THE INFLUENCE OF ENDOGENOUS NITROUS OXIDE IN ORAL REGIONS FOR DRUG TESTING SENSITIVITY AND ACCURACY

Iza Muradin
15 march 2024
Utrecht University
Dinesh Duran-Jimenez & Ruben Kranenburg
In collaboration with TNO and the Dutch police

Index

1. Abstract

Background: Nitrous oxide (N_2O) is a naturally occurring gas produced in the environment by bacteria and by industrial activity. N_2O is used in medical settings and also in a recreational setting. with increasing illegal use in the western world. This leads to incidents, such as its use in traffic with driving under influence. Currently, on-site detection of N_2O by the police is not possible. Oral fluid drug testing could be a potential solution; however, the test results could be influenced by N_2O production by oral bacteria. This review provides an overview of endogenous N_2O production and examines its potential impact on the feasibility of an oral fluid drug test for law enforcement.

Results: The production of N_2O in the oral cavity is caused by various bacteria located in different parts of the oral cavity, mainly at dental plaque sites, and the dorsal side of the tongue. N_2O is produced via denitrification using the key enzyme nitric oxide reductase. This process seems to be influenced by several factor. Biochemical factors, such as the pH, oxygen availability and temperature, nutritional components like diet and nutrient availability, physiological factors like gender, age and individual genetic variability, and lifestyle factors including oral hygiene practices and substance abuse. Therefore, differences between individuals can be diverse. N_2O concentrations in saliva appear to range from nano- to micromolar levels. Oral fluid drug testing is an emerging method where the detection limit is in the range of ng/mL, which suggests that endogenous N_2O could potentially influence an oral fluid drug test result. The potential use of pre-sampling procedures, such as mouthwash, could reduce endogenous N_2O levels, and the establishment of concentration thresholds could help to distinguish between endogenous N_2O and exogenous laughing gas use in a test result.

Conclusion: The presence of endogenously produced N_2O in the oral cavity could possibly pose a significant challenge to the sensitivity of oral fluid drug testing for N_2O. Future research is necessary to better understand the factors affecting N_2O production, determine specific N_2O concentrations, and develop standardized testing protocols to minimize the risk of contamination from endogenous sources. By addressing these challenges, oral fluid drug testing could possibly be implemented as a reliable method for law enforcement, providing quick, non-invasive, and accurate results in drug-related investigations in the future.

2. Introduction

- *Nitrous oxide (N_2O) is a potent greenhouse gas produced naturally by microbial and industrial activity.*
- *N_2O is used recreationally, known as laughing gas, which leads to health and societal concerns due to its psychoactive effects.*
- *The detection of recreational N_2O use, especially in driving under influence cases, is challenging due to the absence of on-site testing methods for law enforcement, but oral fluid drug testing could be a potential as less invasive, alternative method for N_2O testing.*
- *The presence of endogenously produced N_2O in the oral cavity could potentially interfere with oral fluid drug test results and therefore law enforcement, complicating the differentiation between illegal use and endogenous produced N_2O.*
- *This review aims to explore the details of endogenous N_2O production and its effect on the sensitivity and feasibility of oral fluid drug testing for potential use in the future for N_2O. This will be done by identifying the production sources, the influences on endogenous production, and lastly, the current knowledge on forensic oral drug testing methods.*

Nitrous oxide (N_2O), a chemically stable, colorless, and odorless gas, is often noted for its sweet odor under certain conditions (Vinckenbosch et al., 2023). Environmentally, N_2O is a significant atmospheric component at about 310 ppbv. and a greenhouse gas that is many times stronger than CO_2. This molecule is mainly produced by bacteria through microbial activities like denitrification where nitrate (NO_3^-) is reduced to nitrogen gases, and can also be formed by industrial activity, both contributing to the global nitrogen cycle (Bleakley & Tiedje, 1982; Mitsui et al., 1997).

N_2O is used various forms and settings, such as in medical settings for anesthesia and analgesia. In some countries, N_2O is used also used in illegal recreational settings known as laughing gas. When inhaled, typically from balloons using whipped cream patterns, its effects can include dissociation, euphoria, and ataxia, posing risks like neurological and psychiatric conditions with N_2O acting as a NMDA receptor antagonist (Brunt et al., 2022; Vinckenbosch et al., 2023).
Illegal use has seen a rise in recreational use since 2010, particularly among youth, raising societal and health concerns (Aerts et al., 2022). In the Netherlands, a country with significant recreational N_2O use, there has been a considerable rise in police reports related to N_2O-related traffic incidents since 2016 (Vinckenbosch et al., 2023).

Despite the prevalence of recreational N_2O use and its potential for illegal use while driving, currently there are no methods for on-site N_2O detection by law enforcement. This results in a lack of effective identification and prosecution for N_2O drugged driving (DUINO) (Kadehjian, 2005).
The pharmacokinetics of N_2O suggest its recent use could possibly be detected in an individual's breath, blood, and saliva (Vinckenbosch et al., 2023). Saliva, or oral fluid, appears

to be a potentially convenient sample because sampling is less invasive than, for example, a blood test or urine test, and also seems to correlate better with recent use (Drummer, 2006). For certain drugs, oral fluid drug testing has already been developed and is being used by the police. However, for N_2O, there are still no quantitative and qualitative oral fluid testing methods available.

There is a potential issue for oral fluid drug testing of N_2O. Literature suggests that bacteria within the oral cavity can produce endogenous N_2O. N_2O could be produced endogenous in the homeostasis process of signal molecule NO via denitrification (Schreiber, 2009). Details on the production process and concentration of endogenously produced N_2O via denitrification have been limitedly investigated.
The concentration of endogenously produced N_2O could possibly impact the sensitivity of test results in oral fluid drug testing. This 'background level' of endogenous N_2O could have an influence in law enforcement cases with individuals being wrongfully accused of illegal N_2O usage by false positive oral fluid drug testing results, caused by endogenous N_2O concentrations instead of exogenous concentrations by using laughing gas.

This literature review therefore investigates the relationship between endogenously produced N_2O in oral fluid and its potential impact on oral fluid drug detection methods for law enforcement investigations. The central question in this research is:

How might the levels of endogenously produced nitrous oxide, influenced by complex biological pathways and bacterial activity in oral fluid, affect the efficacy and reliability of nitrous oxide detection methods used in law enforcement's drug-related investigations?

The review is structured around several sub-questions, each examining a different aspect of this theme. The first focus area is the identification of specific enzymes and oral bacteria involved in the production of N_2O within different locations the oral cavity. The paper then analyzes the influences of several factors on N_2O production. Examples are biochemical and nutritional properties of saliva—such as pH, ionic strength, and organic composition—alongside physiological, genetic, and lifestyle determinants, including health practices and dietary habits. Combining these two, gives an indication of the concentration of endogenous N2O in oral fluid which will be discussed briefly. Lastly, the review addresses the current knowledge on forensic oral drug testing methods, focusing on their effectiveness, sensitivity and accuracy, and potential challenges and complications in future detecting of N_2O for law enforcement purposes.

3. Method

Search strategy

The first step in this review was the formulation of the main and sub-questions. These questions helped come up with various themes related to the topic. With the main- and sub-questions, the boundaries for the literature search were determined specifically focused on answering these questions. For the literature search, inclusion and exclusion criteria were determined. Several research engines were used for this task, mainly Google Scholar, but also PubMed, ScienceDirect, and databases like ProQuest, JSTOR, and ResearchGate. The different used search terms can be found in the appendix.

Literature selection

During this research, limited information was found on certain topics within the sub-questions specifically focused on N_2O. This led to broadening the scope/inclusion criteria with also considering nitrate, nitrite, and nitric oxide due to their connection to N_2O production. Exploring N_2O production in nature was firstly also considered due to the lack of literature within the inclusion criteria. However, this was ultimately excluded to avoid broadening the scope too much and deviating from the central theme of endogenous N_2O production and its impact on drug testing in law enforcement.

Scoping search inclusion criteria:

- Source types: Scientific articles and government publications that provide insights into N_2O and related compounds in the context of oral production, and scientific articles on oral fluid drug testing.
- Language: Publications in English, given the focus of the research on these sources.
- Publication date: No specific restriction to include a wide range of studies from 1982 to the present.
- Subject focus: Studies focused on endogenous N_2O production in the oral cavity, factors influencing this production, and the impact of N_2O on saliva drug tests.
- Study population: Mainly research involving adult humans, animal studies also included in topics with limited data available.

Scoping search exclusion criteria:

- Language restrictions: Articles not published in English, unless they offer critical and unique information not available in English literature.
- Insufficient data on production location (endogenous): Studies providing information on N_2O production outside of the oral cavity were excluded.
- Language restrictions: Articles not published in English, unless they offer critical and unique information not available in English literature.
- Sub-question relevancy: Studies that did not answer (one of the) sub questions directly were excluded.

Literature was selected by first systematically reviewing papers within these criteria. The studies were first screened on titles, then abstract, and lastly, full text. Screening was done separately for the different sub-questions.

Data extraction

The gathered literature was then again analyzed by full-text evaluation, including the introduction, results, and discussion. The method section was often skipped as this was primarily not needed. With this, the articles were evaluated for relevance to the research questions. Within this analysis, relevant quotes were extracted from each article. Relevant quotes included text from articles that answered one of the sub-questions. The quotes per article were divided in the format below (table 1). Here, the requirements of making a quote relevant to this review are also visible (table 1 on the right). The different groups of relevant quotes corresponding to a sub question were then combined to text and used as information in the different sections of this review.

Table 1. Overview of themes and corresponding sub-questions for the full-text analyses and quote grouping.

Introduction	General information	General information on nitrous oxide (start of review)
		Illegal use (used as a drug)
		N_2O found in oral fluids
	Study significance	Importance in law enforcement
		Relevance in drug testing
Sub question 1	Identification enzymes and bacterial sources	Identification of enzymes
		Identification of bacterial sources
		Location of enzymes/bacteria/process
		Chemical properties of saliva (pH, ionic strength)
Sub question 2	Influences on endogenous N_2O production	Physical properties of saliva (types, production sites
		Physiological influences on N_2O or DNRA/denitrification processes (Circadian rhythms, gender, smoking)
		External stimuli (food intake, stress) influencing nitrous oxide or DNRA/denitrification processes
Sub question 3	Concentration of N_2O	Concentration of N_2O (or other nitrogen components)
Sub question 3	Oral fluid drug testing	Implications for interpreting results
		Challenges in oral fluid collection and analysis: Sensitivity and accuracy
		General information on nitrous oxide (start of review)

Research scope

The scope of the research was defined partly by time constraints and partly by the complexity of the topic. While the primary focus was on the impact of N_2O on oral fluid drug testing, several other themes were discussed. Endogenous N_2O in oral cavity, the recreational use of N_2O and knowledge about oral drug tests in general were also studied. As this review was made in collaboration with TNO and the Dutch police the focus was specifically on oral fluid drug testing relevance and also knowledge gaps relevant to law enforcement. Overall, the study provides an extensive overview of the nitrogen cycle in the mouth leading to endogenous N_2O production, influencing factors in this process, an estimation of the N_2O concentration and lastly its potential impact on oral fluid drug testing.

Limitations and process reflection

Reflecting on the review process, challenges were encountered due to limited data on specific sub-questions, necessitating an expansion of the search to related compounds. This approach risked diluting the focus. The restriction to English-language sources, while practical, may have excluded valuable insights. The limitations underscore the necessity for future research to adopt a more inclusive and detailed methodology to understand the complexity of endogenous N_2O and its influence in oral fluid drug testing.

4. Results

In the results section, the focus is initially placed on identifying sources that can produce N_2O. This includes examining the bacteria, the enzymes, genes coding for these enzymes, and their locations in the oral cavity. Following this, several influences on endogenous N_2O production are explored, including biochemical factors such as pH, oxygen availability, and temperature; nutritional factors including diet composition and nutrient availability; physiological factors like gender and age; variations from genetic and individual differences, including genetic variability and disease states; and lifestyle and behavioral factors, such as oral hygiene practices and substance use, particularly smoking and alcohol. Subsequently, the possible concentration of endogenous N_2O is determined. Lastly, in this section, oral fluid drug testing is also discussed, and finally, the connection to N_2O and the possible implementation of oral fluid drug testing is discussed, linked to the effect of the presence of endogenous N_2O.

Endogenous N_2O production seems to occur in the oral cavity. The oral cavity encompasses the inside of the mouth, including the tongue, teeth, gums, palate, and the opening to the throat. The oral cavity contains a diverse microbial community, being the second most diverse community with 50 to 100 billion bacteria across over 700 different prokaryotic species. The bacteria form a complex community in a matrix, also known as a biofilm. Biofilm formation occurs at various locations in the oral cavity, crucial for oral health and other processes (Bahadoran et al., 2021; Doel et al., 2005; González-Soltero et al., 2020; Paster et al., 2001; Schreiber, 2009).

Oral biofilms are nourished by oral fluids. Oral fluid is primarily saliva, a complex mixture that mainly consists of water and many non-water molecules such as vital electrolytes, immunoglobulins, low protein contents (0.3%), and nitrogenous compounds (Drummer, 2006). These molecules can influence various properties of oral fluid, such as the pH and buffering capacity, which alters drug disposition (Bonardo et al., 2022). Over 90% of oral fluid is produced by three major salivary glands (Lee, 2020). Oral fluids provide nutrition and buffering for bacteria in the biofilms and also actively participate in chemical processes within the oral cavity (Takahashi, 2015).

As mentioned above, there are various regions in the oral cavity with distinctive living conditions for microorganisms, which leads to the formation of different types of biofilms. Around teeth and gums, various types of biofilm can also be found, collectively referred to as dental plaque (Schreiber, 2009). Dental plaque mainly consists of biofilm located above the gums and teeth and also below the gums (Deng et al., 2022). Both have different nutrient sources and microorganism compositions (Takahashi, 2015).
Oral biofilms are also found on the tongue surface, specifically in the crypts. The structure of the crypts forms a reservoir for oral debris and provides protection against chewing and salivary flow (Kroes et al., 1999). There also seem to be various differences between locations on the tongue surface, such as in oxygen availability (Rosier et al., 2022). Bacteria that can form biofilms are also present in circulating saliva itself (Rosier, Buetas, et al., 2020).

