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Environmental adaptations of the oral microbiota and effects on the divers' physiology: analysis of the metagenome and salivary stress biomarkers.

Doctoral thesis

NTNU
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Hva skjer dykkeres munnhuler under kommersiell metningsdykking?

Miljøtilpassinger i oral mikrobiota og effekter på dykkerens fysiologi: analyser av metagenomet og stressbiomarkører i spytt.

Metningsdykking har vært brukt som arbeidsform for undervannsintervensjoner offshore i olje- og gassindustri i Nordsjøen siden 1960-tallet. Høyt omgivelsestrykk og økt partialtrykk av oksygen i de lukkede systemene metningsdykkerne lever er utfordrende, fysiologisk og teknisk. Studier av patofysiologi i metningsdykking har vanligvis vært gjort på blod. Men plebotomi er invasivt, vanskelig å gjennomføre under trykk, og kan føre med seg en risiko for infeksjon på stikkstedet. Ved å bruke spytt som prøvemateriale unngår man disse problemene forbundet med blodprøvetaking. Spytt er også en kilde til mikroorganismer i munnhulen. Mangfoldet av mikroorganismer som lever i og på menneskekropper, mikrobiotaen, kan tenkes å påvirke dykkernes fysiologi. Under metningsdykking vil mikroorganismene i munnhulen påvirkes av de samme omgivelsefaktorene som dykkeren gjør. Den orale mikrobiotaens prosess for akklimatisering til det hyperbare miljøet i metningsdykking kan tenkes å påvirke metabolske prosesser som igjen er involver i fysiologiske balanser hos dykkeren.

Denne avhandlingen tar for seg effekter av langvarig eksponering for høyt omgivelsestrykk med økt partialtrykk av oksygen på orale mikrobiomer hos profesjonelle metningsdykkere. 16S rRNA sekvensering er brukt til å kartlegge diversitet og endringer i bakteriefloraen i munnhulen under og etter et fireukers kommersielt metningsdykk til 200 meters dyp (paper I). De samme sekvenseringsdatene ble videre brukt til å lage en modell av endringer i bakteriell metabolsk aktivitet under og etter metningsdykking (paper II). Til sist ble det gjort en pilotstudie av stressresponser hos metningsdykkere gjennom måling av åtte biomarkører i dykkernes spytt gjennom en toukers dykk til 80 meters dybde (paper III).

Det orale mikrobiomets akklimatisering til økt oksygentrykk i det hyperbare miljøet viste seg i form av transient nedgang i anaerobe og en tilsvarende øking i aerobe og fakultativt anaerobe bakterier, sammen med en nedgang i bakteriell diversitet (paper I). Disse endringene i sammensetning av mikrobiomet later til å føre til en transiente endring i metabolske spor involvert i bakteriell overlevelse og vekst, og også en nedgang i bakteriell biosyntese av vitamin B12. Dette funnet støtter opp under eksisterende anbefalinger om vitamintilskudd i metningsdykkeres diett (paper II). Til slutt viste analysen av biomarkører i spytt en økt mengde av inflammasjonsmediatorer som kan reflektere det autonome nervesystemets respons på det endrede partialtrykk av oksygen. Slike inflammasjonsresponser er i samsvar med funn fra tidligere studier på blod (paper III).

Konklusjonen på det samlede arbeidet er at metningsdykking påvirker sammensetning og diversitet av det orale mikrobiomet, men at disse endringene er reverserbare. Biomarkører i spytt later til å være egnet for ikke-invasiv overvåking av fysiologisk stress under offshore metningsdykking.

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Contents

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Abbreviations

ASV: Amplicon sequence variant

CNS: Central nervous system

CRP: C-reactive protein

DSV: Dive support vessel

EC: Enzyme Commission

HPNS: High-pressure nervous syndrome

IL: Interleukin

kPa: kilopascal

msw: meter of sea-water

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

PO_2: Partial pressure of oxygen

ROS: Reactive oxygen species

TNF-α: Tumor necrosis factor-alpha

WGS: Whole-genome sequencing

Definitions

Biomarker: a characteristic (substance, structure, or process) which is objectively measured and evaluated as an indicator of normal biological or pathogenic states.

Bell-run: the moment the divers commute from the compression chamber to the sea bottom using a diving bell. The pressure inside the bell and the compression chamber are equal.

Compression: the process of increasing gas pressure inside the compression chamber until reaching the pressure of the work site.

Cytokines: molecules that act as messengers between cells and can be produced by several cell types (e.g., immune cells).

Decompression: slow reduction of the ambient pressure until reaching the surface pressure. During this process the high partial pressure of oxygen is raised to facilitate elimination of the inert gas dissolved in the tissues.

Decompression illness: Decompression illness results from a supersaturation with inert gases of the blood and tissues during or after decompression. Decompression illness includes decompression sickness and arterial gas embolism.

Diving bell: submersible compression chamber designed for transport of the divers between the surface (compression chamber) and the work site (sea bottom) under increased pressure.

Hyperbaric: increased ambient pressure.

Hyperoxia: increased partial pressure of oxygen in a breathing gas relative to atmospheric pressure.

Microbial ecology: defines the relationship of microorganisms with one another and with their environment.

Microbiome: the totality of microbial organisms and their genes in a sample or environment where they interact.

Microbiota: the set of microorganisms present in the microbiome.

Pipelines: A series of scripts written to improve the analysis of a dataset in which the outputs of one step are the inputs for the next step.

Supersaturation: refers to the state where the partial pressure of a gas in the tissue exceeds that of the ambient pressure.

Taxa: A population of phylogenetically related organisms.

List of papers

This thesis is based on the following three scientific papers and will be referred to by their roman numerals in the thesis:

I. **Monnoyer, R**., Haugum, K., Lautridou, J., Flatberg, A., Hjelde, A., & Eftedal, I. (2021). Shifts in the Oral Microbiota During a Four-Week Commercial Saturation Dive to 200 Meters. *Frontiers in Physiology,* 12(562). doi:10.3389/fphys.2021.669355

II. **Monnoyer, R.**, Eftedal, I., Hjelde, A., Deb, S.K., Haugum, K. & Lautridou J. (2021). Functional Profiling Reveals Altered Metabolic Activity in Divers' Oral Microbiota During Commercial Heliox Saturation Diving. *Frontiers in Physiology,* 12(1791), doi:10.3389/fphys.2021.702634

III. **Monnoyer, R.**, Lautridou, J., Deb, S., Hjelde, A., & Eftedal, I. (2021). Using Salivary Biomarkers for Stress Assessment in Offshore Saturation Diving: A Pilot Study. *Frontiers in Physiology*, 12. doi:10.3389/fphys.2021.791525

Summary

Commercial saturation diving has been used by the offshore oil and gas industry in the North Sea since the 1960s to perform long-term underwater work safely and efficiently. The high-pressure oxygen conditions of the confined environment of the hyperbaric chamber bring challenging restraints, whether physiological or technical. Studies of basic pathophysiology in saturation diving have usually been done on the blood. However, phlebotomy is an invasive method and may add unnecessary supplementary risks of stress or infection for the divers. Using saliva not only makes it possible to bypass the technical problems specific to blood testing; it also gives access to information on the microorganisms present in the oral cavity. The variety of microorganisms living in and on the diver's body, named microbiota, may influence the diver's state of health. During saturation diving operations, microorganisms inhabiting the oral cavity of the divers are also exposed to the hyperbaric hyperoxic environment. The acclimatization of these microorganisms to the hyperbaric environment can impact central metabolic processes involved in the maintenance of physiological and metabolic homeostasis of the diver.

The thesis addresses the effects of prolonged exposure to the high partial pressure of oxygen on the oral microbiota of thirty occupational saturation divers. Using 16S rRNA gene sequencing, we described the bacterial diversity and changes in the bacterial composition of the oral cavity before, during, and after a 28-day heliox commercial saturation dive at 200 meters deep (paper I). Based on the sequencing data, we used a computational approach to estimate the functional activities associated with the bacterial composition of the oral microbiota during and after saturation diving compared to a pre-dive baseline (paper II). Additionally, we assessed the effects of saturation on the physiology of nine divers by measuring levels of salivary biological markers of oxidative stress and inflammation collected throughout a 14-day heliox saturation dive to a depth of 80 meters (paper III).

Acclimatization of the oral microbiota to the high partial pressure of oxygen during hyperbaric saturation was demonstrated by a transient change in the relative abundance of aerobic versus anaerobic bacteria, resulting in reduced bacterial diversity (paper I). The shift between bacterial communities led to transient changes in several metabolic pathways involved in their survival and growth. A decrease observed in vitamin B_{12} biosynthesis reinforces the findings of the literature about requirements in vitamin B_{12} supplementation as part of the diver's diet. This supplementation could help correct the mild anemia observed in the divers after saturation (paper II). Finally, the salivary biomarkers analysis showed an increased production of salivary inflammatory mediators probably reflecting the response of the autonomous nervous system to the high partial pressure of oxygen. The signs of inflammatory responses strengthen what had already been demonstrated on the divers' blood in previous saturation diving studies (paper III).

In conclusion, this work highlighted the impact of saturation diving on divers both on their oral microbiota and on their physiology via oral stress biomarkers. Our results show that the oral microbiota and its functional activity changed during saturation. Furthermore, we showed that saliva could be used to monitor the physiological stress experienced by the divers during saturation diving operations.

1. Introduction

1.1 Saturation diving

1.1.1 Definition

In saturation diving, saturation refers to the process by which the tissues of the divers are saturated with breathing gases until a state of equilibrium is reached with the ambient pressure of the hyperbaric chamber, meaning that no more gas can be absorbed by the tissues [1]. The breathing gas is a mixture of oxygen and an inert gas used to dilute oxygen to a non-toxic level in the tissues. An inert gas, as the name suggests, is a gas that does not chemically react with any other component under normal conditions of pressure and temperature. In diving, the most used inert gases in breathing gas mixtures are nitrogen and helium. Nitrogen is used together with oxygen in the nitrox mixture, helium in the heliox mixture, and both in the trimix mixture.

