candidate phyla
- Gemmatimonadota
- Calditrichota
- Bacteroidota
- Myxococcota
- Acidobacteriota
- Desulfobacterota
- Campylobacterota
- Pseudomonadota

Nitrogen metabolising genes

- napA/narG (Nitrate reduction)
- nrfA (Ammonification)
- nirK/nirS (Nitrite reduction)
- norB (Nitric oxide reduction)
- Clade I nosZ (Nitrous oxide reduction)
- Clade II nosZ (Nitrous oxide reduction)

Isolates	NO$_3^-$ → NO$_2^-$		NO$_2^-$ → NO		NO → N$_2$O	N$_2$O → N$_2$		
	narG	*napA*	*nirK*	*nirS*	*norB*	Clade I *nosZ*	Clade II *nosZ*	Clade III *nosZ*
Antarctobacter sp.	■		■		■			
Sedimentitalea sp.				■	■			

	NO₃⁻ → NO₂⁻		NO₂⁻ → NO		NO → N₂O	N₂O → N₂		
Isolates	*narG*	*napA*	*nirK*	*nirS*	*norB*	Clade I *nosZ*	Clade II *nosZ*	Clade III *nosZ*
Olleya marilimosa							■	
Winogradskyella sp.					■		■	
Marinobacter adherens	■				■	■		
Oceanimonas sp.			■		■	■		

Aerobic **Anaerobic + N₂O** **Michaelis-Menten Kinetics**