In the various types of biofilms, there are bacteria that can reduce NO_3^- via nitrite (NO_2^-) to nitric oxide (NO) for NO homeostasis. NO is used as a signaling molecule in the body (Ahmed et al., 2021; Morou-Bermúdez et al., 2022). Furthermore, NO also contributes to systemic

health benefits such as blood pressure control and enhanced sports performances (Lundberg et al., 2008; Mitsui & Kondo, 1998; Rosier et al., 2022). In the oral cavity, besides functioning as a signaling molecule, NO also influences bacterial behavior and microbiome compositions (Schreiber et al., 2010).

NO production occurs via the entero-salivary or NO_3^- - NO_2^- - NO pathway (see Fig. 1). In this process, NO_3^- and NO_2^- are inactive byproducts and form reserves for NO bioactivity (Burleigh et al., 2018; Hezel & Weitzberg, 2013; Lundberg et al., 2008). The bacteria contain reductases that can catalyze each step in this process (Hezel & Weitzberg, 2013).
The pathway is supplied with substrate by NO_3^- supplementation via dietary sources (Feng et al., 2023; Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020; Vanhatalo et al., 2018). NO_3^- and NO_2^- are then swallowed and converted into NO (and NO_2^-) by gastrointestinal bacteria, with NO then enters the circulation. NO production also occurs in the mouth, but this process is negligible because the conversion from NO_2^- to NO is slow and NO_2^- is quickly swallowed (Vanhatalo et al., 2018). The salivary glands take up NO_2^- and NO from the systemic circulation, concentrate it, and excrete it into the oral cavity (Burleigh et al., 2018; González-Soltero et al., 2020).

NO can be reduced by bacteria via two pathways. NO can either be reduced to ammonium via the Dissimilatory Nitrate Reduction to Ammonium (DNRA) pathway. An alternative to this is to N_2O and eventually dinitrogen (N_2) via denitrification. Both processes occur under distinct circumstances (Morou-Bermúdez et al., 2022). As mentioned above, the production and concentration of endogenously produced N_2O via denitrification has been limitedly investigated.

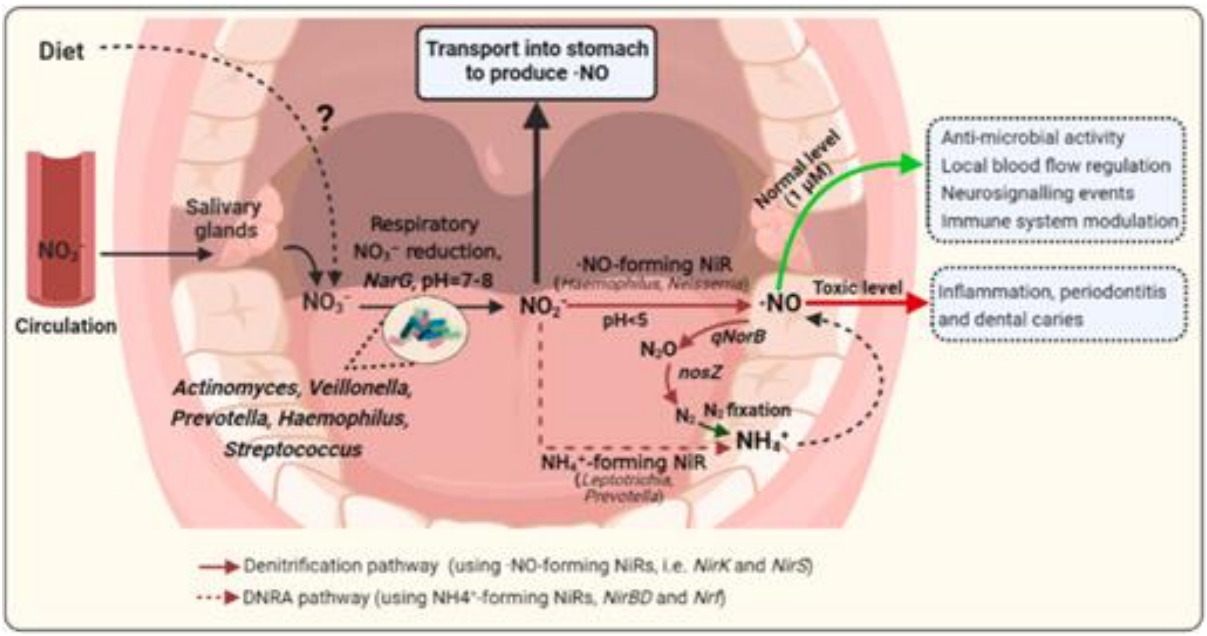

Figure 1. This schematic illustrates the complex NO_3^- - NO_2^- - NO metabolic pathway in the oral cavity, highlighting the role of salivary glands and transport into the stomach for NO production. Additionally, the pathway shows the faith of NO by conversion to either N_2 or NH_4^+ via denitrification and DNRA (Bahadoran et al., 2021)

4.1. Identification of sources

- *Various bacteria have been identified as the primary source in the oral cavity for N_2O production through denitrification. There were no studies that directly reported N_2O production by bacteria. Looking at involved genes, major producers could be Veillonella species, Actinomyces species and Rothia species, with the same species also being major NO_3^- and NO_2^- reducers.*
- *Various enzymes in the oral cavity contribute to denitrification, with the key enzyme for N_2O production being nitric oxide reductase (Nor).*
- *Several production locations in the oral cavity exhibit denitrification enzymatic activity. Dental plaques have been identified as the main sites with the highest N_2O production. In the crypts at the end of the tongue, anaerobic bacteria also seem to produce N_2O, with saliva playing a role by providing nutrients.*

4.1.1. Bacterial sources

In literature, there are many bacteria found that contribute to NO homeostasis. These bacteria can possibly reduce NO further to N_2O via NO reductase in the denitrification process through denitrification and DNRA. In Table 2, a comprehensive overview is made of several bacteria and their role in NO_3^-, NO_2^-, and NO reduction, observed in studies. The table also includes a section on which oral bacteria likely contain genes for this possible reduction step, with data from GenBank (Morou-Bermúdez et al., 2022). The Nar gene encodes the NO_3- reductase, the Nir gene encodes the NO_2- reductase, and lastly, the Nor gene produces the NO reductase. For some bacteria, the location within the oral cavity is also noted if mentioned in literature. See Fig. 2 for an overview of this pathway and corresponding enzymes. For a detailed overview of the key genes, enzymes, and microbial species involved in the reduction processes within the oral cavity, refer to Appendix 6.2.

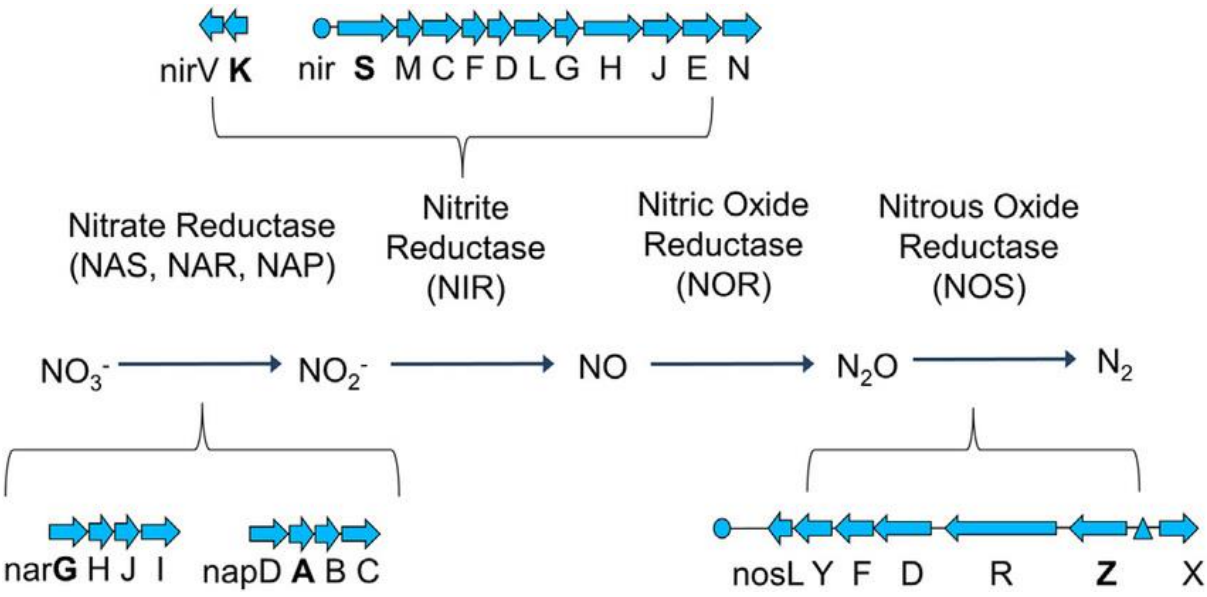

Figure 2. This figure, retrieved from (Levy-Booth et al., 2014) provides a schematic representation of the denitrification pathway, highlighting the sequential enzymes involved and the corresponding genes responsible for the reduction of NO_3^- to N_2.

Table 2. Comprehensive summary of the literature on various oral bacteria in relation to their location and NO_3^-/NO_2^-/NO reducing capabilities. Additionally, the presence of genes causing the reduction steps are also shown per bacterial species with GenBank data (Morou-Bermúdez et al., 2022). Information on NO_3^-/NO_2^-/NO reduction is actually investigated and mentioned in papers, while the information on Nir/Nar/Nor genes were only found in GenBank and not in vivo. "N/A" indicates that no information is available in studies (not available). Major reducers are highlighted in dark green. The Nar gene encodes the NO_3^- reductase, the Nir gene for NO_2^- reductase, and lastly the Nor gene produces the NO reductase.

Type of bacteria	Bacteria genus	Bacteria subspecies	Specific location in oral cavity	NO_3-reduction	NO_2-reduction	NO reduction	Nar gene	Nir gene	Nor gene	DRNA genes	Source
Strict anaerobes, gram-negative	*Veillonella species*	*Veillonella atypica*	Top surface tongue	Yes, major reducer	Yes, major reducer (anaerobic)	Possibly	Yes	Possibly	Yes	Yes	(Ahmed et al., 2021; Burleigh et al., 2018; Doel et al., 2005; Hezel & Weitzberg, 2013; Hyde et al., 2014; Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020; Schreiber et al., 2010; Zhang & Huang, 2023).
		Veillonella dispar	Top surface tongue	Yes, major reducer	Yes, major reducer (anaerobic)	Possibly	Yes	Possibly	Yes	Yes	(Ahmed et al., 2021; Burleigh et al., 2018; Doel et al., 2005; Hezel & Weitzberg, 2013; Hyde et al., 2014; Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020; Schreiber et al., 2010; Zhang & Huang, 2023).
		Veillonella parvula	Teeth area	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Morou-Bermúdez et al., 2022)
	Prevotella species	-	N/A	Yes	N/A	N/A	No	Possibly	Yes	Yes	(Ahmed et al., 2021; Morou-Bermúdez et al., 2022; Rosier et al., 2022)
	Selenomonas species	-	N/A	Yes	N/A	N/A	N/A	N/A	N/A	N/A	(Rosier et al., 2022)
	Fusobacterium nucleatum	-	N/A	No	Yes (unknown)	N/A	No	No	No	Yes	(Morou-Bermúdez et al., 2022)
	Tannerella species	-	N/A	N/A	N/A	N/A	N/A	Yes	N/A	N/A	(Ahmed et al., 2021)
	Oribacterium species	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Morou-Bermúdez et al., 2022)
	Porphyromonas species	-	N/A	N/A	N/A	N/A	No	No	Yes	Yes	(Morou-Bermúdez et al., 2022)
	Eikenella species	*Eikenella corrodens*	Teeth/dental surface (in general)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Kroes et al., 1999)
	Selenomonas species	*Selenomonas noxia,*	Teeth/dental surface (below teeh)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Kroes et al., 1999)
	P. melaninogenicass	-	N/A	N/A	N/A	N/A	N/A	Yes	N/A	N/A	(Feng et al., 2023)
Aerobe, gram-positive	*Nocardia species*	-	N/A	Yes	No	Possibly	N/A	N/A	N/A	N/A	(Bonardo et al., 2022; Hezel & Weitzberg, 2013)
Microaerofile, gram-negative	*Campylobacter concisus*	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Yes	(Feng et al., 2023)