1.1.2 History

The first experimental saturation dive was done by Edgar End and Max Nohl in 1938 at the County Emergency Hospital recompression facility in Milwaukee, Wisconsin, USA [2]. They used a mixture of helium and oxygen as breathing gas instead of the common compressed air. Their goal was to find a way to avoid the impact of nitrogen on the mental deterioration of divers that was initially studied in air diving by Albert R. Behnke, a U.S. Navy Submarine Medical Officer. The use of nitrogen in diving can induce nitrogen narcosis, or *inert gas narcosis*, which is a reversible condition where the consciousness is altered by the anesthetic effect of nitrogen at high pressure while diving at a depth greater than 150 feet (50 meters of sea water) [3]. Following the experiments of End and Nohl, helium was introduced in the breathing gas mixture and became the Navy's standard in deep diving to avoid nitrogen narcosis. Helium is less narcotic than nitrogen and its lower molecular weight offers less breathing resistance and better absorption by the tissues at increased pressures [4]. Today, air and nitrox are commonly used for dives less than 50 meters of seawater (msw) in recreational and military dives, but also in underwater habitats for research purposes. The heliox mixture is used as the main breathing gas for dives deeper than 50 msw like in the hyperbaric chambers during commercial saturation diving operations [1].

The first heliox commercial saturation diving operation was performed in 1965 by Westinghouse Electric Corporation at the Smith Mountain Dam in Virginia, USA. The saturation diving system named Cachalot System was used to stay at 61 msw to work on trash racks without the divers having to undergo daily decompression [5]. Following this operation, this saturation system was more commonly installed in oil field work, with routine saturation periods of up to two weeks.

1.1.3 Commercial saturation diving

In the production sector of the oil and gas industry, commercial offshore saturation diving is used to perform long-term underwater work at greater depths for installing, maintaining, and repairing subsea structures.

During saturation diving operations, the divers live in a hyperbaric chamber system in a heliox atmosphere stationed on a dive support vessel (DSV). Hyperbaric exposure results

in an increase in the partial pressure of oxygen (PO_2). A classic dive profile is made up of a succession of three different phases called "compression", "bottom time" or living depth, and "decompression". The "compression" is the first step in saturation diving where the divers' bodies gradually reach a dynamic equilibrium with the pressurized environment of the hyperbaric chamber allowing them to live there and be ready to work. When the operation is finished, the divers are going through a "decompression" phase. The return to surface pressure is done by slow, gradual reduction of partial pressure of oxygen to avoid rapid decompression [1].

The diving procedures depend on the operation and location of the offshore field and vary between countries. All the different diving sectors have their own regulations. For example, in the North Sea, the Norwegian petroleum industry follows the NORSOK U-100 Standards. The saturation time is limited to 21 days with a stay at living depth that shall not exceed 14 days and be as close as possible to the working depth [6]. Therefore, the ambient pressure in the chamber is approximately equivalent to the depth of the underwater immersion, allowing divers to perform bell-run excursions to work on the ocean floor without experiencing excess decompression stress.

1.1.4 Environmental stress

All living beings are confronted with environmental factors which can trigger physiological stress responses. In 1936, Hans Selye defined stress as "not what happens to you, but how you react to it" [7]. The effects of environmentally imposed stress in the saturation divers' living atmosphere may affect their well-being and health. Preserving health in such harsh environments requires successful acclimation of the human body's inner physiological mechanisms.

1.1.4.1 Adaptative stress responses

Physiological responses that help to restore or maintain the homeostasis of the body are called adaptative stress responses, which involve a close interaction between the immune system and the central nervous system. The central stress response system is represented by the hypothalamic-pituitary-adrenal axis and the autonomous nervous system (sympathetic-adreno-medullar and the parasympathetic systems) [8], both of which interact with the immune system. The state of stress can be the result of many perturbations of physiological, endocrinal, or homeostatic mechanisms involving oxidative or inflammatory reactions with the release of specific stress biological markers [9]. A biological marker, or biomarker, has been defined by the World Health Organization as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [10]. Therefore, the biomarkers have been widely used in clinical care for diagnostic, prognostic, and therapeutic purposes [11-13].

The emergence of biomarkers from other body fluids such as saliva has made it easier to monitor health and disease, especially in occupational and environmental medicine. The main objective of occupational medicine is to determine the causes of stress (risk factors) in the workplace and to identify the positive and negative affective outcomes [14]. The identification of biomarkers of psychological and physiological stress would make it possible to better manage and prevent work-related illnesses and injuries. Several salivary stress biomarkers have already been identified, including cortisol and alpha-amylase, but also pro-inflammatory cytokines [15-19]. The production of pro-and anti-inflammatory cytokines will depend on the stress reactions encountered, either physical or psychological.

Salivary cortisol is a good measure of the adaptation of the hypothalamic-pituitary-adrenal axis to long-lasting stress, known as chronic stress [20]. In contrast, acute stress with a short-term adaptive response is associated with an activation of the sympathetic-adreno-medullary system which is measured with salivary alpha-amylase and chromogranin A [21, 22]. The immune system also responds to acute stress with the production of salivary markers of inflammation including cytokines, tumor necrosis factor-alpha, and C-reactive protein [17].

Saturation divers that live in a hyperbaric chamber are not easily accessible and are exposed to a stressful living environment. Therefore, the use of salivary biomarkers as a simple and non-invasive method is an asset to monitor their health during saturation diving operations.

1.1.4.2 Excess oxidative stress

Saturation divers are exposed to an increased partial pressure of oxygen which triggers excessive production of reactive oxygen species (ROS) to which they must adapt [1]. The production of reactive oxygen species is a natural physiological phenomenon of aerobic metabolism. The levels of ROS are regulated by several antioxidants enzymes participating in the redox homeostasis [23]. In healthy conditions and at low levels, ROS act as signaling molecules of intracellular signaling pathways involved in different biological reactions and immune responses [24, 25]. The oxidative stress occurs when the redox homeostasis is disrupted by an imbalance between the antioxidants and prooxidants, where the latter predominates. This perturbation leads to oxidative damages of cellular components, changes of cellular signaling pathways, and alterations of gene expression associated with cellular responses to oxidative stress [26]. At the cellular level, ROS can also damage the structures of proteins, lipids, or even nucleic acids [27].

1.1.5 Health implications

Working under high ambient pressure requires good physical condition which must be validated by a medical practitioner. According to the NORSOK standard, the certificate of medical fitness for each diver must also indicate their previous work experience, including diving exposure data, and diving-related illnesses and injuries. The health of the divers is monitored using techniques that are easy to implement and interpret, safe and non-invasive, e.g., hearing test (audiometry), lung function test (spirometry), and questionnaires [6].

1.1.5.1 Diving disorders

During decompression, the body will undergo a decrease in oxygen pressure with the elimination of inert gas in the tissues. One of the most common issues in saturation diving is related to rapid decompression resulting in decompression illness. Decompression illness is the consequence of the body's blood and tissues being supersaturated with inert gases during or after decompression. It encompasses two different conditions: decompression sickness and arterial gas embolism [28]**.** Decompression sickness is the consequence of bubble formation in tissues and blood vessels during decompression, which can result in mild symptoms (e.g., skin rash, joint pains) to more severe (e.g., lung damage, paralysis, or death) [29]. Arterial gas embolism refers to venous bubbles introduced directly in the arterial circulation, either through a small opening between the right and the left atrium of the heart (called a patent foramen ovale), or when a large number of these bubbles are not all effectively filtered by the lungs [30].

Conversely, the rate of compression during heliox dives deeper than 150 msw may cause high-pressure neurological syndrome (HPNS). This condition is characterized by neurological, psychological, and electroencephalographic abnormalities [31]. This term was first defined by Dr. Ralph W. Brauer in 1968 following a heliox dive in a hyperbaric chamber at 362 meters done by the company COMEX in Marseille, France [4]. He observed symptoms of psychomotor disorders and electroencephalographic changes. Clinical manifestations also include headaches and tremors, which were described as "helium tremor" in 1965 by Dr. Peter Bennett. In addition, come also confusion and nausea [32]. The prevalence and severity of symptoms are related to the speed of compression. The faster the speed of compression rate and the higher the pressure, the more severe the clinical presentation will be [33]. A slower compression during the dives may help prevent HPNS, and, if necessary another option is to alter the composition of the breathing gas.

1.1.5.2 Oxygen toxicity

Oxygen toxicity occurs in response to the high partial pressure of oxygen with an excessive concentration of free radical intermediates, such as reactive oxygen species. These byproducts can cause cellular damage to the brain, lungs, and eyes [34]. In the late 19th century, two pioneer studies described the toxic effects of high oxygen pressure. Paul Bret first demonstrated in 1878 the existence of central nervous system (CNS) oxygen toxicity in larks [35]. The pulmonary oxygen toxicity was next described in 1899 by James Lorrain Smith in mice [36]. Several signs and symptoms of oxygen toxicity have been reported in saturation diving. As CNS toxicity affects the brain and spine, it results in a wide range of neurologic effects including convulsions, muscular twitching, nausea, or tunnel vision. On the other hand, pulmonary oxygen toxicity can lead to lung inflammation and fibrosis of lung tissue with a loss of diffusion capacity [37, 38]. Divers must consider the oxygen partial pressure and the duration of exposure to prevent any toxicity damages.

1.1.5.3 Anemia and fatigue

During the diving operations, the increase in PO_2 in the heliox environment between the storage depth at 40 kilopascal (kPa) and work excursions (bell-runs) at 60-80 kPa is perceived by the body as relative hyperoxia. Conversely, when the diver finishes his underwater work and returns to the hyperbaric chamber at 40 kPa, the decrease in the PO_2 is perceived as relative hypoxia [39]. These variations of PO_2 trigger the erythropoietin production which appears to adjust the production of erythrocytes (red blood cells), thus keeping the hemoglobin levels in a normal range [40]. However, during the decompression, the PO_2 is constantly kept at 50 kPa to facilitate the elimination of the inert gas (i.e., helium) in the divers' body. In the last 13 meters of the decompression, the PO_2 is gradually reduced until reaching 21 kPa. The drop of oxygen pressure is associated with hypoxia by which the body responds by increasing erythropoietin production [41]. However, it is not instantaneous. This may explain the mild anemia and the fatigue experienced by the saturated divers up to one week after decompression [42]. These symptoms have been reported after questionnaires and interviews [43]. This reversible state may be explained by the acclimatization of the divers to the variations of PO_2 during saturation and decompression.

1.1.5.4 Infectious diseases

In the 1990s, skin and other superficial infections were encountered during saturation diving [44-46]. The living conditions unique to saturation diving most likely have an impact on the development of microbial growth. The confined environment of the hyperbaric chamber in

combination with elevated temperature and humidity can be responsible for alterations in the microflora of the divers and increase the risk of infections. In addition, cross-contamination between divers can easily happen due to the promiscuity between members of the same working team [47].

One of the main microbial infections among occupational divers is external otitis caused by the bacterium *Pseudomonas aeruginosa*, followed by staphylococcal and fungal infections of the skin [44, 46]. Microbial contamination in the hyperbaric chamber can also be related to water, either from the freshwater supply or from seawater. Bacteria, such as *Pseudomonas* spp., are naturally present in fresh and seawater, and fungi are known to thrive in warm and humid conditions. Consequently, life in the hyperbaric chamber improves the proliferation of these microbes. However, it is possible to avoid infectious diseases by following precautionary measures regarding several aspects of diving. This includes the water quality, the quality of equipment, the hygiene of the divers, and the cleaning of the chamber and the diving equipment. Despite these procedures, divers can be more susceptible to infections due to the fatigue they experience after a deep dive [48].

1.2. Microbiome and microbiota

1.2.1 Definition

The human body is host to a vast number of microbial organisms, including bacteria, fungi, viruses, archaea, and protozoa. In 2001, Lederberg and McCray first defined the term microbiome as the set that includes all symbiotic, commensal, and pathogenic microorganisms, their genes, and the environment where they interact [49]. The microbiota is the set of microorganisms that comprises the microbiome. The close interaction (symbiosis) between microorganisms have different forms of symbiotic relationships, including mutual, parasitic or commensal [50]. The organisms involved in a symbiotic relationship are qualified as symbionts. Symbiosis most often implies a mutualistic relationship, in which both organisms benefit from the association. Other classes of relationships between organisms include parasitism, where one species benefits from the other species by harming it; or commensalism in which one species benefits while the other species are not affected.

1.2.2. Taxonomy rank

Taxonomy is the science that studies the relationships between living organisms, in which they are classified, identified, and named [51]. The classification groups together organisms according to their genetic and phenotypic similarities within the same set, or taxa. The identification of bacteria is based on their morphological, cultural, or biochemical characteristics [52, 53]. In the current classification of life, organisms are classified into three domains: Eukaryotes, Bacteria, and Archaea. Viruses are currently not a part of the tree of life because they are unable to live or replicate without a host. Each organism is identified and a name is assigned for each different species. Bacterial nomenclature is governed by the International Code of Nomenclature of Prokaryotes, which applies to all prokaryotes [51, 54]. The nomenclature of prokaryotes is defined only up to the rank of class. At present, the rank of the phylum is not controlled by the Prokaryotic Code but is being debated for inclusion [55]. An example of the nomenclature of the common bacterium *Escherichia coli* according to the hierarchical system is described in Table 1.

Table 1. Taxonomic classification of *Escherichia coli*

Rank	Suffix	Example
Domain		*Bacteria*
Phylum		*Proteobacteria*
Class		*Gammaproteobacteria*
Order	-ales	*Enterobacteriales*
Family	-aceae	*Enterobacteriaceae*
Genus		*Escherichia*
Species		*coli*
Strain		NCTC 90001 / ATCC 11775

1.2.3 Microbial diversity

The Convention on Biological Diversity defines biological diversity as “the variability among living organisms and the ecological complexes to which they belong” [56]. Microbial diversity includes diversity within species, diversity between species, and diversity of ecosystems. In the 1960s, Hutchinson [57] and MacArthur [58] developed mathematical methods to predict the microbial diversity and dominance of a species in a given ecosystem. Later in 1972, Whittaker introduced the terms alpha and beta diversity [59], which are indices of variations in microbial diversity over space and time. The composition of an ecological community, or diversity, depends on the environment from which it is sampled.

The alpha diversity is characterized by the number (richness) and the relative abundance (evenness) of species present within a given sample or environment (Figure 1). Different indices or measures are available to describe and compare the structure of a microbial community present within the sample from a studied environment.

- The Shannon-Weaver and Simpson diversity indices are both based on species richness and relative abundance of each species [60]. The Shannon index emphasizes species richness while the Simpson index emphasizes species evenness.
- Non-parametric methods focus on rare species which contain almost all the information about undetected species richness compared to abundant species [61]. Chao1 [62] and ACE (Abundance-based Coverage Estimator) [63] are estimators of species richness for low-abundance species.

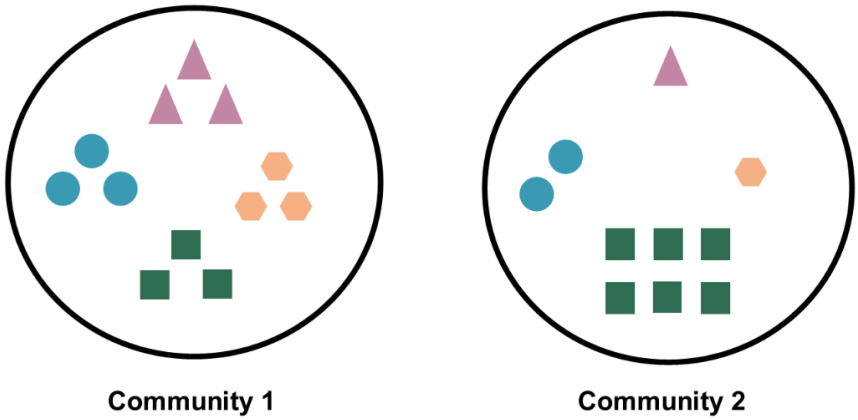

Figure 1. **Species richness and evenness.** Both communities 1 and 2 have the same species richness, four species each. However, in terms of abundance, the organisms in community 1 are more evenly distributed than in community 2.

The beta diversity measures the diversity of species between samples or environments. It calculates the number of species that are different in two communities [64]. A high number of shared species between two samples or environments indicates a low beta diversity and vice versa. Visualization of clusters of similar communities or separation of different communities commonly uses principal coordinates analysis and non-metric multidimensional scaling [65]. The measure of beta diversity is commonly based on calculations of distance matrices such as Bray-Curtis dissimilarity and Jaccard index of species. They are based on the presence or absence of species between two samples. The beta diversity can be also calculated using a phylogenetic distance called UniFrac.

- Bray-Curtis dissimilarity index, as the name suggests, assesses the dissimilarity between two given samples based on the abundance of taxa present in each sample [66]. Jaccard index compares the number of shared species (presence or absence) to the total number of species between two samples [67]. Bray-Curtis is considered a measure of relative similarity based on abundance, while Jaccard is based on incidence.
- UniFrac, or unique fraction, calculates differences between microbial communities based on phylogenetic information, such as the phylogenetic distance between sets of organisms observed in a phylogenetic tree [68]. The weighted UniFrac (quantitative) reports the abundance of organisms observed, whereas the unweighted UniFrac (qualitative) considers only their presence or absence, highlighting both abundant and rare taxa.

1.2.4 The human microbiome

The body is made up of approximately 10^{13} eukaryotic cells and ten times more bacterial cells, which represent nearly 10^{14} cells. A large majority of these bacterial cells reside in the gastrointestinal tract and mouth. With an average of 100,000 billion bacteria comprising over 400 different species for the gut, and 10 billion bacteria with 700 different species for the

mouth, these are the two most complex ecosystems in the body [69]. Microorganisms colonize various tissues of the human body where they will acclimatize according to the inhabited site. A predominance of facultative anaerobic bacteria is found in the gastrointestinal tract, while most aerobic bacteria occupy the respiratory tract, nasal cavity, and skin surface [70].

The human microbiome can be categorized into a core and a variable microbiome. The important role of microorganisms in human health and disease depends on the composition and function of the host microbiota. The core microbiome is found in most healthy individuals that share a similar set of microbial genes. The variable microbiome is exclusive to an individual and evolves in response to a unique lifestyle, genotypic characteristics (e.g., specific ethnic groups), pathobiology (disease status of the host), and living or working in a particular environment [71, 72]. All these combined factors are displayed in Figure 2.

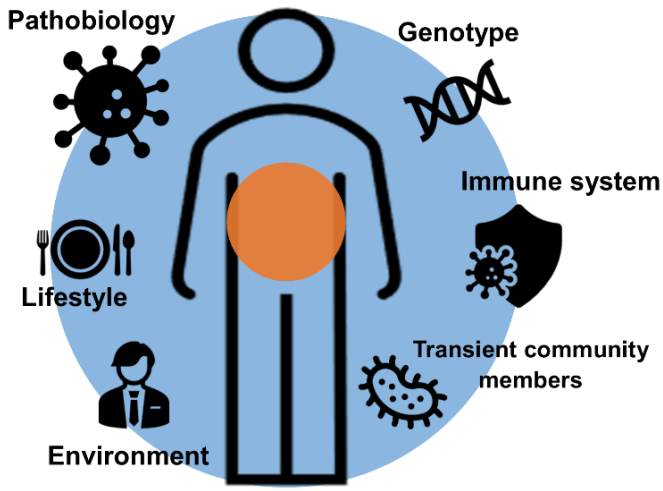

Figure 2. The human microbiome. The core microbiome (orange) and the variable microbiome (blue) are important to the host's state of health and disease. Several factors are susceptible to influence the composition of the variable microbiome and lead to disease development.

In the last 40 years, the gut microbiome has been the most studied human microbiome. The impact of the gut microbiota on metabolic disorders has been used to develop disease-specific diagnostics, with a focus on key mechanisms. The knowledge generated by these studies made it possible to discriminate bacteria involved in the host's state of health and disease [73]. The microorganisms constituting the human microbiome have been characterized through the Human Microbiome Project. The project aimed to identify the role of these microorganisms in health and predisposition to diseases, which could help to develop personalized clinical care (e.g., human microbiota manipulation or transplantation and use of probiotics) [71].

1.2.4.1 The Human Microbiome Project

The Human Microbiome Project is the logical continuation of the Human Genome Project, which completed the entire identification and mapping of human genes in 2003. The complete characterization of the human microbiome that began in 2008 was the mission of the National Institutes of Health [74]. The goal was to help answer several biological questions about the evolution of the microbiota of an individual during his life, the existence of a core microbiome and how is it acquired and transmitted, but also the factors influencing the genetic diversity of the microbiome and therefore the adaptation of microorganisms to various physiological or pathophysiological states. The study cohort for this project was comprised of 239 individuals who were sampled at 18 different body habitats among five body sites: the skin, oral and nasal cavity, gastrointestinal and genitourinary tract. One of the biggest challenges was dealing with unclassified or newly identified organisms. Thus, specific databases like the Human Oral Microbiome Database took care of the taxonomic classification for the unnamed members of the oral microbiome. The database is based on samples from healthy volunteers collected in nine sites of the oral cavity and oropharynx: the tongue, hard palate, oral mucosa (cheek), gums, palatal tonsils, throat, supra- and subgingival dental plaque, and finally saliva [75].

1.2.4.2 The oral microbiome

As described earlier, the oral microbiome is the second most complex and diverse human microbiome after the gut microbiome, where the oral cavity comprises over 700 species. The Human Oral Microbiome Database provides descriptions of oral bacterial taxa and a web repository of oral bacterial genome sequences (www.homd.org). The database includes 619 taxa distributed in 13 phyla: 96% of the total oral bacteria belong to six major phyla *Firmicutes, Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Spirochaetes*, and the remaining 4% to *Chlamydiae*, *Chloroflexi*, *Euryarchaeota*, *Synergistetes*, *Tenericutes*, and SR1 and TM7 (Figure 3) [76-79].