Type of bacteria	Bacteria genus	Bacteria subspecies	Specific location in oral cavity	NO_3-reduction	NO_2-reduction	NO reduction	Nar gene	Nir gene	Nor gene	DRNA genes	Source
Facultative anaerobes, gram-positive	*Actinomyces species*	*Actinomyces odontolyticus*	Top surface tongue (saliva, teeth also)	Yes, major reducer	Yes, major reducer (aerobic and anaerobic)	N/A	Yes	No	Yes	Yes	(Feng et al., 2023; Morou-Bermúdez et al., 2022; Schreiber et al., 2010, 2012; Tribble et al., 2019).(Rosier, Moya-Gonzalvez, et al., 2020)
		Actinomyces naeslundii	Top surface tongue (saliva, teeth also)	Yes, major reducer	Yes, major reducer (aerobic and anaerobic)	N/A	Yes	No	Yes	Yes	(Feng et al., 2023; Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020; Schreiber et al., 2012; Tribble et al., 2019)
	Rothia species	*Rothia mucilaginosa*	Tongue surface (saliva, teeth and other oxygen-rich regions)	Yes, major reducer	Yes, major reducer (aerobic)	N/A	Yes	No	Yes	Yes	(Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020; Schreiber et al., 2010).
		Rothia aeria	Teeth/dental surface (saliva, tongue also)	Yes, major reducer	Yes, major reducer (aerobic)	N/A	N/A	N/A	N/A	Yes	(Rosier, Moya-Gonzalvez, et al., 2020)
		Rothia dentocariosa	Teeth/dental surface (saliva, tongue also)	Yes, major reducer	Yes, major reducer (aerobic)	N/A	Yes	No	Yes	Yes	(Morou-Bermúdez et al., 2022; Rosier, Moya-Gonzalvez, et al., 2020)
	S. aureus species	-	N/A	Yes	N/A	N/A	N/A	N/A	N/A	N/A	(Bonardo et al., 2022)
	S. epidermis species	-	N/A	Yes	N/A	Possibly	N/A	N/A	N/A	N/A	(Bonardo et al., 2022; Hezel & Weitzberg, 2013)
	Corynebacterium species	*Corynebacterium pseudodiptheriticum*	N/A	Yes	Yes (oxygen independent)	Possibly	Yes	No	Yes	Yes	(Bonardo et al., 2022; Hezel & Weitzberg, 2013; Kroes et al., 1999; Morou-Bermúdez et al., 2022)
Facultative anaerobes, gram-negative	*Neisseria species*	-	N/A	Yes	N/A	N/A	Yes	Yes	Yes	Yes	(Morou-Bermúdez et al., 2022; Rosier et al., 2022)
	Haemophilus species	-	N/A	Yes	Yes (oxygen independent)	N/A	Yes	Yes	Yes	Yes	(Morou-Bermúdez et al., 2022; Rosier et al., 2022)
	Granulicatella species	-	N/A	Yes	N/A	N/A	N/A	N/A	N/A	N/A	(Rosier et al., 2022)
	Staphylococcus species	-	N/A	N/A	Yes (oxygen independent)	Possibly	N/A	N/A	N/A	N/A	(Hezel & Weitzberg, 2013; Rosier, Moya-Gonzalvez, et al., 2020)
	Streptococcus species	*Streptococcus mutans*	N/A	N/A	Yes (unknown)	Possibly	Possibly	No	Possibly	Yes	(Ahmed et al., 2021; Hezel & Weitzberg, 2013; Morou-Bermúdez et al., 2022)
		Streptococcus mitis	N/A	N/A	N/A	N/A	No	No	No	Yes	(Morou-Bermúdez et al., 2022)
		Streptococcus parasanguinis	N/A	N/A	N/A	N/A	No	No	No	Yes	(Morou-Bermúdez et al., 2022)
	Aggregatibacter species	-	N/A	N/A	N/A	N/A	Yes	Yes	Yes	Yes	(Morou-Bermúdez et al., 2022)
Total species involved				**12**	**8**	**0***	**7**	**5**	**9**	**14**	

Table 2 provides a broad overview of various species involved in NO homeostasis. When examining observed reduction steps in literature, NO_3^- reduction appears to be the most common, with 12 species capable of this step. NO_2^- reduction also occurs frequently, with 8 species performing the conversion to NO. The major NO_3^- and NO_2^- reducers are the strict anaerobe, gram-negative *Veillonella species*, and the facultative anaerobe, gram-positive *Actinomyces species* and *Rothia species*. These species are highlighted in dark green in the table. The rate of NO_3^- and NO_2^- reduction is linked to the presence of reducing species, where the amount of reducing species is correlated with the presence of reduction substrate NO_3^- and NO_2^- (Bonardo et al., 2022; Doel et al., 2005).
Reduction of NO_2^- accounts for the production of NO, which can also be formed by nitric oxide synthase, detected only in a few, mostly gram-positive bacterial species (Schreiber et al., 2012).
NO can further be reduced to N_2O. In soil bacteria, N_2O production primarily occurs in the stationary growth phase of their life cycle. This could suggest that the same might be true for oral bacteria. (Bleakley & Tiedje, 1982). However, literature does not provide data on specific bacteria that perform the reduction step from NO to N_2O. Six species, including *Veillonella species*, are described as bacteria that could possibly do this through denitrification.

Table 2 also provides an overview of which oral bacteria likely have genes in their genome that encode for reductase genes according to GenBank. There seem to be significant differences in the presence of involved genes among oral bacteria. Genes related to DNRA seem to be more common, present in 14 species, than those for denitrification (Nar, Nir, Nor) in 5 to 9 species (Morou-Bermúdez et al., 2022).
The Nor gene, encoding enzymes that perform the reduction step to N_2O, interestingly appears to be the most common, with 9 species containing this gene. The major NO_3^- and NO_2^-reducing species *Veillonella species, Actinomyces species,* and *Rothia species* also seem to contain the Nor gene, which could indicate that these species may also play a significant role in N_2O production. Interestingly, some species, including *Rothia* and *Actinomyces*, show NO_2^- reduction but, according to GenBank data, do not have the Nir gene in their genome. *Rothia* bacteria specifically also appear to have genes that encode for NO_3^- transport, DNRA, and denitrification but lack genes for the complete denitrification to N_2 (Rosier, Moya-Gonzalvez, et al., 2020).

Fungi in the oral cavity are also capable of NO_3^- reduction, but their role in oral health or disease is not well understood. Interactions between fungi and bacteria in the oral cavity are gaining importance in recent studies, indicating a potential area for future research about the extent of NO_3^- reduction by oral fungi and its impact on fungal-bacterial interactions, which could also possibly affect endogenous N_2O production (Rosier et al., 2022).

4.1.2. Enzymes

In the oral cavity, several bacterial enzymes are involved in the reduction of NO_3^- to N_2O and N_2 through denitrification (Morou-Bermúdez et al., 2022). Humans lack the enzymatic machinery to reduce NO_3^- to NO_2^- and thus rely on commensal bacteria for this reduction (Bryan et al., 2022; Lundberg et al., 2008; Qu et al., 2016). In Fig. 3, a complete overview of the denitrification steps is given. See appendix 2 for a complete overview of the involved enzymes.

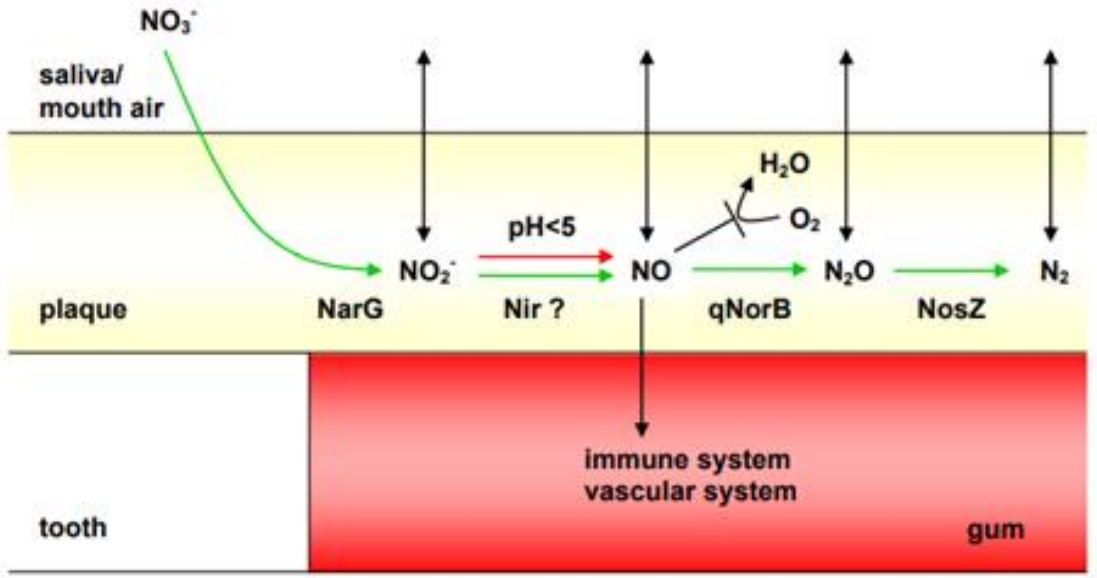

Figure 3. This diagram, adapted from (Schreiber, 2009), outlines the enzymatic conversion pathway of salivary NO_3- to NO_2-, and further to NO, N_2O and N_2 within dental biofilms.

For the two-electron reduction of NO_3^- to NO_2^-, several oral bacteria use the nitrate reductase (NR or Nar) enzyme. Bacterial nitrate reduction in the oral cavity involves three types of nitrate reductases: cytoplasmic assimilatory (Nas), membrane-associated cytoplasmic (NarG, NarZ), and periplasmic (Nap) (Bahadoran et al., 2021).

NO can be formed in two ways: through the reduction of NO_2^- by the nitrite reductase (Nir) enzyme, or synthesis via arginine by NO synthases (eNOS) (Ahmed et al., 2021; González-Soltero et al., 2020; Schreiber et al., 2012). This pathway is being associated with age, with the eNOS gene being polymorphic and can become potentially dysfunctional with age (Tribble et al., 2019).

NO can also be formed by a number of mammalian cells through enzymatic and non-enzymatic processes. Enzymes such as molybdenum-containing enzymes like xanthine oxidoreductase, aldehyde oxidase, and sulfite oxidase are found in the liver and kidneys and can catalyze this reduction. Mitochondrial complexes III and IV also reduce NO_2^- to NO, although their significance in nitrogen metabolism is (Hezel & Weitzberg, 2013).

The fate of NO is either being converted back to NO_2^- for the NO_3^- - NO_2^- - NO pathway or further reduction via denitrification or DNRA. Reduction via denitrification to N_2O is catalyzed by the nitric oxide reductase (Nor) enzyme. In Fig. 4, this reduction reaction is visible.

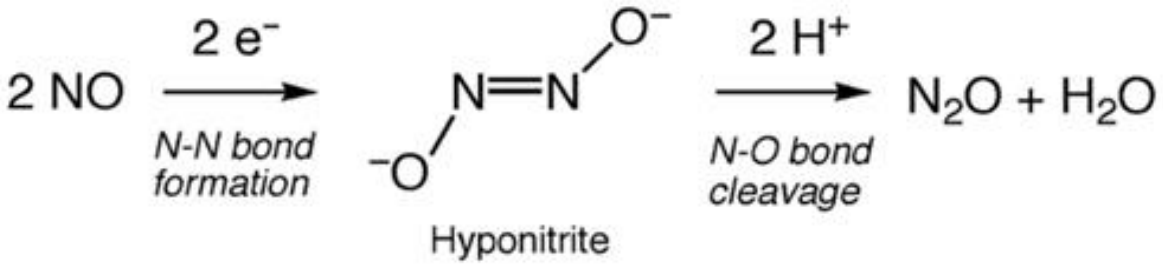

Figure 4. Presented by (Hino et al., 2010), this reaction scheme shows the two-step chemical process converting NO to N_2O. The initial step involves the formation of the intermediate hyponitrite ($O=N-N=O^-$).

In this reaction, NO is converted using two electrons and two protons, facilitated by a hydrogen-bonding network involving three Glu residues, enabling N-O bond cleavage for N_2O and H_2O production, as shown in Fig. 4. (Hino et al., 2010). Several types of Nor enzymes have been identified. qNor, a respiratory NO reductase, plays a role in NO detoxification and bacterial survival, and is primarily found in pathogenic bacteria (Schreiber, 2009; Schreiber et al., 2012). cNor, more commonly found in oral bacteria, contains a cytochrome c subunit. It is an integral membrane enzyme with two subunits, responsible for the conversion of NO to N_2O (Hino et al., 2010).
The produced N_2O can either be converted to N_2 by the multi-copper nitrous oxide reductase (Nos) enzyme, or be exhaled, affecting the bioavailability of NO (Morou-Bermúdez et al., 2022; Schreiber et al., 2012).

4.1.3. Production location

There are various locations within the oral cavity where denitrification and therefore N_2O production occurs. Fig. 5 provides a summary of this distribution throughout the oral cavity. For most specific bacteria species, the exact location within the oral cavity is not known. See table 3 for an overview.

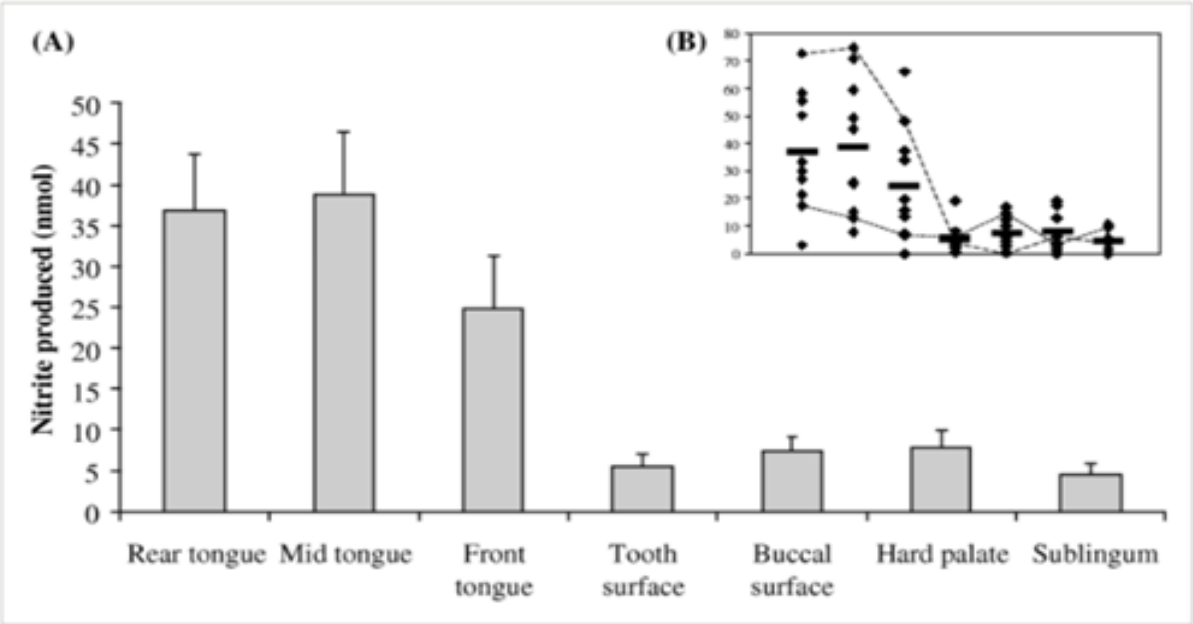

Figure 5. Extracted from (Doel et al., 2005), this figure demonstrates the variability in NO_2^- production and denitrification activity across different oral sites. (A) presents the average NO_2^- produced at various locations within the oral cavity. The rear and mid tongue regions show a notably higher NO_2^- production compared to the front tongue, tooth surface, buccal surface, hard palate, and sublingual areas. (B) The individual data points of graph A.