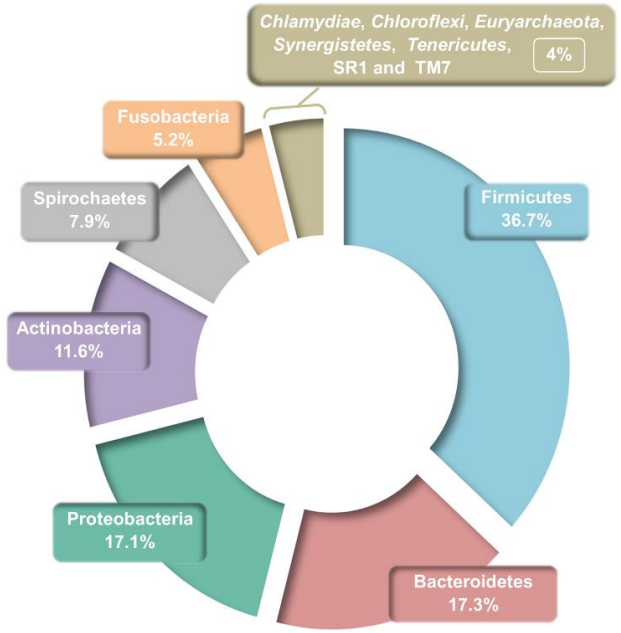

Figure 3. Oral microbiome. Proportions of the different phyla found in the Human Oral Microbiome Database.

The biofilm attached to the various soft- and hard-tissue surfaces of the oral cavity contains microorganisms that contribute to the oral homeostasis by protecting the oral cavity from pathogens and preventing oral infectious disease development [80]. The microbial ecology of the oral cavity is also diverse in its composition due to its continuous contact with the external environment, but also due to the host's lifestyle (diet, medications, or smoking) and the environment of the oral cavity (temperature and pH) [81].

Depending on their location in the mouth, from tooth surfaces to supragingival and subgingival regions, oral bacteria can be classified according to their oxygen needs. During the development of the oral biofilm, the environment changes from aerobic to anaerobic, thus explaining the predominance of obligate anaerobic bacteria in the mouth which have a growth advantage over facultative aerobes [82]. The modification in the oxygen concentration within the surrounding environment can affect the composition and performance of the bacterial communities, depending on whether they are aerobic or anaerobic. The formation of reactive oxygen species and reactive nitrogen species during an inflammatory immune response is mediated by neutrophils and macrophages in a process called oxidative burst [83]. The latter is involved in the elimination of undesirable microorganisms, but can however have harmful effects for the host if there is an overproduction of reactive oxygen and nitrogen species or a deficiency of endogenous antioxidant defenses [84]. The development of a highly regulated complex of antioxidant defense enzymes (e.g., catalase or superoxide dismutase) as well as other enzymatic and non-enzymatic defense mechanisms is crucial for aerobic and anaerobic bacteria survival to overcome the toxic effects of reactive oxygen species [85, 86].

1.2.5 The human microbiota in health and disease

1.2.5.1 Immunity and inflammation

The human immune system is a complex biological defense system made up of a coordinated set of recognition elements that discriminate the self from the non-self. It is inherited from birth but is also autonomous and adaptive. Thus, the immune system is composed of adaptative and innate defense mechanisms, where cells and organs work together to protect and react to infections and diseases [87-89]. The cells and mechanisms involved in the innate immune system provide an immediate response against infectious agents. It does not require cell division. The innate immune system uses pattern recognition receptors, either transmembrane (toll-like receptors) or cytoplasmic (nucleotide oligomerization domain (NOD)-like receptors) [90]. During infection, the receptors expressed by the immune cells recognize specific and conserved structures on pathogens called pathogen-associated molecular patterns (PAMPs). However, all microorganisms express these structures, either pathogens or those living in the microbiota. To initiate an inflammatory response, receptors must not only recognize PAMPs but also molecules specific to damaged cells (e.g., oxidative stress and heat shock proteins) [87]. Conversely, the adaptive immune system depends on the clonal expansion of T- and B- lymphocytes which will provide longer-lasting protective effects. This process allows the establishment of a memory response, on which the principle of vaccination is based, hence the term adaptive immunity. Innate immune responses, on the other hand, are not specific to a particular pathogen [91].

1.2.5.2 Immune homeostasis

In early life, the gut microbial colonization of the host's mucosal surfaces is partly responsible for the formation of a strong human immune system, as our adaptive immune system develops. These early interactions between the immune system and the host microbiota are crucial for susceptibility to infectious and inflammatory diseases later in life [92, 93]. Due to this coevolution over time, the immune system has become accustomed to foreign antigens in our body, thus contributing to immune homeostasis [94]. Among the environmental factors that have an impact on the development of systemic diseases, one that stands out is the microbiota. The state of health of the host is then defined by the capacity of the immune system to distinguish beneficial microbes of the microbiota from pathogens, but also environmental antigens to coordinate appropriate immune responses. Maladaptive immune responses may result in pathologies such as allergies, autoimmune and inflammatory disorders [95].

1.2.5.3 Alteration in the human microbiota

"*The states of health or disease are the expressions of the success or failure experienced by the organism in its efforts to respond adaptively to environmental challenges*"
(Renée Dubos, 1965)

The healthy and homeostatic state of the microbiota, known as "eubiosis", is relatively robust and composed of a variety of organisms that are beneficial for the development and health of the host. However, alteration in the microbial composition of the human microbiota can result from the genetic background and diet of the host, diseases and infections, or the use of antibiotics [96]. The change in the homeostatic balance, known as "dysbiosis", can be observed through a reduction in the bacterial diversity with a loss of beneficial microbes and

an increase of pathobionts [97, 98]. The term pathobiont applies to an overgrowth of commensals or a change in the symbionts characteristics due to altered specific genetic or environmental conditions [99]. Thus, any changes in the host microbiota (e.g., domination by pathobionts) can promote inflammation and trigger the development and progression of autoimmune and chronic diseases [100].

In addition to its symbiotic relationship with the human microbiota, the immune system is also sensitive to the host's diet [101, 102]. Nutrients, such as sugars, fats, and vitamins, play important roles in the microbial diversity of the oral microbiome [103]. In the oral cavity, the disturbance of the homeostatic balance usually causes a series of oral infectious diseases including dental caries or periodontal diseases. A change in the composition of the oral microbiota is responsible for the shift from healthy periodontal conditions (with a predominance of the genus *Neisseria*) to periodontal diseases (with the predominance of the genera *Prevotella* and *Veillonella*) [104].

1.2.5.4 The oral microbiota and non-oral diseases

The oral cavity is a gateway to the respiratory and digestive systems, and its proximity to the bloodstream could help oral bacteria to infect other sites in the body via gingival crevices and untreated carious lesions. According to epidemiologic studies, the risk of developing cardiovascular disease is higher in people with periodontal disease, aside from common risk factors (e.g., smoking, age, and sex) [105]. Any changes in the oral microbiota have been associated not only with oral diseases but also with many other systemic diseases associated with inflammation: heart disease, pneumonia, cystic fibrosis, or rheumatoid arthritis to name a few [96, 106-108]. The human oral microbiota has recently become a new focus of research as a potential biomarker in the diagnosis of some systemic diseases by helping to develop disease treatment and personalized medicines [109].

According to the World Health Organization, cancer remains today the second leading cause of death in the world with almost 10 million deaths. Each year, 657,000 new cases of cancers of the oral cavity and pharynx are estimated resulting in more than 330,000 deaths. In some cases, pathogens responsible for the development of cancer in the gastrointestinal tract also originate from the oral cavity. Thereby, periodontal pathogens of the oral cavity such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* can be used as biomarkers for oral, colorectal, and even pancreatic cancers. Oral bacteria can influence the pathogenesis of cancer by leading to chronic inflammation, acting as antiapoptotic agents, and producing carcinogenic substances [110].

1.3. Human microbiome study tools

Studies of the composition, function, and dynamics of the human microbiota help to better define the host-microbial communities interactions involved in health and disease [72]. Before the development of new generation sequencing techniques, the identification of microbes was done by more traditional techniques such as in vitro culture [111]. Since then, several techniques called "meta-omics" have emerged. A meta-omic study encompasses the study of microorganisms, their genes, metabolic pathways, or functions. A microbiome study involves biological data from DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics), or metabolites (metametabolomics) from a microbiome sample

and its environment or host [112]. These various approaches have distinct objectives depending on the research questions. Metagenomics focuses on investigating the composition of a microbial community under different conditions. Metatranscriptomics helps identify which genes are expressed under different conditions. Metaproteomics studies the metabolism of microbial communities while metametabolomics focuses on which metabolites are produced under different conditions [113].

1.3.1 16S rRNA gene sequencing

The most common sequencing approach to analyze the microbiome is 16S rRNA gene sequencing. PCR primers are designed to bind to the highly conserved regions of the 16S rRNA gene, which is specific to bacteria and present in almost all bacterial species. Bacterial 16S rRNA genes usually include nine hypervariable regions (V1 to V9), interspersed between conserved regions To determine the microbial phylogeny of a sample, bacterial species will be discriminated using the different variable regions of the gene. The DNA samples are amplified and fragmented into random sequences by a "shotgun" method and then sequenced either by conventional Sanger sequencing or by next-generation sequencing (NGS) for large-scale sequencing projects. Each sequence is assigned to a microbial taxon (bacteria, archaea, or lower eukaryotes) at different taxonomic levels (phylum, class, order, family and genus). 16S rRNA gene sequencing is a fast, simple analysis even with host-contaminated samples and cost-effective for a low-resolution view of a microbial community. However, the choice of primers and variable regions does not provide full access to functional information and limits the taxonomic resolution at the genus level at best [114].

1.3.2 Whole-genome sequencing

With improvements in high-throughput sequencing, metagenomics studies have moved from 16S amplification to whole-genome sequencing (WGS) which uses random primers to sequence overlapping regions of a genome. This approach provides a general view of the entire genome of bacteria present in a sample. Its high resolution also makes it useful for sequencing any species of microbe associated with disease [115]. In addition, WGS can provide functional biological profiles for microbial communities with associated metabolic pathways with a higher taxonomic resolution where 16S rRNA sequencing only determines the presence or abundance of bacterial species [114]. On the other hand, it makes it quite expensive in the preparation, sequencing, and analysis of samples. The invaluable volume of data generated for one study also brings bioinformatic challenges in data analysis.