Table 3. This table summarizes the variability in N_2O production potential across different locations within the oral cavity, highlighting the specific bacteria identified in each area.

Location	**N_2O production chance**	**Bacteria found**
Rear/Mid Tongue	High	*Veillonella, Actinomyces, H. parainfluenza, various proteobacteria, Rothia mucilaginosa*
Front Tongue	Low	Unknown
Teeth	High	*Veillonella parvula, Corynebacterium species, Selenomonas noxia, Eikenella corrodens, Rothia aeria, Rothia dentocariosa*
Cheek	Low	Unknown
Roof of mouth	Low	Unknown
Under the Tongue	Low	Unknown
Saliva	Medium	*Actinomyces*
Dental plaque	Very high	*Actinomyces, Veillonella parvula, Corynebacterium species, Selenomonas noxia, Eikenella corrodens, Rothia aeria, Rothia dentocariosa*

The tongue is one of the places where denitrification occurs, specifically in the deep crypts on the top and back of the tongue (Takahashi, 2015). The back of the tongue is rich in bacteria such as *Veillonella* and *Actinomyces* (Doel et al., 2005). The processes that take place here are primarily under anaerobic conditions (Li et al., 1997; Morou-Bermúdez et al., 2022; Qu et al., 2016).
The top area of the tongue demonstrates the highest NO_3^- reduction activity in the oral cavity (Kroes et al., 1999). This area is abundant with bacteria like *H. parainfluenza* and several proteobacteria (Bonardo et al., 2022). Specific bacteria like *Rothia mucilaginosa* are found to be 100 times more prevalent on the tongue compared to other locations, specifically in oxygen-rich regions (Rosier et al., 2022).

Saliva also contains various bacteria capable of denitrification (Burleigh et al., 2018; Morou-Bermúdez et al., 2022). *Actinomyces species*, found in both saliva and dental plaque, are significant producers of NO_2^- in the presence of oxygen in saliva (Rosier et al., 2022).

On teeth surfaces (dental biofilms), there also appears to be denitrification activity under both aerobic and anaerobic conditions (Morou-Bermúdez et al., 2022). This location is determined to be the main sites of N_2O production in the human mouth (Schreiber et al., 2010). Several bacterial isolates have been identified in dental plaque. The composition of dental plaque is predominantly Gram-positive rods (73%), but it also harbors a more diverse flora compared to other oral sites. Unique bacterial species identified in dental plaque include *Veillonella parvula, Corynebacterium species, Selenomonas noxia,* and *Eikenella corrodens* (Kroes et al., 1999). *Rothia aeria* and *Rothia dentocariosa* species are significantly more prevalent in dental plaque, with *Rothia dentocariosa* isolates species over 100 times more common than in other locations. There is less known about the specific differences between activity in biofilms on the top and bottom of the teeth (Rosier, Buetas, et al., 2020).

4.2 Influences on endogenous N_2O production

Endogenous N_2O production is influenced by numerous factors. In the table below a summary of these influences can be found.

Factor type	Factor	Effect on N_2O production	N_2O production increase with
Biochemical	pH	N_2O production via denitrification favors a low pH, and also affects microbiome composition this pH.	Low pH
	Oxygen availability	Denitrification and N_2O production favor aerobic conditions but is also possible in an anaerobic environment (in which DRNA is usually preferred).	Aerobic conditions
	Temperature	Higher temperatures are linked with higher N_2O production via denitrification (but also DRNA).	Higher temperatures
Nutritional	Diet composition	A NO_3^- rich diet, containing vegetables, enhances denitrification and therefore N_2O production by increasing denitrifying species.	NO_3^- rich diet
		A low-sugar diet appears to positively influence denitrification and thus N_2O	Low-sugar diet
	Nutrient availability	Low carbon source availability, which leads to a low C/NO_3^- ratio, favors denitrification.	Low carbon source availability
		Higher NO_3^-/NO_2^- ratios promote accumulation of N_2O by favoring denitrification.	High nitrate source availability
Physiological	Gender	Females have higher normalized N_2O production, possibly by sex hormones influencing gene regulation	-
	Age	There is a bell-shaped relationship between oral N_2O production and age, increasing after puberty	-
	Individual differences	Individual differences such as ethnicity, diseases, and geographic location effect variations in oral microflora, causing different endogenous N_2O production	-
Lifestyle and behavioral	Substance use	Smoking example inhibits denitrification and alcohol creates an environment that favors DRNA.	No smoking or alcohol use
	Oral hygiene practices	Oral hygiene significantly impacts N_2O production with. Effective plaque control leads to enhanced denitrification resulting in N_2O production. Mouth cleansing itself reduces exhaled N_2O by decreasing oral bacteria.	Good oral hygiene practices
		After using mouthwash, about half the levels of N_2O are still found in exhaled air, indicating other sources in the body for N_2O production.	-

This chapter examines the different factors impacting endogenous N_2O production, from biochemical factors such as pH, oxygen availability, and temperature, to nutritional components like diet composition and nutrient availability. It also addresses physiological aspects including gender, age and individual differences like genetic variability and disease states, and lifestyle and behavioral factors including oral hygiene practices and substance use involving smoking and alcohol. See Fig. 7 for an overview of the different pathways and their influencing factors.

4.2.1. Biochemical factors

The pH of saliva has a significant impact on the production of N_2O by chemically altering the saliva. Usually, an acidic pH favors the formation of N_2O by favoring denitrification, while DNRA is mostly performed in a high pH environment (Bonardo et al., 2022; Morou-Bermúdez et al., 2022).
The pH affects each reduction step differently due to the variation in optimal pH values for the highest activity of each involved enzyme. It is not known which step is the rate-limiting step in this process. The optimal pH for enzymatic activity of NO_3^- reductase is around 8 (Vanhatalo et al., 2018). It has been determined that an acidic environment around a pH of 6 consistently stimulates NO_2^- reduction (Rosier, Moya-Gonzalvez, et al., 2020). Furthermore, a pH of 3 results in 80% N_2O production, while a pH of 9.5 led to insignificant N_2O production (Schreiber et al., 2012). This further implies that N_2O production by NO reduction is enhanced under acidic conditions.
Additionally, oral pH plays a significant role in shaping the composition of biofilm communities. For instance, there's a negative correlation between *Veillonella* and pH, while *Neisseria* shows a positive correlation (Rosier, Buetas, et al., 2020). In terms of dietary influence, beetroot juice, for example, can increase oral pH from 7.0 to 7.5, impacting microbial composition (Vanhatalo et al., 2018).

Oxygen availability in oral sites also seems to influence NO reduction pathways, and therefore N_2O production, by favoring either denitrification or DNRA. DNRA is energetically favored in areas with limited oxygen availability. Denitrification usually therefore occurs under more (micro)aerobic conditions, such as in dental plaque (Morou-Bermúdez et al., 2022). However, N_2O production via denitrification is also possible anaerobic conditions in several studies (Bleakley & Tiedje, 1982; Doel et al., 2005; Rosier et al., 2022). Overall, there can be said that denitrification occurs in oxygen-rich regions and DRNA in oxygen-depleted regions.
In the literature, it is also noted that NO_3^- reduction is especially sensitive to oxygen levels. On the tongue, an increase in NO_2^- production under lower oxygen levels is observed, caused by an enhanced expression of respiratory NO_3^- reductase enzymes translation (Li et al., 1997).
In dental or teeth areas, oxygen availability is linked to biofilm thickness. Thicker biofilms contribute to reduced oxygen levels locally, making DNRA more common, particularly in cases of poor oral hygiene (Morou-Bermúdez et al., 2022).

Lastly, characteristics of saliva can also influence the production of endogenous N_2O. The temperature of saliva, for example, plays a role, with temperatures over 30°C generally favoring DNRA (Morou-Bermúdez et al., 2022). Notably, there is also a correlation between lower N_2O production and lower temperatures, as identified in multiple studies (Cressey & Cridge, 2022). As the average temperature of oral fluid is approximately 30 degrees Celsius, temperature seems to favor DNRA in most cases.

Saliva clearance can also impact reduction pathways by molecule clearance from the oral cavity. This process refers to the rate at which substances are removed from the mouth by salivary flow, either naturally or through mechanical actions like swallowing. The influence of saliva clearance on N_2O production depends on several factors, such as the clearance rate and the adherence of bacteria to oral tissues, their position in the mouth, and the solubility of substances in saliva. The clearance time can range from a few minutes to several hour (Doel et al., 2005).

Enzymatic activity regulation and transport of molecules can also influence endogenous N_2O production. For example, in aerobic conditions the expression of nitric oxide reductase (Nor) may be inhibited by the nitrate reductase and nitric oxide reductase regulator (NNR), leading to NO accumulation. As mentioned before, enzyme activity also varies with pH. The denitrification NO_2^- reductase works best at a pH below 7, while the ammonia-producing NO_2^- reductase is most active above pH 7.5 (Morou-Bermúdez et al., 2022).
The salivary glands use sialin 2 NO_3^-/H^+ transporters to increase plasma NO_3^- concentration in saliva. The role of transporters, specifically sialin transporters, is vital in concentrating and regulating NO_3^- concentrations and could therefore also influence N_2O production. This process enriches saliva with NO_3^- from the plasma, affecting the oral microbiome and its metabolic processes (Rosier, Moya-Gonzalvez, et al., 2020). The functioning and defects of sialin affects can alter NO_3^- transport and thus influence N_2O production (Qu et al., 2016).

4.2.2. Nutritional factors

Several nutritional factors can influence endogenous N_2O production. One of these factors is nutrient availability provided by saliva to the oral biofilms. Concentrations of delivered nutrients and substrates, such as carbon and nitrogen sources, influence the preference for DNRA and denitrification. Other influences on N_2O production include concentrations of molecules like sulfide (S_2^-) and iron (Fe_2^-) (Morou-Bermúdez et al., 2022).
The nitrogen sources provide NO_3^- as a substrate for both processes, while different carbon sources (fermentable sources such as glucose or lactate, or nonfermentable sources such as acetate) act as electron donors. The C/NO_3^- ratio is crucial and favors DNRA with high ratios and denitrification with low ratios. For example, with lactate as a carbon source, a ratio of Lac/ NO_3^- of 2.97 favors DNRA, and a lower ratio of Lac/ NO_3^- of 0.63 favors denitrification (Morou-Bermúdez et al., 2022).
When the C/ NO_3^- proportions are equal, the NO_3^-/NO_2^- ratio determines the pathway (Morou-Bermúdez et al., 2022). A high NO_3^-/NO_2^- ratio favors denitrification and causes N_2O accumulation, as visible in Fig. 6a (Schreiber, 2009). Ammonium levels, produced by DNRA, do not influence N_2O production (Bleakley & Tiedje, 1982).

The influence of diet on NO and therefore N_2O production is diverse. Several beverages contain NO_3^- and NO_2^- with vegetables being the primary dietary source of NO_3^- and NO_2^-as illustrated in Table 4. In some countries, NO_3^- is also found in drinking water. Consuming vegetables high in NO_3^-, such as beetroot, spinach, and kale, stimulates NO_3^- metabolism (Hezel & Weitzberg, 2013). Vitamin C and polyphenols, dietary reducing compounds, can also enhance the reduction of NO_2^- to NO (Lundberg et al., 2008).
A NO_3^- rich diet appears to enhance N_2O production via denitrification, while a diet low in vegetables but high in processed meats lowers the NO_3^-/NO_2^- ratio, favoring DNRA. A low-sugar diet seems to have a positive influence on the NO production which in combination with

a NO_3^- rich diet increases NO and N_2O levels through denitrification (Morou-Bermúdez et al., 2022). Specifically, beetroot juice consumption notably increases the rate of oral N_2O accumulation due to its high NO_3^- content. Drinking 200 mL of beetroot juice can increase the oral N_2O accumulation rate by 3.8 to 9.1 times (Schreiber et al., 2010), with other studies confirming this correlation of N_2O levels increasing after beet juice consumption (Bonardo et al., 2022).

Dietary patterns significantly alter the microbiome's composition, with NO_3^- rich diets increasing denitrifying species like *Neisseria* and decreasing DNRA organisms (Morou-Bermúdez et al., 2022). To conclude, N_2O accumulation by denitrification seems to occur after dietary intake of NO_3^- (Schreiber et al., 2010).

Table 4. (Burleigh et al., 2018) present a detailed overview of the NO_3^- and NO_2^- content in common dietary products, measured in milligrams per 100 grams of food. Additionally, a categorization of vegetables based on their NO_3^- content is provided, ranging from 'Very Low' to 'Very High'.

Average NO_3^- and NO_2^- contents of vegetables, fruits, and processed meat samples

Vegetables (mg/100 g)			Fruits (mg/100 g)			Meats/Processed meats (mg/100 g)		
Type	*NO_3^-*	*NO_2^-*	*Type*	*NO_3^-*	*NO_2^-*	*Type*	*NO_3^-*	*NO_2^-*
Spinach	741	0.02	Apple	0.3	0.008	Bacon	5.5	0.38
Tomato	39.2	0.03	Banana	4.5	0.009	Ham	0.9	0.89
Salad mix	82.1	0.13	Fruit mix	0.9	0.08	Hot dog	9.0	0.05
Broccoli	39.5	0.07	Orange	0.8	0.02	Pork loin	3.3	0

Classifying vegetables according to NO_3^- content

Nitrate content (mg/100 g fresh weight)				
Very Low, <20	*Low, 20 to 50*	*Middle, 50 to 100*	*High, 100 to 250*	*Very High, >250*
Asparagus, eggplant, garlic, onion, green bean, pea, potato, sweet potato, tomato, watermelon	Broccoli, carrot, cauliflower, cucumber, pumpkin	Cabbage, dil, turnip, savoy, cabbage	Chinese cabbage, fennel, leek, parsley	Celery, cress, lettuce, red beetroot, spinach, rucola

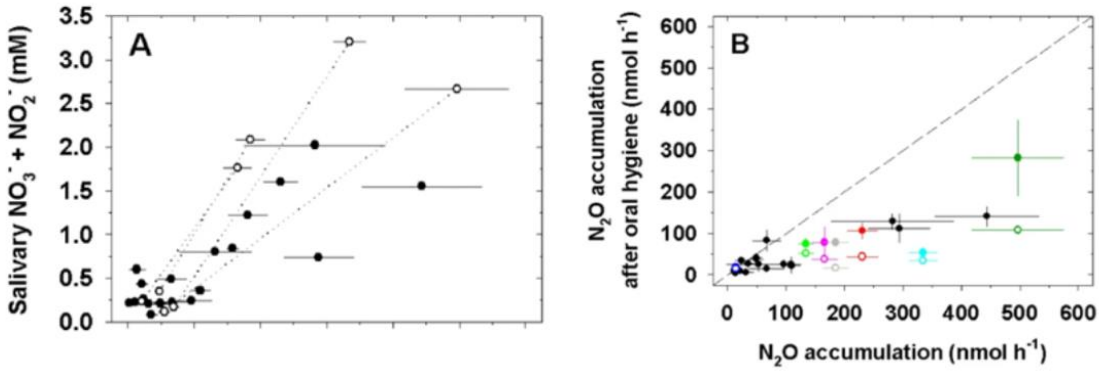

Figure 6. (Schreiber, 2009) present the correlation between salivary NO_3^-/NO_2^- ratios and N_2O production in the oral cavity, alongside the impact of oral hygiene practices on N_2O accumulation. Panel (A) shows the relationship of oral N_2O production with salivary NO_3^-/NO_2^- concentration in individuals with unbrushed teeth. Panel (B) shows the effect of tooth brushing on the rate of N_2O accumulation.

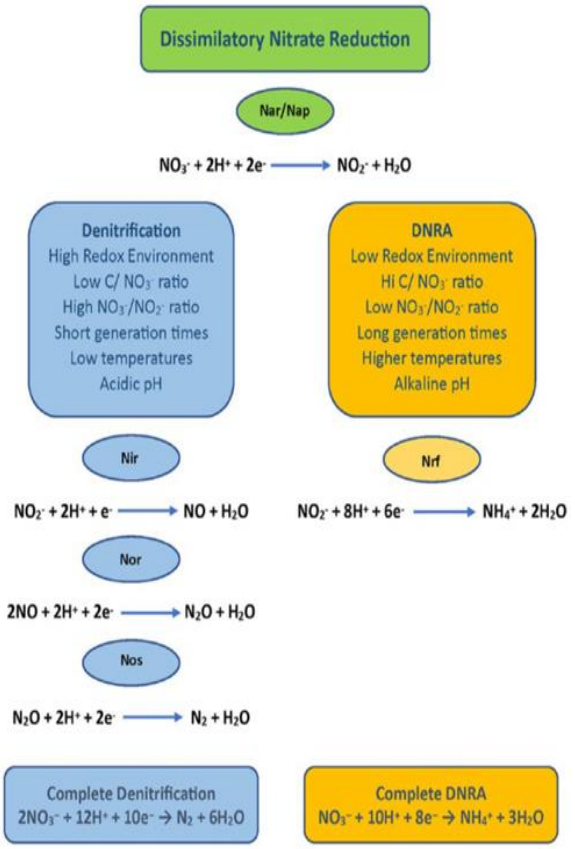

Figure 7. (Morou-Bermúdez et al., 2022) provide a visual summary of the pathways and environmental conditions influencing DRNA and denitrification. The left side (blue) describes denitrification under high redox conditions, leading to the complete reduction of NO_3^- to N_2, while the right side (yellow) illustrates DNRA prevalent in low redox environments, resulting in the formation of ammonium (NH_4). Environmental factors such as C/NO_3^- ratio, NO_3^- /NO_2^- ratio, temperature, and pH that dictate the preference of either pathway are also indicated.

4.2.3. Physiological factors

Numerous physiological factors can affect endogenous N_2O production. Firstly, gender differences significantly impact reductase capabilities and can therefore affect N_2O production. Females generally exhibit higher normalized oral NO_3^- reductase activity and baseline NO_2^- levels compared to males, suggesting a greater capacity for oral NO_3^- reduction. This is shown in Fig. 8, and can suggest a higher N_2O production as a high NO_3^-/NO_2^- ratio seems to favor denitrification. This difference in reductase activity may be caused by female sex hormones, particularly estradiol, which could enhance bacterial NO_3^- and NO_2^- reductase expression (Kapil et al., 2018). Estradiol or other sex hormones could cause varying levels of NaR and NiR gene expression, coding for NO_3^- reducing enzymes (Bahadoran et al., 2021). Sex hormone influences could therefore cause gender-based differences in reduction processes.

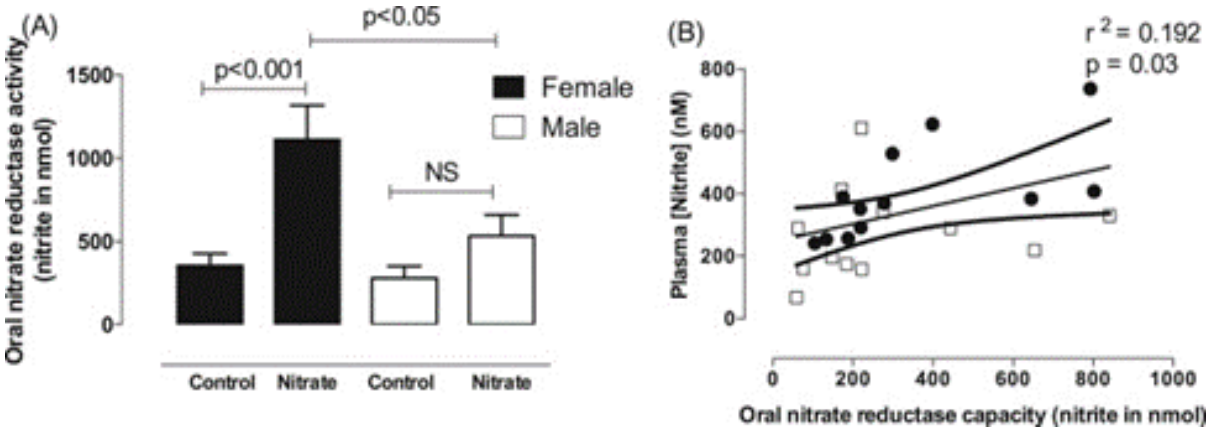

Figure 8. (Kapil et al., 2018) shows gender differences in the oral processing of (dietary) NO_3^-. Panel (A) compares oral NO_3^- reductase activity between males and females with and without NO_3^- supplementation, indicating significantly higher activity in females after NO_3^- intake. Panel (B) correlates the oral NO_3^- reductase capacity with plasma NO_2^- levels, showing a positive association.

N_2O production also appears to be influenced by age. First of all, oral NO_3^- reductase activity and age share a bell-shaped relationship, being extremely low at birth, peaking in middle age, and then declining in older age (see Fig. 9) (Ahmed et al., 2021). Although reductase activity decreases with age, concentrations of salivary NO_3^- and NO_2^- do keep increasing, also visible by an increase of dietary intake effect on NO_3^- and NO_2^- concentrations as age increases. Changes in NO_3^- reduction are proportional to the number of bacteria across all ages (Ahmed et al., 2021).

For oral N_2O levels there also is a significant correlation between age and endogenous concentrations. The percentage of N_2O producers decreases during puberty (ages 13-19), increases with age post-puberty, and reaches approximately 90% at the age of 60 (Mitsui & Kondo, 1998). A study found that older adults showed a higher percentage of N_2O producers (94.1%) compared to young adults (20.0%) after mouth cleansing, with mouth cleansing itself reducing exhaled N_2O in both groups (Mitsui et al., 1997). Changes in the immune system with aging may also influence N_2O production, with the immune system becoming more functional during puberty and deteriorating after puberty (Mitsui et al., 1997).

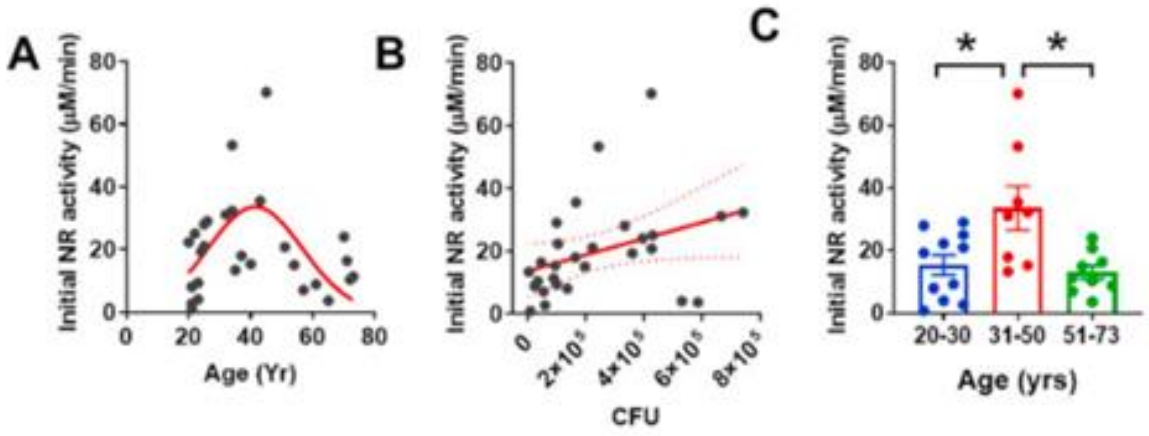

Figure 9. (Ahmed et al., 2021) show the correlation between age and nitrate reductase activity in the human oral cavity. Panel A depicts the non-linear regression of NO_3^- reductase (NR) activity across a wide age range. Panel B illustrates the linear relationship between NO_3^- reductase activity and colony-forming units (CFU). Panel C compares average initial NO_3^- reductase activity across three distinct age groups, with significant differences of NO_3^- reductase activity between age groups.

Several individual differences influence N_2O production, although the significance of these individual differences is not yet known.
Diseases, for example, can influence N_2O production. Individuals with Down Syndrome (DS) exhibit altered NO dynamics and thus N_2O production due to factors like oral and teeth anatomy, mouth breathing, motoric disability, dietary changes, genetics, and immune defects (Bonardo et al., 2022). In periodontitis or gum disease*, Rothia species*. and *Neisseria species* are more abundant healthy individuals compared to those with periodontitis, indicating higher oral N_2O levels (Rosier, Buetas, et al., 2020). In chronic gingivitis or gum irritations, NO_3-rich lettuce juice consumption increased the presence of *Neisseria and Rothia* indicating this same increase in N_2O concentrations (Rosier et al., 2022). Lastly, children with autism spectrum disorder (ASD) seem to generally have lower levels of *Prevotella,* bacteria that may be capable of producing N_2O (Sato et al., 2020).
The composition of the oral microbiome is also crucial for various aspects of oral health, including N_2O production. Microbiome diversity varies in both quality and quantity by ethnicity and geographic location, with significant individual variation, especially in older individuals (Hyde et al., 2014; Mitsui et al., 1997). NO_3^- can affects the microbiome composition by its function that can protect the oral microbiome against acidification from sugar fermentation. (Rosier et al., 2022).

4.2.4. Lifestyle and behavioral influences

Oral hygiene also significantly influences oral N_2O production. Poor oral hygiene promotes DNRA by forming thicker biofilms with lower redox potential and longer bacterial generation times, favoring NO_3^- reduction to NH_4 (Morou-Bermúdez et al., 2022). Conversely, effective plaque control leads to faster bacterial growth and enhanced denitrification with endogenous N_2O production. N_2O accumulation rates in mouth air serve could therefore as sensitive indicators for assessing oral hygiene practices, potentially useful in dental settings (Schreiber, 2009). Caries-associated species like *Streptococcus* and *Veillonella* are significantly reduced in the presence of NO_3^- (Rosier, Buetas, et al., 2020).
Regular tongue cleaning enriches NO_3^- reducing bacteria like *H. parainfluenza* and other Proteobacteria, enhancing NO_2^- reduction by denitrification (Tribble et al., 2019). Less frequent tongue cleaning leads to a microbiome primarily performing DRNA (Morou-Bermúdez et al., 2022). Furthermore, oral odor production also correlates with bacterial load, with high odor producers exhibiting significant increases in total bacterial abundance and specific bacterial groups, including gram-negative anaerobes which could perform denitrification (Hartley et al., 1996).
Mouthwash use, especially chlorhexidine, impacts the oral microbiome and inhibits the increase in salivary and plasma NO_2^- after NO_3^- consumption. Chlorhexidine use leads to a decrease in bacteria on the tongue and creates a more acidic environment (Bryan et al., 2022; Tribble et al., 2019). The last circumstance favors the production of N_2O. However, mouthwash and antiseptics also significantly decrease oral bacterial counts, impacting N_2O production in a negative way directly after use (Schreiber et al., 2010). What is also interesting is that a study found that about half the levels of N_2O are still found in exhaled air after mouth cleansing, suggesting other sources of N_2O production in the body (Mitsui & Kondo, 1998).

Lastly, exercise and substance (ab)use also affect NO pathways. Exercise, combined with dietary changes, can modify the oral microbiome to enhance NO production from NO_3^- sources (Vanhatalo et al., 2018). Cardiovascular benefits of exercise might be partly attributed to

increased NO levels due to stimulated NO pathways from exercise-induced shear stress (Bryan et al., 2022). An increase in NO production leads to more denitrification activity and an increase in N_2O production.

Substance use appears to have an impact on N_2O production. A study shows that methamphetamine use leads to changes in the oral microbiome. This was not specific to other bacteria but in the abundance of all types: more gram-negative bacteria like *Veillonella* were found, and gram-positive bacteria decreased. This could mean that N_2O production potentially increases (Deng et al., 2022).