1.3.3 *In silico* functional analysis

An alternative to WGS for functional analysis is to use bioinformatics approaches. One of them is PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). This software was developed to predict microbial community functions based on taxonomic profiles from 16S rRNA gene sequencing data [116]. This is done by mapping the amplicon sequences against different gene family databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs) and Enzyme Commission numbers (EC numbers) [117, 118].

KEGG (https://www.genome.jp/kegg/pathway.html) is a database for methodical analysis of gene functions, linking genomic data with functional information [119]. The KO (KEGG Orthology) database is a database of molecular functions represented by functional

orthologs. Orthologs genes are genes present in different species that are derived from a common ancestral gene.
An alternative to KEGG database is to use MetaCyc which infers pathways through mapping of EC numbers [120]. EC numbers are a type of gene family characterized by the chemical reactions they catalyze (e.g., EC:1.1.1.1 corresponds to alcohol dehydrogenase). MetaCyc references metabolic pathways involved in both primary and secondary metabolism, as well as associated metabolites, reactions, enzymes, and genes from all domains of life (https://metacyc.org/).

The amplicon-based predictions performed by PICRUSt2 can help generate biological hypotheses but should always be interpreted cautiously. Therefore, several limitations arise when using this approach:

- The accuracy of predictions is based on the content of existing reference genomes. Some environments are better characterized than others but the increased high-quality available genomes tend to avoid this limitation. It is therefore possible for the user to create a personalized reference database with its own genomes to generate predictions for the environment of interest.
- Only the genes from organisms that have been targeted by the primers will be predicted. The software uses 16S amplicon data and thus cannot give functional prediction to the strain level.
- Predictions of genes families are limited to the input function tables based on KEGG orthologs (KO) and EC numbers.
- Mapping of pathways depends on the accuracy in the annotation of the pathways or nominations of gene function available in the database.

2. Aims

The main objective of the thesis was to provide a better understanding of the effects of commercial saturation diving on the oral cavity of saturation divers. This thesis focused on the environmental adaptations of the oral microbiota by investigating its composition and activity during saturation. The thesis also investigated the effects of imposed environmental stress on the diver's physiology through an analysis of salivary biomarkers.

These objectives were addressed through three studies done on biological material from commercial saturation divers:

1) A taxonomic profiling using 16S rRNA gene sequencing described the composition of the oral microbiota among professional saturation divers. The aim was to identify the predominant bacterial communities and changes in relative abundances before, during, and after heliox saturation diving (paper I). The hypothesis was to verify if the bacterial diversity would be impacted by the high partial pressure of oxygen, thus favoring aero-tolerant bacteria.

2) A functional profiling using an *in silico* approach predicted changes in metabolic pathways before, during, and after heliox saturation diving (paper II). The aim was to determine whether these changes, based on the bacterial composition of the diver's oral microbiota, could affect the divers' health and fitness.

3) An analysis of physiological and psychological stress responses used a non-invasive collection method to monitor the health of the divers during saturation diving operations. The aim was to determine if saliva would be appropriate to measure the levels of salivary biomarkers linked to oxidative stress and inflammation (paper III).

3. Methodology

3.1 Ethical approval

The material presented in the thesis was collected during two commercial saturation diving operations conducted aboard TechnipFMC's DSV: the Deep Arctic. One operation was done in the Mediterranean Sea during spring 2018 (March-April), and one in the North Sea in the fall of 2019 (September-October).
The study protocol was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REK), approval number 2018/1184. All participants were informed of the aim and scope of the respective studies and provided written consent before inclusion. Data and samples collection was conducted according to the Declaration of Helsinki principles for ethical human experimentation.

3.2 Study population

Certified saturation divers, all male non-smokers, were enrolled in the studies for the duration of a 14 or 28-day work assignment in hyperbaric heliox saturation. To be eligible for participation, divers had to pass the pre-saturation medical examination and be committed to working in saturation onboard the DSV.

3.3 Diving profile

The divers were organized in four working teams of three divers. Each team worked on a shift of 12 hours, seven days per week, rotating every 6 hours throughout the day to ensure constant underwater work. The storage depths (i.e., the pressure in the divers' living chambers) depended on the depth at which the dives took place.

Samples for the taxonomic and functional analysis in paper I and paper II respectively were from a commercial saturation diving operation that took place in the Mediterranean Sea. The divers were compressed over 6 hours to a storage depth of 178-192 meters of seawater (msw) where they remained for 21 days and were decompressed afterward over a period of eight days which makes a total saturation period of 28 days.

Samples for the salivary biomarkers analysis in paper III were from the commercial saturation diving operation that took place in the Norwegian sector of the North Sea. Here, the diving profile was 2 hours descent to a living depth at 73 msw. The maximum working depth was 85 msw with a total bottom time of up to 11 days, and the return to the surface was performed with a decompression period of 4 days.

4. Experimental protocol

All saliva samples were collected during the different phases of the saturation dive and in two different vials depending on the planned analysis. All samples were kept frozen on board the DSV, and transported frozen to the lab at NTNU prior to analyses as described below.

4.1 Saliva sampling

4.1.1 Paper I and paper II

The first saliva sampling was conducted in spring 2018 from thirty divers. We performed the sampling at four time points: at the surface (day 0), during the bottom phase (day 2 and day 5), and immediately after the decompression (day 28). A total of one hundred and thirteen samples were collected for the oral microbiome sequencing with an all-in-one OMNIgene ORAL OM-505-tubes collection kit (DNA Genotek Incorporation, Ottawa, Canada). The tubes are specifically designed for the collection and stabilization of microbial nucleic acids (DNA and RNA) from saliva. The saliva samples were used for the taxonomic and functional analyses.

4.1.2 Paper III

The second saliva sampling was conducted in fall 2019 from nine divers. We collected the samples throughout the dive (surface (day 0), bottom phase (day 1, 2, and 4), decompression (day 11, 12, 13, and 14), and post-decompression (day 15)). The samples were collected via passive drool using SalivaLab cryotubes (Salimetrics LLC, Carlsbad, CA, USA). The sampling resulted in a total of 60 saliva samples that were used for the analysis of biomarkers of inflammation and stress responses. The following salivary biomarkers were measured: C-reactive protein, alpha-amylase, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), cortisol, and secretory immunoglobulin A (SIgA).

4.2 16S rRNA gene sequencing

In papers I and II, saliva samples were collected from 30 divers at four time points. From the collected material, 92 saliva samples from 23 divers were used for the taxonomic and functional analyses.

4.2.1 DNA isolation

As a first attempt for microbial DNA and RNA purification, we used the QiAmp MinElute Virus Spin Kit (QIAGEN). However, due to the final poor yield of DNA and RNA after the purification, we chose to extract DNA and RNA separately. After several non-successful tries for purifying RNA using the RNEasy Mini kit (QIAGEN), we decided to keep working only on DNA. Several studies comparing DNA isolation kits in saliva samples have been published [121-123]. Based on the results from these studies, we chose to use QIAmpDNA Microbiome Kit (QIAGEN). Although this extraction kit worked well on fresh samples, the DNA yield decreased once we used our frozen samples. As a final solution, all DNA extractions were performed with the QIAmp PowerFecal Pro DNA kit (QIAGEN).

Before DNA isolation, all frozen samples were incubated at 50 °C for 1 hour, based on suggestions from DNA Genotek. Furthermore, instead of the recommended 250 mg of stool sample, we used 250 µL of saliva. Some adjustments were made to the manufacturer's protocol, starting with the homogenization in step 2 where we used the Precellys 24 tissue homogenizer (Bertin Technologies) at 5000 rotations per minute instead of the PowerLyzer24 Homogenizer as suggested in the protocol. In addition, during the final elution step (step 16), we reduced the volume to 25 µL. Following the DNA extraction, all saliva samples were run on a Bioanalyzer and Nanodrop to verify the quality, integrity, purity, and quantity of DNA prior to sequencing.

4.2.2 Sequencing

Following DNA extraction, the 16S rRNA gene for bacteria and archaea encoding the small subunit ribosomal RNA was amplified using PCR with primers targeted towards highly conserved regions of the gene. Within the 16S rRNA genes, there are regions that are highly conserved, but there are also other regions that are more variable, and which are unique to certain microbial groups or genera. Among the nine existing hypervariable regions of the bacterial 16S rRNA gene, the chosen target regions for paper I were the V2-V3 and V4-V5 regions. In ecological and human microbiome studies, the choice of hypervariable regions of the 16S rRNA gene is important to assess the phylogenetic diversity of microbes, as it affects results from sequencing on the structure of the microbiota and taxonomic identification [122, 124-126].

The process of library preparation includes fragmentation of the DNA, binding of primers specific to the region of interest, and amplification by PCR with overhang adapters attached (Figure 4). Next, 16S rRNA gene metagenomic sequencing libraries were prepared according to the QIAseq 16S Region Panel protocol (Qiagen, Hilden, Germany). This will generate a pool of PCR amplicons, which is derived from as many of the bacterial species present in the original sample as possible.

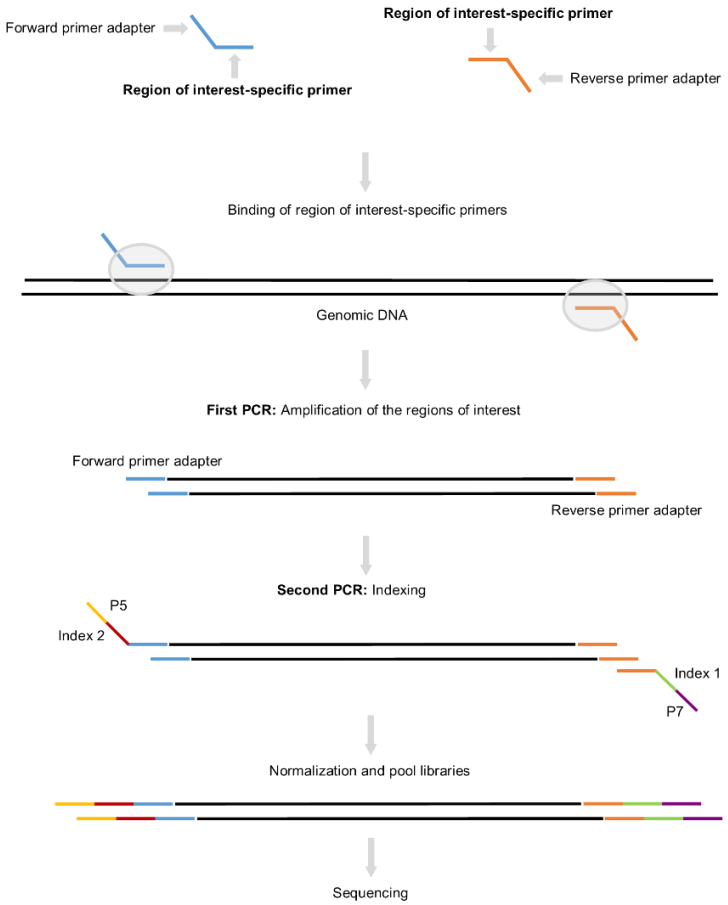

Figure 4. Library preparation. Binding of forward and reverse primers (blue and orange respectively) specific to the region of interest of the bacterial 16S rRNA gene. Then, sample-specific index sequences (green and red) and regions complementary to the flow cell oligos (P5 and P7) are ligated to the flanking DNA. Index tags ensure that individual samples can be identified after sequencing. Libraries are then normalized and pooled. Figure modified from Illumina 2013, 16S Metagenomic Sequencing Library Preparation [127].