The extent of alcohol use can also affect N_2O production. Alcohol consumption leads to lower saliva production, a lower pH in the mouth, and an oxygen-starved environment. On one hand, a lower pH is positive for N_2O production, but DRNA is more energetically favored in areas with limited oxygen. Several studies have found that alcohol consumption also leads to a difference in the oral microbiome, both qualitatively and quantitatively, at different locations in the mouth between drinkers and non-drinkers. *Prevotella*, for example, is enriched in alcohol drinkers, a bacterium that possesses the genes to produce N_2O (Liao et al., 2022).

Smoking also seems to influence N_2O production. For the tongue microbiome, there appears to be a significant difference between current smokers and non-smokers. However, this does not seem to be permanent, as there was no difference between former smokers and non-smokers. Various pathways seem to be influenced by smoking behavior. Bacteria, including *Neisseria*, are less abundant, which means that there may be less N_2O-producing bacteria in the mouth. Smoking further creates a more anaerobic environment, in which DRNA is stimulated (Sato et al., 2020). In another study, it was found that smoking significantly decreases NO_3- reductase activity, with smokers exhibiting approximately 70% decreased activity compared to non-smokers (see Fig. 10). This decline, coupled with higher bacterial counts and a nearly 90% lower NO_3^- reductase activity per colony-forming unit (CFU), underscores the inhibitory role of cigarette smoking on the enterosalivary pathway (Ahmed et al., 2017). These findings indicate that smoking inhibits the formation of endogenous N_2O.

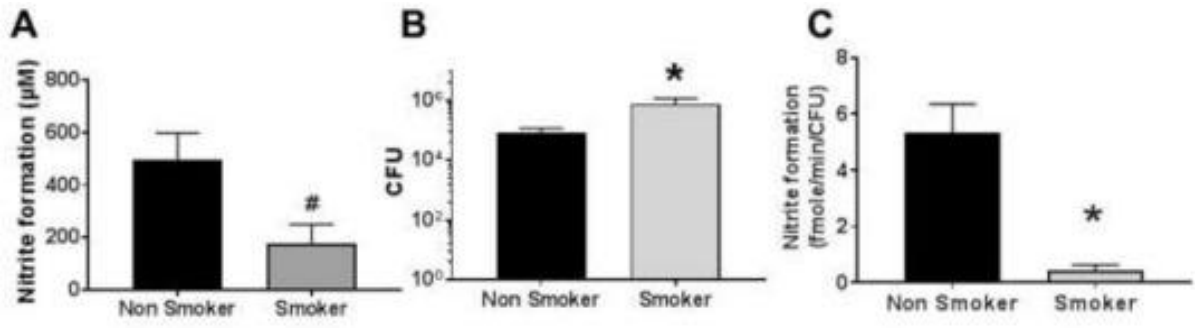

Figure 10. (Ahmed et al., 2017) shows the impact of smoking on oral NO_3^- reduction. Tongue scrapings from non-smokers and smokers reveal significant differences in NO_3^- reductase activity (Panel A), bacterial count (Panel B), and the initial rate of NO_3^- reduction activity normalized with colony-forming unit (CFU) counts (Panel C). The study indicates a reduction in NO_3^- reductase activity and a lower bacterial count in smokers.

4.3. Endogenous nitrous oxide concentrations

- *The exact concentration of oral N_2O is not known.*
- *Literature suggests that oral N_2O concentrations should be in the range of nanomolar to micromolar and increases after diet consumption.*

In the reviewed literature, specific data on endogenous N_2O concentrations was limited. However, concentrations of other related compounds were frequently mentioned. In table 5 an overview of data in literature is given with average determined concentration ranges.

Table 5. Comprehensive overview of literature data on different concentrations mentioned in literature of NO_3^-, NO_2^-, NO and N_2O. An average concentration range of each molecule is also estimated.

Molecule	Concentration mentioned in literature	Average concentration range	Source
NO_3^-	Ingestion of beet juice (BJI) significantly elevates salivary NO_3^- levels to approximately 23.7 mM.	Millimolar to micromolar	(Burleigh et al., 2018)
	Under basal conditions, saliva generally exhibits NO_3^- concentrations about 10 times higher than in plasma, typically within the range of 100–500 µM.		(Rosier, Moya-Gonzalvez, et al., 2020)
	During fasting, salivary NO_3^- concentrations are around 100–500 µM, while plasma NO_3^- levels are significantly lower, approximately 10–50 µM. After consuming a NO_3^- rich meal, the concentrations is between 5–8 mM.		(Rosier, Moya-Gonzalvez, et al., 2020).
NO_2^-	Typically, after a high NO_3^- diet, human saliva can contain around 1000 nmol/ml of NO_2^-.	Millimolar to nanomolar	(Li et al., 1997)
	The ingestion of beet juice markedly increases salivary NO_2^- levels to 14 mM.		(Burleigh et al., 2018)
	Fasting plasma NO_2^- levels are usually found in the 50–100 nM range, significantly lower (100 to 1000 times) than salivary NO_2^- concentrations. This difference becomes even more pronounced after NO_3^- intake.		(Hezel & Weitzberg, 2013)
	The use of mouthwash impacts NO_3^- reduction, with an observed average reduction rate of 85.4 ± 15.9 nmol NO_2^- min−1 after using a 10 ml 1 mm KNO_3^- mouthwash.		(Doel et al., 2005)
NO	No data in literature	-	-
N_2O	N_2O concentrations seem to increase in after BJI.	Micromolar to nanomolar	(Burleigh et al., 2018).
	The yields of N_2O production from NO_3^- respiring bacteria have been recorded to range between 3% to 36%. With an initial 3.5 mM NO_3^- concentration.		(Bleakley & Tiedje, 1982)

NO_3^- concentrations are typically the highest found in this cycle, which aligns with the fact that NO_3^- is the starting point and is supplemented by dietary ingestion. Different concentrations were found in literature. Table 5 shows NO_3^- concentrations in the range of micro- to millimolar. Regarding NO_2^- concentrations, also several concentrations were found in literature. NO_2^- concentrations in the oral cavity are usually in the range of nano- to millimolar. Lastly, no data was found on NO and N_2O concentrations. However, literature suggests that oral N_2O concentrations could in the range of nano- to micromolar.

4.4. Oral fluid drug testing

Oral fluid drug testing in general:

- *Oral fluid drug testing is sensitive and minimally invasive, suitable for remote and on-site applications like DUID, delivering both qualitative and quantitative results.*
- *Accurately correlates with plasma drug levels but is less impacted by N_2O's typically low plasma concentrations.*
- *Sampling can by done by various collection methods, including spitting, pad foams, and tongue wipes, with ng/mL detection thresholds.*
- *Effectiveness varies by drug type and the method faces certain collection and testing limitations.*

Oral fluid drug testing for N_2O:

- *Endogenous N_2O may impact drug testing outcomes as levels seem to impass the ng/mL threshold, with the estimated concentration range of nanomolar to micromolar*
- *Qualitative tests with concentration thresholds can mitigate endogenous effects for law enforcement.*
- *Pad foams and mouthwash are recommended to decrease endogenous N_2O in samples.*
- *Individual differences and detection sensitivity remain areas for future research.*

In this chapter, the current knowledge about oral fluid testing will be discussed. Firstly, oral fluid in general will be discussed along with the advantages of this method compared to other methods. After this, the focus will be on oral fluid collection methods and their analysis, including cut-off values, sensitivity, and specificity. Complications of this method will also be highlighted. Lastly, the potential link will be made to N_2O and oral fluid drug testing, including limitations, implications and possible standard procedures.

4.4.1. Oral fluid testing in general

Oral fluid testing seems to be gaining popularity in recent years due to scientific and regulatory advancements, potentially making it a reliable alternative testing method (Lee, 2020). Its current use is primarily focused on testing in the workplace or for alcohol testing (Kadehjian, 2005). Oral fluid drug testing is also being explored as a possibility for various drugs, such as amphetamines, cocaine (metabolites), opioids, and cannabis (Drummer, 2006).

Several studies highlight the advantages of oral fluid drug testing. See table 6 for a comparison between the available drug testing methods regarding several characteristics. Table 6 shows that oral fluid drug testing is a method with advantages compared to blood and urine tests. The first advantage is that oral fluid drug testing is highly sensitive and can specifically measure the parent drug instead of potential metabolites (Drummer, 2006; Lee, 2020). Another advantage of oral fluid testing is its correlation with blood plasma, reflecting recent drug use more accurately than urine tests (Kadehjian, 2005). Specifically basic drugs have similar amounts in oral fluid as in plasma (Drummer, 2006). Table 7 shows the ratios of various drugs' concentrations in oral fluid to blood.

Recent research is also being conducted on remote oral fluid drug testing, where the processing of results is done remotely, and the oral fluid collection itself is carried out by clients. Remote testing could also have a role in law enforcement. Remote testing has advantages such as speed, minimal invasiveness, and measurement of very frequent drug use. This makes the use of this method potentially possible for law enforcement. Regarding validation and reliability limited data is available (Khazanov et al., 2023).

Table 6. This table outlines the relative invasiveness, immediacy, and accuracy of blood, breath, and oral fluid tests used by law enforcement for on-site evaluations.

	Blood test	**Breathing test**	**Oral fluid test**
Invasiveness	Invasive	Non-invasive	Non-invasive
Sample collection	Medical professional needed	Officer can administer on-site	Officer can administer on-site
Time to detect	Can take hours (transport to lab + analysis)	Results within minutes	Results within minutes to a few hours
Accuracy	High (gold standard in many regions)	Moderate to high (depends on substance)	Moderate to high
Selectivity	High (can differentiate between substances)	Moderate (possible cross-reactivity)	High (with specific immunoassays)
Reproducibility	High (standardized procedures)	High (with proper calibration)	High (with controlled use)
Complications	Requires medically trained personnel, delay in testing	Few; requires calibration	Few
Sample storage	Possible	Not possible	Possible

Table 7. Retrieved from (Drummer, 2006). shows the average oral fluid to blood concentration ratios for several drugs. The average ratios are indicative and can vary due to various factors such as pH, protein binding, the degree of contamination of the membrane in the oral cavity.

Drug (type)	**Average oral fluid to blood concentration ratio**
Alcohol (ethanol)	1.07
Barbiturates	0.3
Buprenorphine	1
Codeine (basic)	4
Methamphetamine (basic)	2
MDMA (basic)	7
Cocaine (basic)	3
Diazepam (acidic)	0.01–0.02
Methadone (basic)	1.6
Morphine (basic)	0.8
Δ9- Tetrahydrocannabinol (neutral)	1.2

4.4.2. Oral fluid sample collection

Oral fluid is a convenient matrix to measure because it primarily consists of a large water component and has a lower protein content compared to blood, which does not make the recovery of drugs a limiting factor (Drummer, 2006). The collection method is also non-invasive in terms of collection compared to blood or urine tests (Lee, 2020). Various collection methods are available for obtaining oral fluid.

Firstly, it's possible to collect oral fluid through expectoration, or spitting. A disadvantage is that this often results in neat oral fluid which is very viscous and difficult to work with. It can also often be contaminated with food or other debris, making a centrifugation step needed. Frequently there isn't enough volume obtained by expectoration, as often 1 mL is required (Drummer, 2006).
Another method is collection via an absorbent pad/foam that can be held against the inside of the cheek for fluid absorption. This can then be mixed with a diluent, which is then squeezed out into the measuring device (Drummer, 2006; Khazanov et al., 2023).
Tongue wipes or scrapings can also be used (Drummer, 2006). This is also a testing method used for measuring nitrate reductase activity, for example (Mitsui et al., 1997). However, a disadvantage here is the physical constraint due to the gag reflex, which disadvantages testing at the back of the tongue (Hartley et al., 1996).

Oral fluid collection has several implications. Firstly, there is often a lack of fluid. This can be resolved by stimulating saliva production, for example by eating. However, this changes the pH and concentration of the drug in the oral fluid. Literature provides various examples of a decrease in drug concentration after saliva stimulation: codeine concentrations decrease by about two- to six-fold, two- to four-fold for methamphetamine, and about five-fold for cocaine. Dry mouth syndrome can also occur more frequently. Due to anxiety and dehydration, little oral fluid is produced, which extends the collection time several minutes for 1 mL sample volume (Drummer, 2006).
Furthermore, literature has also investigated a number of possible influencing factors. Foodstuff and toothpaste seem not to change the concentration of drugs of abuse. Some drugs can modify the production of oral fluid, which affects the concentration. Mouthwash, however, seems to have no effect on the concentration of drugs. Alcohol consumption can lower the concentration of other drugs. This is the case, for example, for THC 1h post-dose. This could be solved by waiting 20 mins before collection (Drummer, 2006).

4.4.3. Oral fluid sample analysis

The analysis of the collected drugs is often performed using mass spectrometry (MS), and more specifically liquid chromatography (LC)-MS, due to the low sample volumes and low detection limits (Lee, 2020). Sample sizes of 0.1 to 0.5 mL are often maintained (Drummer, 2006).
Drugs are usually detectable in oral fluid at ng/mL levels within 24 hours, so the cut-off for tests is often set in this range (Kadehjian, 2005). Table 8 lists different cut-offs for various drugs that have been determined from the literature. For THC for example, one study found that THC concentrations in oral fluid were at 26 ng/mL after high passive exposure (Drummer, 2006).
In terms of sensitivity, a study determined that oral fluid testing has a higher specificity (and

NPV) for various drugs but a low sensitivity and PPV, meaning not all positive drug test results are recognized. Specifically for cannabis, these numbers were lower, and the following values were found: a specificity of 0.84–0.89 and sensitivity of 0.07–0.23. Results varied for all drugs, and in the case of cannabis, this is also due to the detection window for oral drug testing (Khazanov et al., 2023).

Table 8. Retrieved from (Drummer, 2006), you can see the various drugs, the analysis method used, and the limit of quantification (LOQ) for measuring these drugs, all of which are in the range of ng/mL.

Drug	Method Type	LOQ (volume of specimen) ng/Ml
Amphetamines (AM, MA, MDMA, MDEA etc)	GC-MS	20 (100 µL)
	LC-MS	2 (50–200 µL)
Benzodiazepines	LC-MS	500 µL (0.1–0.2 ng/mL)
Cocaine*	LC-MS	2 (200–250 µL)
	LC-MS2 (APCI)	1 (200 µL)
THC	LC-MS	2 (500 µL)
Morphine/6-AM	LC-MS	2 (200–250 µL)
	LC-MS2 (APCI)	1 (200 µL)
Methadone	LC-MS2 (APCI)	1 (200 µL)
	GC-MS	

4.4.4. Oral fluid drug test complications

Oral fluid drug testing method has various complications. The pharmacokinetics of drugs in oral fluid are very complex, partly due to the complexity of oral fluid as a matrix and also individual variations within and between subjects should be considered (Drummer, 2006; Lee, 2020).

First, there are several testing complications. The detection time is limited and depends on the dose and frequency of use, and the time since the last intake (Drummer, 2006; Kadehjian, 2005). Complications can also occur in the administration of the drug. For the analysis of the samples, cutoff concentrations must be considered, and account must be taken of drug formulation impurities, medical conditions affecting oral fluid, secondhand exposure (Lee, 2020).
The drug source in oral fluid must also be considered. The drug source in oral fluid isn't limited to saliva; secretions from the nasal cavity and esophagus, as well as contamination from various administration routes, must be considered as this can cause elevated drug concentrations (Lee, 2020).
The application of oral drug testing differs per drug and depends on various drug characteristics. The concentration of the drug in the fluid depends on the stability of the drug. This is influenced by many factors, such as saliva composition, pH, and flow rate, drug pKa, protein binding affinity, lipophilicity, molecular size (Lee, 2020). Studies have shown that drugs with rapid bioconversion also show metabolites on the oral fluid drug test. For cocaine, for example, benzoylecgonine and ecgonine methyl ester have been found (Drummer, 2006).

When a drug is administered, there is a local increase in concentration in this absorptive phase. For THC, this is the case due to its high fat solubility, easy membrane penetration and low partitioning from blood to oral fluid. This can give a distorted picture of the overall concentration (Drummer, 2006).

4.4.5. Nitrous oxide and oral fluid drug testing

Currently, there is no literature on oral fluid drug testing methods for N_2O. Should a method be implemented, the concentration of endogenous N_2O should be considered, as according to chapter 4.3 should be in the range of nano- to micromolar. This concentration range of endogenous N_2O can influence the results of an oral fluid drug test, considering the detection limit mentioned (in chapter 4.4) of ng/mL, meaning that an oral fluid drug test could also detect endogenous N_2O levels only. The final chapter of the results discusses the method considerations and limitations for the implementation of oral fluid drug testing for N_2O.

As described in the introduction, recreational N_2O use seems to necessitate an oral fluid drug test within law enforcement. In the literature, oral fluid drug testing appears to focus primarily on quantification methods. However, for law enforcement, qualification for N_2O is important.
The problem is that endogenous N_2O can influence a qualitative oral fluid drug test result. A method consideration for this could be to maintain a concentration threshold, which should ensure that endogenous N_2O does not affect drug test results. However, research must be conducted on the concentration of exogenous N_2O after drug use and more research on the specific concentrations of endogenous N_2O to determine whether one specific threshold works, or if individual differences in N_2O concentrations make a qualitative test unfeasible.

In determining the best possible sample collection method for N_2O, pad foams against the cheek could be an option. Since N_2O is primarily produced around dental plaques, sampling at these sites should ideally be avoided to minimize contamination. Tongue wipes might also be a potential method, but they come with limitations, as N_2O is produced on the dorsal side of the tongue, and the gag reflex could make sampling challenging. Stimulating saliva production with food should decrease the concentration of N_2O in the oral cavity. As mentioned earlier, mouthwash does not seem to affect exogenous N_2O concentrations but does lower endogenous N_2O concentration. Incorporating this into the testing procedure could reduce endogenous N_2O levels. Alcohol consumption can decrease the concentration of other drugs, but this issue could be addressed by waiting 20 minutes before collection. If alcohol is detected when N_2O usage is also suspected, wait 20 minutes before collecting samples in the testing procedure, but keep in mind that waiting could also reduce the exogenous N_2O concentration in oral fluid by swallowing.

There are some possible limitations for N_2O oral fluid drug testing. As previously discussed, individual differences can significantly impact endogenous N_2O production. However, considering individual factors is challenging due to the complexity and variability among individuals, but it's essential to keep in mind for oral drug testing regulations and data interpretation.
As N_2O has a very low half-life, it is expected that this compound presents a low detection window, potentially complicating drug analysis as it would be necessary to measure exogenous N_2O immediately after use, which is impractical in real-world scenarios.
Factors as the pH can also impact both the testing process and the production of substances,

just as gender and age influence endogenous N_2O production. Future research is however needed on the exact influence of individual influences.
Another characteristic, usually a limitation of oral fluid drug testing is the local increase in drug concentration immediately after use, which can be significantly higher than the overall concentration, especially for THC. Such characteristics could be advantageous for exogenous N_2O detection, potentially resulting in a high exogenous N_2O concentration peak in detection that could be significantly higher than the concentration of endogenous N_2O.
Lastly, some drugs can influence oral fluid production, thereby affecting saliva dynamics. In dental settings, N_2O inhibits saliva production and clearance, so illegal N_2O usage might increase the endogenous N_2O concentration ((z.d.), z.d.).

5. Conclusion

In this review, the potential influence of endogenously produced N_2O on oral fluid drug testing results for law enforcement has been examined. To investigate this, an effort was made to outline the production of endogenous N_2O.

4.1. Identification of sources It is found that endogenous N_2O is produced by oral bacteria via nitric oxide reductase (NOR) primarily at dental plaque and tongue sites.
4.2. Influencing factors on N_2O production This endogenous N_2O process is influenced by several factors. Intrinsic factors such as pH, oxygen, temperature, and nutrient levels, with low pH and carbon, aerobic conditions, higher temperatures, and higher nitrate-to-nitrite ratios particularly enhancing denitrification and N_2O output. Endogenous N_2O production is also modulated by genetic and lifestyle factors, including gender, age, diet, oral hygiene practices, and substance use, each influencing the denitrification process differently.
4.3. Endogenous N_2O concentration Although information about the concentration of N_2O produced in oral fluids is limited, the findings suggest that N_2O concentrations should be in nano- to micromolar ranges.
4.4. Oral fluid drug testing: General Oral fluid drug testing offers a sensitive, less invasive way to measure drugs directly, the possibility of saliva storage, with concentrations aligning well with plasma levels. Despite its utility, especially for on-site law enforcement use like in DUID cases, there are practical challenges in sample collection and analysis. This method can be used with various collection techniques and operates with detection limits in the ng/mL range but faces limitations in its application across different substances.
4.4. Oral fluid drug testing: N_2O For law enforcement, a qualitative N_2O oral fluid drug test would be optimal. Endogenous N_2O, potentially exceeding the ng/mL detection limit, can influence oral fluid drug testing results, particularly with samples at sites like dental plaque or the tongue where its N_2O production is high. Implementing procedures like using mouthwash and avoiding high N_2O production areas, alongside setting a concentration threshold, can minimize its impact on results. Sampling with pad foams is recommended.
Future research Limitations in the research included the lack of age, ethnicity, and gender information of subjects, and many studies also had small sample sizes. Direct information on endogenous N_2O production is scarce. Future research on the sources could, for example, involve specific studies on NO reduction to N_2O via Nor in the oral cavity, the role of oral fungi, and regulation at gene and enzymatic levels. For factors influencing production, further exploration is needed on the impact of oxygen levels and temperature on N_2O production, and the research should also investigate other sites in the body that could contribute to N_2O production. Lastly, research into the actual concentration of N_2O is crucial to determine its influence with certainty and to establish specific test procedures and thresholds in oral fluid drug testing. The sensitivity and specificity of oral drug testing are drug-specific, indicating the need for research specifically focusing on N_2O and oral fluid drug testing.

In conclusion, interpreting N_2O oral fluid drug tests involves several challenges such as determining a threshold concentration, addressing low detection times and minimizing individual differences. To reduce the effect of endogenous N_2O on oral fluid drug test results, a suitable method would require high accuracy, sensitivity, and specificity, incorporating an N_2O concentration threshold and a (pre) sampling procedure to reduce endogenous N_2O effects on oral fluid test results. This makes oral fluid drug testing a viable option in law enforcement for N_2O testing due to its quick, reliable, and non-invasive results, with many advantages compared to other methods.

6. Appendix

6.1. Used search terms

Used search terms for conducting this research:

- *"bacterial nitrate reduction oral cavity" for exploring bacterial processes of nitrate conversion in the mouth.*
- *"oral microbiome nitrite reduction pathways" for investigating the mechanisms by which oral bacteria reduce nitrites.*
- *"nitric oxide reduction oral bacteria" for understanding how oral microorganisms contribute to nitric oxide turnover to nitrous oxide.*
- *"non-bacterial oral nitrous oxide sources " for identifying non-microbial contributors to nitrous oxide production in the oral environment.*
- *"oral nitrate and nitrite reductases" for identifying enzymes responsible for nitrate and nitrite reduction in saliva.*
- *"nitric oxide reductase activity in oral cavity" for exploring the role of nitric oxide reductase in the mouth's biochemical processes.*
- *"salivary nitrous oxide reductase enzymes" for examining the presence and function of nitrous oxide reductase in saliva.*
- *"gene expression oral nitrous oxide production " for studying genetic factors influencing nitrous oxide levels in oral fluid.*
- *"nitrous oxide production sites tongue" for identifying specific areas of the tongue involved in nitrous oxide synthesis.*
- *"salivary glands nitrous oxide" for assessing the role of saliva-producing glands in nitrous oxide production.*
- *"dental biofilms and nitrous oxide" for exploring the impact of dental health and biofilms on nitrous oxide levels.*
- *"saliva pH impact nitrous oxide" for understanding how saliva's acidity or alkalinity affects nitrous oxide production.*
- *"oxygen tension oral nitrous oxide production" for studying the effect of oxygen levels in the mouth on nitrous oxide synthesis.*
- *"enzymatic regulation oral reductases" for identifying how enzyme activity in saliva modulates nitrous oxide production.*
- *"nutrient availability nitrous oxide" for assessing the impact of available nutrients in the oral cavity on nitrous oxide synthesis.*
- *"genetic factors oral nitrous oxide production" for exploring how genetics may influence nitrous oxide levels in the mouth.*
- *"gender oral nitrous oxide levels" for assessing differences in nitrous oxide production between genders.*
- *"oral diseases oral nitrous oxide production" for understanding how various oral health conditions impact nitrous oxide levels.*
- *"age oral nitrous oxide" for exploring how nitrous oxide production in the mouth changes with age.*
- *"dietary influence nitrous oxide" for investigating how different foods impact nitrous oxide concentrations in saliva.*
- *"oral hygiene oral nitrous oxide" for assessing the impact of oral health practices on nitrous oxide production.*
- *"lifestyle habits oral nitrous oxide" for exploring how lifestyle choices like exercise and smoking affect nitrous oxide levels in the mouth.*
- *"oral fluid drug testing" for understanding the overall methodologies and applications of testing saliva for various substances.*
- *"Oral fluid collection" for examining the techniques and best practices for collecting saliva samples effectively and efficiently.*
- *"Oral fluid sample analysis" for exploring the methods used to analyze saliva samples for the presence of various compounds.*
- *"Oral fluid drug test complications" for identifying potential issues and challenges associated with testing saliva for drugs and other substances.*

6.2. Identification sources overview

This appendix provides a comprehensive overview of genes, bacteria, and enzymes mentioned throughout the text, detailing their roles, functions, and endogenous N_2O production.

Table 9. The characteristics of various oral microbiome species, highlighting their gram stain properties, oxygen tolerance, typical locations within the oral cavity, reduction capacities for nitrogenous compounds, and their significance in the nitrogen cycle and overall oral health.

Gram Stain / Oxygen Tolerance	**Species Examples**	**Reduction Capacities (NO_3^-/NO_2^-/NO/N_2O)**	**Relevance in Review**
Gram-negative, strict anaerobe	*Veillonella atypica, Veillonella dispar, Veillonella parvula, Prevotella species, Selenomonas species, Fusobacterium nucleatum, Tannerella species, Porphyromonas species, Eikenella corrodens*	YES/YES/Possible/Possible	These species are major reducers of NO_3^- and NO_2^-, with *Veillonella species* being highly relevant to N_2O production due to their potential for reducing NO to N_2O.
Gram-positive, facultative anaerobe	*Actinomyces odontolyticus, Actinomyces naeslundii, Rothia mucilaginosa, Rothia aeria, Rothia dentocariosa, Corynebacterium species (e.g., Corynebacterium pseudodiphtheriticum), Streptococcus species (e.g., Streptococcus mutans, Streptococcus mitis, Streptococcus parasanguinis)*	YES/YES/Not determined/Possible	This group is capable of both aerobic and anaerobic reduction, indicating a flexible response to oxygen levels in the oral cavity.
Gram-negative, microaerophile	*Campylobacter concisus, and other Campylobacter species*	NO/NO/NO/YES (limited information)	Though data is limited, microaerophiles like *Campylobacter* may influence N_2O levels.
Gram-positive, aerobe	*Nocardia species*	YES/NO/Possible/Not determined	Aerobes are less studied in the context of oral denitrification but could be relevant under specific microaerophilic conditions.

Table 10. This table summarizes the key genes and their corresponding enzymes responsible for the sequential reduction of nitrogen compounds in the denitrification process within the oral cavity. Each letter in the genes section indicates a different location in the cell.