Once the sample libraries were pooled and normalized, the amplicons were sequenced with a MiSeq V3 flowcell on a MiSeq instrument, according to the manufacturer's instructions (Illumina, Inc. San Diego, USA). During a sequencing reaction, the DNA fragments first hybridize with short oligo DNA bound to the flow cell. Next, the DNA fragments are amplified in clusters by "bridge amplification" (Figure 5).

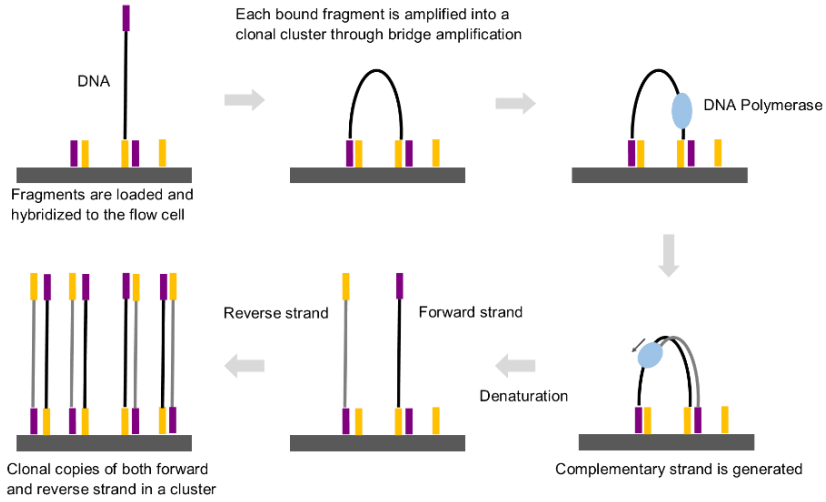

Figure 5. Cluster generation by bridge amplification. The DNA attaches to the flow cell via complementary sequences. The strand bends over and attaches to a second oligo forming a bridge. A polymerase synthesizes the reverse strand. The two strands release and straighten. Each forms a new bridge (bridge amplification). The result is a cluster of DNA forward and reverse strand clones.

At the end of clonal amplification, the flow cell is washed of all of the reverse strands, leaving only the forward strands. Sequencing begins when a primer attaches to the forward strand's adapter primer binding site, and a polymerase adds fluorescently tagged nucleotides (dNTPs) to the DNA strand. Each dNTP is tagged with a fluorescent reversible terminator, allowing the incorporation of only one base per cycle. Each time a new base is attached, the fluorescent signal unique to the base is imaged and recorded, then the reversible fluorophore is cleaved and new fluorescently tagged dNTPs are added over the flow cell (Figure 6).

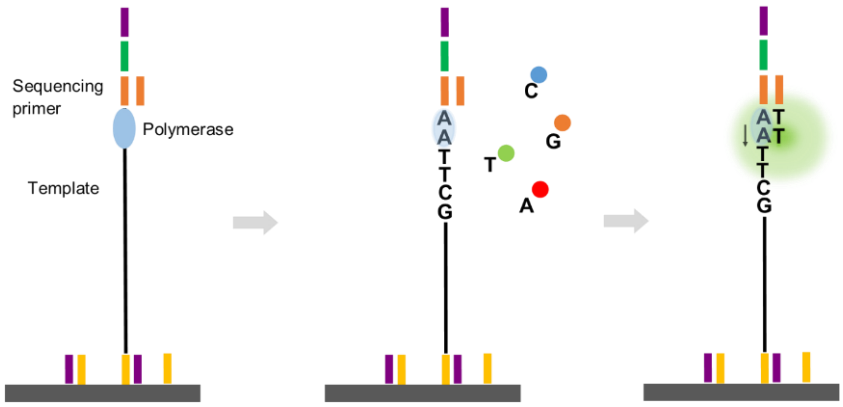

Figure 6. Sequence by synthesis. Tagged nucleotides are added over the flow cell. Each of the four nucleotides has a specific fluorescent label. At each cycle, a nucleotide is added by the polymerase to the DNA strand and excited by a light source. The emission wavelength along with the signal intensity determines the base call. A computer records all of the emissions. The number of cycles determines the length of the read.

4.3 Bioinformatics

The following steps are the result of bioinformatic analysis, using different pipelines and software packages for the analysis of the sequencing data. The taxonomic analysis of 16S rRNA gene sequences (paper I) includes pre-processing and quality control of the sequencing reads, a taxonomic assignment of the generated amplicons sequences, and statistical analysis of the data [128]. The functional analysis uses an *in silico* approach based on the 16S rRNA gene sequencing data (paper II).

4.3.1. Taxonomic analysis

1. Preprocessing of raw sequencing reads

The raw sequencing reads are available as fastq files and are subject to quality check and pre-processing before any downstream analysis. This step includes quality control, adapter trimming (demultiplexing), and quality filtering of the sequencing data, and was conducted in the Quantitative Insights Into Microbial Ecology (QIIME2, version 2019.10) [129] environment using QIIME2 plugins. QIIME is a free and open-source platform using the user's raw sequencing data and easily produce preliminary measurements of inter-and intra-sample diversity. Fastp (version 0.20.0) [130] was used to perform a quality check through a set of analyses, such as “per base sequence quality”, “per sequence quality score”, “sequence length distribution”, or “adapter content”. The Divisive Amplicon De-noising Algorithm (DADA2) [131] uses the demultiplexed fastq files as input and from which the adapters have been previously removed. The pipeline will identify and correct any sequencing errors, low quality or chimeric sequences. The generated output is an amplicon sequence variant (ASV) table, which reports the number of times each amplicon sequence variant was observed in each sample. The term amplicon sequence variant (ASV) refers to single DNA sequences from a 16S rRNA marker gene analysis. The table was used as input for taxonomic assignment.

2. Taxonomic assignment

Preprocessed and denoised raw sequencing reads are assigned into groups according to their homology, i.e. operational taxonomic units (OTUs), with a sequence similarity of at least 97 %. The taxonomic classification is obtained by comparing amplicon sequence variants to microbial 16S rRNA reference databases. We used reference sequences from the SILVA database (version 132) [132] to extract sequences at a similarity threshold of 99 % with locus-specific sequences from V2-V3 and V4-V5 QIAseq 16S primers. A phylogenetic tree was created using the FastTree program [133], a QIIME2 phylogeny plugin, based on the alignment of sequences. The resulting table with the taxonomy classification from FastTree was filtered to include only assigned reads of the taxonomic kingdom Bacteria and exclude reads assigned to mitochondria or chloroplasts. The final phylogenetic analysis based on the generated BIOM file (abundance profile) from QIIME and phylogenetic trees from FastTree was done on R studio using the Phyloseq [134] package for comparison of bacterial diversity and visualization of the microbial composition throughout the saturation dive.

3. Statistics

To assess and compare bacterial communities in the divers' oral microbiota in paper I, two diversity measurements were calculated in R studio using the Phyloseq package [134]. The alpha diversity was characterized by the Shannon index using the function estimate_richness and the beta diversity by the Bray-Curtis dissimilarity index using the function ordinate. A principal coordinate analysis, a statistical method used to explore similarities between samples, visualized the differences in microbial communities on a scatterplot. Any significant difference between the different groups (based on the four time-points of the dive) of the first two dimensions was estimated by permuted analysis of variance (permANOVA) from the vegan package.

A statistical analysis was performed to measure any change in the Shannon Index and bacterial relative abundance throughout the dive (IBM SPSS Statistics Software Version 26.0). First, the data distribution was visually examined using Quantile-Quantile plots. To test if the data were normally distributed, Shapiro-Wilk normality tests were calculated for each time point. A resulting $p > 0.05$ meant the data were normally distributed. If not, the data were transformed to better fit the distribution. After transformation, if the data were still non-normal, a non-parametric analysis was applied. Then, a one-way repeated measures ANOVA with a *post hoc* Bonferroni adjustment for multiple comparisons was used to assess the alpha diversity within the divers between the four time points. First, on the Shannon index, and then on the relative abundances at the phylum and genera level. P-values of < 0.05 were considered statistically significant. Mauchly's sphericity test assumed homogeneity for $p > 0.05$, and if this assumption was violated a correction using Greenhouse-Geisser was applied. When the data were non-normal, a non-parametric ANOVA using Friedman's test was calculated and pairwise comparisons were performed with a Bonferroni correction for multiple comparisons.

4.3.2. Functional analysis

The functional analysis of bacterial communities of paper II was based on the ASV (Amplicon sequence variant) table and taxonomic classifications from paper I using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [116]. PICRUSt2 is a predictive tool designed to assign metabolic functions to 16S rRNA genes previously identified during the taxonomic analysis.

The PICRUSt2 workflow is shown in Figure 7. The method consists of the phylogenetic placement of ASVs; gene content prediction for each ASV; metagenomic prediction per sample and inference of pathway abundances.

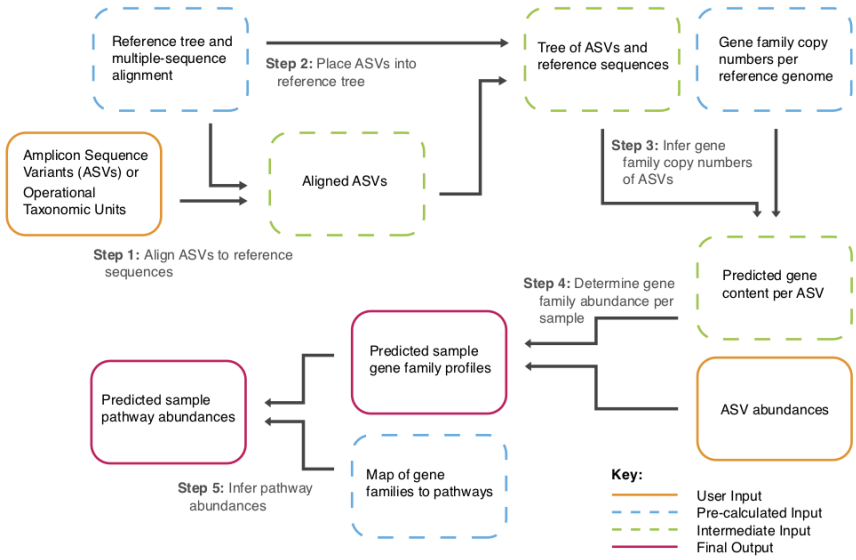

Figure 7. PICRUSt2 workflow. Amplicon Sequence Variants are used as input, and gene family and pathway abundances are output. Figure from Douglas *et al*. [116].

Step 1-2. Alignment of ASVs and placement into a reference phylogenetic tree

The study sequences (ASVs) are aligned with 16S reference sequences. Once aligned, the study sequences are placed into a reference phylogenetic tree. By default, the reference tree contains 16S rRNA genes from bacterial and archaeal genomes from the Integrated Microbial Genomes database [135]. The phylogenetic tree is then used to predict individual gene-family copy numbers for each ASV. Gene families are groups of related genes that share a common ancestor.

Step 3. Prediction of gene family copy numbers of ASVs (gene content prediction)

This step estimates the gene content of all microorganisms present in the phylogenetic tree. The output gives the predicted marker gene abundances (16S rRNA gene) and the predicted gene family abundances (e.g., Enzyme Commission (EC) numbers). It is also possible to calculate the nearest-sequenced taxon index values for each ASV, which defines how close an ASV is to the existing 16S reference sequence.

Step 4. Prediction of gene family abundance per sample (metagenome prediction)

Microbes have variable copy numbers of 16S rRNA gene which is species/strain-dependent. For example, an organism with seven copies of the 16S rRNA gene would be predicted at a higher relative abundance compared to an organism with one copy of the 16S rRNA gene, even if they are at the same absolute abundance in the sample. Therefore, ASVs are divided by their predicted 16S rRNA gene copy number for normalization to avoid any issues in the interpretation of the results. It results in relative abundances of organisms rather than the relative abundance of the 16S rRNA gene itself.

Following copy number normalization, each ASV is multiplied by their predicted gene family copy number (functional abundance) such as EC numbers. The sum of all ASVs in a sample gives the predicted metagenome.

Step 5. Prediction of pathway abundances

This final step predicts pathway abundances using MetaCyc database [120]. The pathway abundances are calculated on the gene families abundances. One of the gene families abundances used is EC number abundances. EC numbers are regrouped to MetaCyc reactions where they are organized by their functions (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases). EC numbers are used to associate these reactions within MetaCyc pathways. It first helps identify if the pathway is present or not. If present, the pathway abundance is then calculated. The pathway abundance table indicates the participation of each ASV in the abundance of pathways at the whole community level, rather than the prediction of the pathway abundance within the predicted genome of that ASV alone.

For this study, the list of the different predicted pathways was used to predict the functional profile of the oral microbiota throughout the heliox saturation dive. The significance in activated pathways was calculated using paired t-tests as implemented in the voom method in the limma R package [136] with an adjusted p-value < 0.05. The first time-point (day 0, at the surface) was used as a reference for comparison in the pathway abundance with the other time-points during the dive (day 2 and day 5 of the bottom phase and day 28, after decompression). Significantly different and biologically relevant pathways were plotted in agreement with the biological hypotheses of the study.

4.4. Immunoassay data

Paper III is a pilot study on the feasibility of using saliva biomarkers as indicator of inflammation and stress responses in saturation divers to high partial pressure of oxygen. The samples were assayed at the Salimetrics' SalivaLab (Carlsbad, CA) using their own Salimetrics Salivary Assay Kits for each biomarker: C-reactive protein, alpha amylase, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), cortisol and salivary immunoglobulin A (SIgA). The raw data was then used for statistical analysis.

4.4.1 Statistical analysis

Statistical analysis on the selected eight salivary biomarkers was done using the same steps as paper I 's statistical analysis. The distribution of the data was verified using Shapiro-Wilk normality tests ($p > 0.05$). The differences in biomarkers levels between surface and the bottom phase of the dive were calculated using one-way repeated measures ANOVA with a *post hoc* Bonferroni adjustment for multiple comparisons and Mauchly's sphericity test for homogeneity ($p > 0.05$). In the case data were non-normally distributed, the differences in biomarkers levels between surface and the bottom phase of the dive were calculated using a non-parametric test (Friedman) and pairwise comparisons with a Bonferroni correction for multiple comparisons ($p < 0.05$).

5. Summary of the results and discussion

Saturation diving impacts the divers' physiology in many aspects. The confined and high-pressure oxygen environment in which saturation divers are exposed may impact the physiological and metabolic homeostasis, and therefore their health and performance [1]. The main pathologies associated with saturation diving, namely decompression sickness, high-pressure nervous syndrome, and oxygen toxicity, have been extensively described to better understand their pathophysiology. Several studies focused on the inflammation process that occurs during different types of diving and how it can be linked with the development of these pathologies [26, 137-140]. The microorganisms that live in and on our bodies have an important role in health and disease. Their interactions with the immune system may affect primary metabolic processes, making the human microbiome a new tool for investigating its important link with disease. Oral bacteria living in the divers' mouth are in direct contact with hyperbaric breathing gas and these external conditions can affect the composition as well as the metabolic functions of the oral microbiota. The main objective of this thesis was to investigate the effects of the high partial pressure of oxygen during commercial saturation diving on the composition and metabolic functions of the oral bacterial communities after 16S rRNA gene sequencing and to measure salivary biomarkers representative of this stressful environment.

5.1 Paper I and II: Taxonomic and functional analysis of the oral microbiota during saturation diving

While gut microbiota has been widely studied over the last 40 years, the oral microbiota and its influence on health is a relatively recent area of study from systemic diseases to psychological disorders. Lately, a few studies have focused on the effects of saturation diving on the human microbiota. In our study, the environmental conditions of saturation diving led to transient changes in the taxonomic and functional profiles of the oral microbiota.

In paper I, we identified 10 bacterial phyla in the 23 divers' saliva using 16S rRNA gene sequencing. The distribution of these phyla was similar to the distribution found in a healthy core oral microbiota meaning that the oral microbiota of the divers was not altered before the dive. Therefore, all changes observed in the oral microbiota during the dive were due to the inherent conditions of saturation diving. We also observed a reduction in the alpha diversity during saturation, meaning that the complexity in the composition of the oral microbiota changed. We observed an increase in the relative abundance of aerobic and facultative anaerobic bacteria belonging to the phyla Proteobacteria and Actinobacteria due to their most abundant genera *Neisseria* and *Rothia* respectively. Conversely, we observed a lower relative abundance of the phylum Fusobacteria (genus *Fusobacterium*) during saturation. Our findings are in contrast with a study from Yuan *et al*., that described the changes in the composition of gut microbiota in saturation diving with living and excursion depths at 55–134 meters underwater [141]. They reported no change in alpha diversity but a decreased abundance in the genus *Bifidobacterium* of the phylum Actinobacteria. They hypothesized that one possible contributing factor of the decrease of *Bifidobacterium* was due to hyperoxia. This hypothesis is even more relevant for our study where the oral microbiota is directly in contact with the breathing gas mixture during the dive. Thereby, oral bacteria need to adapt to the frequent changes in pressure and oxygen availability. Several

studies have already described the adaptation of bacteria to changes in oxygen concentration, either in high altitude (hypoxia) in gut microbiota [142, 143] or during hyperbaric oxygen therapy (hyperoxia) in lung microbiota [144]. These observations are in concordance with our results where the bacterial composition in the oral microbiota of the divers was dominated by aerobic bacteria (phyla Actinobacteria and Proteobacteria) which filled the niche left by the decreasing anaerobes (phylum Fusobacteria).

In paper II, we aimed to elucidate whether transient changes in bacterial diversity could impact the diver's health during saturation, especially by investigating the functional activities in the diver's oral microbiota. The functional analysis used PICRUSt2 based on the previous 16S rRNA gene sequencing data from which the metabolic changes were predicted.

The predicted metabolic pathways that significantly changed during the bottom phase were involved in the bacterial growth and survival associated with their oxygen requirements. These results are consistent with the taxonomic analysis of microbial diversity and the oxygen-driven shift of bacterial abundance observed in paper I. Indeed, there was a significant increase in energetic metabolic pathways linked to the predominance of aerobic bacteria. They are able to use oxygen as an energy molecule in reactions involved in the tricarboxylic acid cycle and respiratory chain. Furthermore, a decrease in anaerobic pathways such as fermentation has also been observed.

Bacterial survival may also imply oxidative stress responses against the increase of the partial pressure of oxygen. Several enzymatic defense mechanisms that help bacteria manage oxygen toxicity and ROS production are described in paper I. Additionally, non-enzymatic bacterial defense mechanisms were estimated to increase during saturation such as pentose phosphate pathway and the biosynthesis of guanosine tetraphosphate pp(G)pp and mycothiol. The changes in metabolic pathways involved in bacterial growth and survival are not expected to have an impact on the diver. They are merely the sign that aerobic bacteria were predominant due to the high partial pressure of oxygen during the bottom phase.

Diet is another possible factor contributing to changes in oral microbiota diversity and therefore microbial activity. Several studies have recommended the use of antioxidants and vitamins (C, E, B_9, and B_{12}) in saturation diving [138, 145, 146]. Antioxidants, such as vitamin C and E, may prevent cellular damages caused by oxidative stress during a deep saturation dive [147]. Vitamins such as folate (B_9) and adenosylcobalamin (B_{12}) are important in erythropoiesis [148] and may help reduce mild anemia and fatigue experienced among divers after decompression [43, 145, 149]. Vitamin B_{12} biosynthesis, either anaerobic or aerobic, is confined to a few bacteria and archaea. We did not find any significant change in the superpathway of biosynthesis and salvage of tetrahydrofolate during the bottom phase, but we did observe a decrease in the biosynthesis of adenosylcobalamin. A new source of vitamin B_{12} can be added to the diver's diet to compensate for this decrease. This result is in agreement with a vitamin B_{12} supplementation previously recommended for saturation divers [145].

5.2 Paper III: Environmental stress assessment on the diver's physiology using saliva

Limitations in offshore saturation diving studies are mainly due to technical and logistics restraints. These studies worked on the blood as it is considered the best choice for studying physiological changes [137-140]. However, blood is challenging to collect during saturation diving. It requires trained personnel and the samples need to be pre-processed onboard the dive support vessel by using a centrifuge to prepare serum or plasma. Although blood biomarkers have been extensively used in clinical care, the biomarkers are also available in urine or saliva [150]. The aim of paper III was to examine whether salivary biomarker analysis might be a feasible approach for the evaluation of environmental stress in offshore saturation diving.