Gene	Codes for enzyme	Function	Relevance in Review
NR or Nar (G,H,I,J) Nap (A,B,C,D) Nas	Nitrate reductase	Catalyzes the reduction of NO_3^- to NO_2^-	Central to denitrification; presence in major reducers suggests an active NO_3^- reduction in the oral cavity
Nir (S,M,C,F,D,L,G,H,J,E,N)	Nitrite reductase	Reduces NO_2^- to NO	Though less frequently present, plays a crucial role in the production of NO, which is a precursor to N_2O
Nor (qNor, cNor)	Nitric oxide reductase	Converts NO to N_2O	Most prevalent reductase, indicating its importance in N_2O production within the oral cavity
Nos (L,Y,F,D,R,Z,X)	Nitrous oxide reductase	Converts N_2O to N_2	Essential for reducing N_2O to N_2, completing the denitrification process and maintaining nitrogen balance.
eNOS	Nitric oxide synthase	Produced NO from arginine	Alternative way of generating NO, that can be reduced to N_2O

7. References

Aerts, L., Morais, J., Evans-Brown, M., Jorge, R., Gallegos, A., Christie, R., Néfau, T., Planchuelo, G., Sedefov, R., Victorri-Vigneau, C., Povilanskienė, R., Grasaasen, K., Palmqvist, D., Mongan, D., Killeen, N., Duarte, O., & Santos, A. (2022). *Recreational use of nitrous oxide: A growing concern for Europe*. https://doi.org/10.2810/2003

Ahmed, K. A., Kim, K., Ricart, K., Van Der Pol, W., Qi, X., Bamman, M. M., Behrens, C., Fisher, G., Boulton, M. E., Morrow, C., O'Neal, P. V., & Patel, R. P. (2021). Potential role for age as a modulator of oral nitrate reductase activity. *Nitric Oxide : Biology and Chemistry*, *108*, 1-7. PubMed. https://doi.org/10.1016/j.niox.2020.12.001

Ahmed, K. A., Nichols, A. L., Honavar, J., Dransfield, M. T., Matalon, S., & Patel, R. P. (2017). Measuring nitrate reductase activity from human and rodent tongues. *Nitric Oxide : Biology and Chemistry*, *66*, 62-70. PubMed. https://doi.org/10.1016/j.niox.2017.04.001

Bahadoran, Z., Mirmiran, P., Carlström, M., & Ghasemi, A. (2021). Inorganic nitrate: A potential prebiotic for oral microbiota dysbiosis associated with type 2 diabetes. *Nitric Oxide*, *116*, 38-46. https://doi.org/10.1016/j.niox.2021.09.001

Bleakley, B. H., & Tiedje, J. M. (1982). Nitrous oxide production by organisms other than nitrifiers or denitrifiers. *Applied and Environmental Microbiology*, *44*(6), 1342-1348. PubMed. https://doi.org/10.1128/aem.44.6.1342-1348.1982

Bonardo, B., Estu, A., Maulani, B., dewi, A., & Saskianti, T. (2022). Role of Salivary Nitric Oxide on Caries Status of Children with Down Syndrome. *Journal of International Dental and Medical Research*, *14*, 1611-1616.

Brunt, T. M., van den Brink, W., & van Amsterdam, J. (2022). Mechanisms Involved in the Neurotoxicity and Abuse Liability of Nitrous Oxide: A Narrative Review. *International Journal of Molecular Sciences*, *23*(23), 14747. PubMed. https://doi.org/10.3390/ijms232314747

Bryan, N. S., Burleigh, M. C., & Easton, C. (2022). The oral microbiome, nitric oxide and exercise performance. *Nitric Oxide*, *125-126*, 23-30. https://doi.org/10.1016/j.niox.2022.05.004

Burleigh, M. C., Liddle, L., Monaghan, C., Muggeridge, D. J., Sculthorpe, N., Butcher, J. P., Henriquez, F. L., Allen, J. D., & Easton, C. (2018). Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. *Free Radical Biology and Medicine*, *120*, 80-88. https://doi.org/10.1016/j.freeradbiomed.2018.03.023

Cressey, P., & Cridge, B. (2022). Exposure to nitrate from food and drinking-water in New Zealand. Can these be considered separately? *Food Additives & Contaminants: Part A*, *39*(5), 838-852. https://doi.org/10.1080/19440049.2022.2037725

Deng, Z., Guo, K., Cao, F., Fan, T., Liu, B., Shi, M., Liu, Y., & Ma, Z. (2022). Altered dental plaque microbiota correlated with salivary inflammation in female methamphetamine users. *Frontiers in Immunology*, *13*, 999879-999879. PubMed. https://doi.org/10.3389/fimmu.2022.999879

Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M., & Allaker, R. P. (2005). Evaluation of bacterial nitrate reduction in the human oral cavity. *European Journal of Oral Sciences*, *113*(1), 14-19. https://doi.org/10.1111/j.1600-0722.2004.00184.x

Drummer, O. H. (2006). Drug testing in oral fluid. *The Clinical Biochemist. Reviews*, *27*(3), 147-159.

Feng, J., Liu, J., Jiang, M., Chen, Q., Zhang, Y., Yang, M., & Zhai, Y. (2023). The Role of Oral Nitrate-Reducing Bacteria in the Prevention of Caries: A Review Related to Caries and Nitrate Metabolism. *Caries Research*, *57*(2), 119-132. https://doi.org/10.1159/000529162

González-Soltero, R., Bailén, M., de Lucas, B., Ramírez-Goercke, M. I., Pareja-Galeano, H., & Larrosa, M. (2020). Role of Oral and Gut Microbiota in Dietary Nitrate Metabolism and Its Impact on Sports Performance. *Nutrients*, *12*(12), 3611. PubMed. https://doi.org/10.3390/nu12123611

Hartley, M. G., El-Maaytah, M. A., McKenzie, C., & Greenman, J. (1996). The Tongue Microbiota of Low Odour and Malodorous Individuals. *Microbial Ecology in Health and Disease*, *9*(5), 215-223. https://doi.org/10.3109/08910609609166462

Hezel, M., & Weitzberg, E. (2013). The oral microbiome and nitric oxide homoeostasis. *Oral Diseases*, *21*(1), 7-16. https://doi.org/10.1111/odi.12157

Hino, T., Matsumoto, Y., Nagano, S., Sugimoto, H., Fukumori, Y., Murata, T., Iwata, S., & Shiro, Y. (2010). Structural Basis of Biological N 2 O Generation by Bacterial Nitric Oxide Reductase. *Science*, *330*(6011), 1666-1670. https://doi.org/10.1126/science.1195591

Hyde, E. R., Andrade, F., Vaksman, Z., Parthasarathy, K., Jiang, H., Parthasarathy, D. K., Torregrossa, A. C., Tribble, G., Kaplan, H. B., Petrosino, J. F., & Bryan, N. S. (2014). Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: Implications for nitric oxide homeostasis. *PloS One*, *9*(3), e88645-e88645. PubMed. https://doi.org/10.1371/journal.pone.0088645

Kadehjian, L. (2005). Legal issues in oral fluid testing. *Forensic Science International*, *150*(2-3), 151-160. https://doi.org/10.1016/j.forsciint.2004.11.024

Kapil, V., Rathod, K. S., Khambata, R. S., Bahra, M., Velmurugan, S., Purba, A., S. Watson, D., Barnes, M. R., Wade, W. G., & Ahluwalia, A. (2018). Sex differences in the nitrate-nitrite-NO• pathway: Role of oral nitrate-reducing bacteria. *Free Radical Biology and Medicine*, *126*, 113-121. https://doi.org/10.1016/j.freeradbiomed.2018.07.010

Khazanov, G. K., Ingram, E., Lynch, K., Trim, R., McKay, J., & Oslin, D. W. (2023). Validity and reliability of in-person and remote oral fluids drug testing compared to urine drug testing. *Drug and Alcohol Dependence*, *250*, 110876. https://doi.org/10.1016/j.drugalcdep.2023.110876

Kroes, I., Lepp, P. W., & Relman, D. A. (1999). Bacterial diversity within the human subgingival crevice. *Proceedings of the National Academy of Sciences of the United*

States of America, *96*(25), 14547-14552. PubMed. https://doi.org/10.1073/pnas.96.25.14547

Lee, D. (2020). Oral Fluid Testing. *Principles of Forensic Toxicology*, 629-656. https://doi.org/10.1007/978-3-030-42917-1_38

Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry*, *75*, 11-25. https://doi.org/10.1016/j.soilbio.2014.03.021

Li, H., Duncan, C., Townend, J., Killham, K., Smith, L. M., Johnston, P., Dykhuizen, R., Kelly, D., Golden, M., Benjamin, N., & Leifert, C. (1997). Nitrate-reducing bacteria on rat tongues. *Applied and Environmental Microbiology*, *63*(3), 924-930. PubMed. https://doi.org/10.1128/aem.63.3.924-930.1997

Liao, Y., Tong, X.-T., Jia, Y.-J., Liu, Q.-Y., Wu, Y.-X., Xue, W.-Q., He, Y.-Q., Wang, T.-M., Zheng, X.-H., Zheng, M.-Q., & Jia, W.-H. (2022). The Effects of Alcohol Drinking on Oral Microbiota in the Chinese Population. *International Journal of Environmental Research and Public Health*, *19*(9), 5729. PubMed. https://doi.org/10.3390/ijerph19095729

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. (2008). The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery*, *7*(2), 156-167. https://doi.org/10.1038/nrd2466

Mitsui, T., Kato, N., Shimaoka, K., & Miyamura, M. (1997). Effect of aging on the concentrations of nitrous oxide in exhaled air. *Science of The Total Environment*, *208*(1-2), 133-137. https://doi.org/10.1016/s0048-9697(97)00267-2

Mitsui, T., & Kondo, T. (1998). Effects of mouth cleansing on the levels of exhaled nitrous oxide in young and older adults. *Science of The Total Environment*, *224*(1-3), 177-180. https://doi.org/10.1016/s0048-9697(98)00353-2

Morou-Bermúdez, E., Torres-Colón, J. E., Bermúdez, N. S., Patel, R. P., & Joshipura, K. J. (2022). Pathways Linking Oral Bacteria, Nitric Oxide Metabolism, and Health. *Journal*

of Dental Research, *101*(6), 623-631. PubMed. https://doi.org/10.1177/00220345211064571

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., & Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *Journal of Bacteriology*, *183*(12), 3770-3783. PubMed. https://doi.org/10.1128/JB.183.12.3770-3783.2001

Qu, X. M., Wu, Z. F., Pang, B. X., Jin, L. Y., Qin, L. Z., & Wang, S. L. (2016). From Nitrate to Nitric Oxide. *Journal of Dental Research*, *95*(13), 1452-1456. https://doi.org/10.1177/0022034516673019

Rosier, B. T., Buetas, E., Moya-Gonzalvez, E. M., Artacho, A., & Mira, A. (2020). Nitrate as a potential prebiotic for the oral microbiome. *Scientific Reports*, *10*(1), 12895-12895. PubMed. https://doi.org/10.1038/s41598-020-69931-x

Rosier, B. T., Moya-Gonzalvez, E. M., Corell-Escuin, P., & Mira, A. (2020). Isolation and Characterization of Nitrate-Reducing Bacteria as Potential Probiotics for Oral and Systemic Health. *Frontiers in Microbiology*, *11*, 555465-555465. PubMed. https://doi.org/10.3389/fmicb.2020.555465

Rosier, B. T., Takahashi, N., Zaura, E., Krom, B. P., MartÍnez-Espinosa, R. M., van Breda, S. G. J., Marsh, P. D., & Mira, A. (2022). The Importance of Nitrate Reduction for Oral Health. *Journal of Dental Research*, *101*(8), 887-897. https://doi.org/10.1177/00220345221080982

Sato, N., Kakuta, M., Uchino, E., Hasegawa, T., Kojima, R., Kobayashi, W., Sawada, K., Tamura, Y., Tokuda, I., Imoto, S., Nakaji, S., Murashita, K., Yanagita, M., & Okuno, Y. (2020). The relationship between cigarette smoking and the tongue microbiome in an East Asian population. *Journal of Oral Microbiology*, *12*(1), 1742527-1742527. PubMed. https://doi.org/10.1080/20002297.2020.1742527

Schreiber, F. (2009). Detecting and Understanding Nitric Oxide Formation during Nitrogen Cycling in Microbial Biofilms. *http://elib.suub.uni-bremen.de/diss/docs/00011534.pdf*.

Schreiber, F., Stief, P., Gieseke, A., Heisterkamp, I. M., Verstraete, W., de Beer, D., & Stoodley, P. (2010). Denitrification in human dental plaque. *BMC Biology*, *8*, 24-24. PubMed. https://doi.org/10.1186/1741-7007-8-24

Schreiber, F., Wunderlin, P., Udert, K. M., & Wells, G. F. (2012). Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: Biological pathways, chemical reactions, and novel technologies. *Frontiers in Microbiology*, *3*, 372-372. PubMed. https://doi.org/10.3389/fmicb.2012.00372

Takahashi, N. (2015). Oral Microbiome Metabolism. *Journal of Dental Research*, *94*(12), 1628-1637. https://doi.org/10.1177/0022034515606045

Tribble, G. D., Angelov, N., Weltman, R., Wang, B.-Y., Eswaran, S. V., Gay, I. C., Parthasarathy, K., Dao, D.-H. V., Richardson, K. N., Ismail, N. M., Sharina, I. G., Hyde, E. R., Ajami, N. J., Petrosino, J. F., & Bryan, N. S. (2019). Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate. *Frontiers in Cellular and Infection Microbiology*, *9*, 39-39. PubMed. https://doi.org/10.3389/fcimb.2019.00039

Vanhatalo, A., Blackwell, J. R., L'Heureux, J. E., Williams, D. W., Smith, A., van der Giezen, M., Winyard, P. G., Kelly, J., & Jones, A. M. (2018). Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. *Free Radical Biology & Medicine*, *124*, 21-30. PubMed. https://doi.org/10.1016/j.freeradbiomed.2018.05.078

Vinckenbosch, F. R. J., Durán Jiménez, D., Helmerhorst, H., Dahan, A., Aarts, L., Bikker, F., Theunissen, E., & Ramaekers, J. G. (2023). The prevalence, risks, and detection of driving under the influence of nitrous oxide. *WIREs Forensic Science*. https://doi.org/10.1002/wfs2.1508

(z.d.). (z.d.). *When Dental Nitrous Oxide May Be Recommended for Your Dental Visit* [Dentist website]. ttps://smilesonmichigan.com/

Zhang, S.-M., & Huang, S.-L. (2023). The Commensal Anaerobe Veillonella dispar Reprograms Its Lactate Metabolism and Short-Chain Fatty Acid Production during

the Stationary Phase. *Microbiology Spectrum*, *11*(2), e0355822-e0355822. PubMed. https://doi.org/10.1128/spectrum.03558-22