As described earlier, saturation diving is considered stressful for the body. Environmental stress factors may alter the cellular homeostasis and lead to adaptive stress responses through the activation of the hypothalamic-pituitary-adrenal axis and autonomous nervous system, both of which interact with the immune system [8]. Interactions between the immune system and the diver's physiology have been studied in saturation diving using blood biomarkers of oxidative stress and inflammation [138]. Oxidative stress damages have also been measured in saliva during saturation diving, including the production of reactive oxygen species and total antioxidant capacity [150]. The transient increase that we have observed in the levels of salivary inflammatory mediators including CRP, TNF-α, IL-1β, and IL-8 during saturation could be the result of an activation of the sympathetic nervous system responsible for the stimulation of the salivary glands. We observed no changes in IL-6, salivary cortisol, and alpha-amylase levels during saturation. These results represent the involvement of a physiological stress associated with systemic inflammation due to the high oxygen pressure environment, rather than a general stress response.

6. Study limitations

Several limitations arose when analyzing the data from the metagenomic study. First of all, since it was the first study to analyze saliva samples that went through decompression, we had to ensure the quality of the samples. The samples were kept frozen at -80°C at the NTNU before being checked for quality. Unfortunately, even if the saliva collection kit was designed for both DNA and RNA, RNA wasn't stable enough to come out for decompression without being deteriorated.

Second, following the bioinformatic analysis on the 16S rRNA gene sequencing data, the taxonomic analysis revealed interesting results on bacterial abundance evolution during the dive. When designing the study, based on hematology data, the results ensured that most changes would occur during the first week of saturation, with little change later in the dive. However, it would have been interesting to have a saliva sample on day 14 (mid-bottom phase) to verify this statement.

Finally, studies on the oral microbiome emphasize the importance of diet and oral health on bacterial composition. No recommendations were made on the divers' lifestyle regarding diet or use of toothpaste.

Regarding the computational approach, PICRUSt2 software is not capable of visualizing changes in expression from individual bacteria, as it uses 16S rRNA gene sequencing data for functional predictions. It does not determine changes in metabolic activity within individual bacteria but predicts however functional changes at the whole bacterial community level. The predicted changes in functional diversity are based on 16S rRNA gene sequencing data which was present in the reference database.

7. Conclusion

In this thesis, we aimed to provide a better understanding of the effects of heliox commercial saturation diving on the oral commensal microbiota of the divers during commercial diving operations.

I. The taxonomic analysis using 16S rRNA gene sequencing revealed that the oral bacterial diversity was reduced during the bottom phase of saturation diving. The oral microbiota composition showed a significantly higher relative abundance of aerobic bacteria and a lower relative abundance of anaerobic bacteria. This transient change in composition indicates a growth advantage of aero-tolerant bacteria over anaerobes following the increase in the pressure of the breathing gas during the saturation phase and therefore in oxygen concentrations.

II. The functional profiling of the oral bacterial communities based on the 16S rRNA gene sequencing data was performed using a computational approach: PICRUSt2. The software estimated changes in several metabolic pathways involved in the survival and growth of bacteria. These results were consistent with the findings in paper I with the oxygen-driven bacterial shift. An increase in defense mechanisms against oxidative stress has also been predicted. Furthermore, the observed reduction of bacterial vitamin B_{12} biosynthesis may affect the diver's physiology. This result supports nutritional recommendations already considered for vitamin B_{12} as part of the diet of divers during diving operations.

III. Environmental stress was measured using salivary biomarkers that can highlight transient pro-inflammatory responses, most likely triggered by the saturation diving. Saliva seems to be suitable for a daily assessment of the effects of the hyperbaric environment on the divers as a minimally invasive approach that removes the need for direct access to personnel trained in biological sample collection.

8. Further perspectives

Interactions between bacteria and the physiology of the diver is a relatively new area of study. In paper II, we assessed metabolic changes in oral bacteria and speculated on the impact of the reduction in B_{12} biosynthesis on the blood transport capacity of the divers. In future work, it would be interesting to measure these relationships by acting on external factors such as the diver's diet or his vitamin and antioxidant supplementation.

It would also be interesting to evaluate the salivary stress biomarkers in other diving conditions, e.g., for deeper dives, in order to improve procedures for decompression for example. This method could be useful to bring on the same level the different existing diving procedures between the different countries. This would require setting up on-site tests that would allow immediate measurement of the marker in question.

9. References

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10. Appendix: Paper I - III

Paper I

frontiers in Physiology

ORIGINAL RESEARCH
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Shifts in the Oral Microbiota During a Four-Week Commercial Saturation Dive to 200 Meters

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During commercial saturation diving, divers live and work under hyperbaric and hyperoxic conditions. The myriads of bacteria that live in and on the human body must adjust to the resultant hyperbaric stress. In this study, we examined the shifts in bacterial content in the oral cavity of saturation divers, using a metagenomic approach to determine the diversity in the composition of bacterial phyla and genera in saliva from 23 male divers before, during, and immediately after 4 weeks of commercial heliox saturation diving to a working depth of circa 200 m. We found that the bacterial diversity fell during saturation, and there was a change in bacterial composition; with a decrease at the phylum level of obligate anaerobe *Fusobacteria*, and an increase of the relative abundance of *Actinobacteria* and *Proteobacteria*. At the genus level, *Fusobacterium*, *Leptotrichia*, *Oribacterium*, and *Veillonella* decreased, whereas *Neisseria* and *Rothia* increased. However, at the end of the decompression, both the diversity and composition of the microbiota returned to pre-dive values. The results indicate that the hyperoxic conditions during saturation may suppress the activity of anaerobes, leaving a niche for other bacteria to fill. The transient nature of the change could imply that hyperbaric heliox saturation has no lasting effect on the oral microbiota, but it is unknown whether or how a shift in oral bacterial diversity and abundance during saturation might impact the divers' health or well-being.

Keywords: acclimatization, decompression, heliox saturation, microbiome, metagenomic, bacterial phyla, bacterial genera

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INTRODUCTION

Commercial saturation diving is used to perform long-term subsea work at greater depths. During saturation diving operations, the divers live within a pressurized, hyperbaric chamber system in a heliox atmosphere (a mix of oxygen and helium) for longer periods, normally limited to 28 days (DMAC, 2006). The divers commute to work from the hyperbaric chamber system to the sea bottom in a diving bell. Although saturation diving is generally considered safe, the environmental conditions encountered by the divers are still a matter of health concern (Brubakk et al., 2014). Preserving health in harsh environments requires successful

acclimation of the body's physiological mechanisms. In this respect, the multiple microorganisms living in or on the body are also involved; i.e., the various microbiota that constitutes the human microbiome. Since the first appearance of the term microbiota in 2001 (Lederberg and McCray, 2001) and the recent development of metagenomics study tools, a large number of studies have emerged emphasizing the dual role of microorganisms inhabiting the human body in health protection as well as the development of diseases (Scannapieco, 2013; Wade, 2013; Yamashita and Takeshita, 2017; Sharma et al., 2018; Zheng et al., 2020). They interact with the host's immune system and affect central metabolic processes (Chu and Mazmanian, 2013; Belkaid and Hand, 2014).

Divers are exposed to several stress factors during hyperbaric saturation (Bosco et al., 2018), which may also affect the composition and activity of the microbial communities. Bacteria residing in the divers' oral cavity, the oral microbiota, come directly into contact with the hyperbaric breathing gases. It has been proposed that oral bacteria contribute to their host's health and fitness beyond the oral cavity. For instance, they may be involved in the control of cardiovascular function by nitric oxide (NO) *via* their essential function in the regulation of nitrate (NO_3^-) production (Hyde et al., 2014; Cutler et al., 2019), and are thought to play a role in autoimmune disease susceptibility (Nikitakis et al., 2017). The effects of hyperbaric heliox saturation on the human oral microbiome have yet to be determined.

This study was designed to examine the effects of commercial saturation diving on the bacterial content of the oral microbiota. A metagenomic approach was used to determine the composition of bacterial phyla and genera in divers' saliva before, during, and immediately after 4 weeks of commercial heliox saturation diving.

MATERIALS AND METHODS

Ethics

The study was conducted during a commercial saturation diving operation in the Mediterranean Sea, March–April 2018. The protocol was approved in advance by the Norwegian Regional Committee for Medical and Health Research Ethics (REK), approval number 2018/1184. Divers who passed the pre-saturation medical examination and were committed to saturation onboard the Dive Support Vessel (DSV) Deep Arctic were eligible for participation. They were informed of the aim and scope of the study and provided written consent before inclusion. The experimental procedures were conducted according to the Declaration of Helsinki principles for ethical human experimentation.

Study Subjects

Initially, 30 certified saturation divers, all male non-smokers, were enrolled in the study for the duration of a 28-day work assignment in hyperbaric heliox saturation. All held valid health certificates for work in saturation and fulfilled the operator's requirement for aerobic fitness with maximum oxygen uptake capacity $VO_{2max} \geq 40$ L/min. **Table 1** describes the study subject characteristics prior to saturation. The study did not interfere with the divers' lifestyle, whether diet, activity outside of operational requirements, nor the use of dentifrice. In the final analysis, we included only divers from whom four sets of saliva samples were successfully obtained, resulting in data from 23 of the 30 divers.

TABLE 1 | Study subject characteristics prior to saturation (n = 23).

	Mean	Range
Age (years)	44	31–60
BMI (kg/m^2)	26.8	20.2–31.2
VO_2 max (L/min)	48	44–60

BMI, body mass index; VO_2 max, maximum oxygen uptake capacity.

Saturation Diving

Saturation diving was performed according to the contractor's procedures as previously described (Łuczyński et al., 2019). In summary, the divers were compressed in a heliox atmosphere over a period of about 6 h to a storage depth of 178–192 meters of seawater (msw). They remained at storage depth throughout the bottom phase for 21 days, during which time they performed daily shifts of underwater work in teams of three at depths of 191–207 msw. Each shift lasts 12 h, 7 days per week. A dive bell was used to transport the divers between the pressure chamber and the underwater work site. When the bottom phase was completed, the divers were decompressed back to atmospheric pressure over a period of 8 days.

During the bottom phase, the partial pressure of oxygen (ppO_2) was kept at 40 kPa in the pressure chamber and 60–80 kPa during the bell-runs. During decompression, the ppO_2 was increased to 50 kPa until a depth of 13 msw was reached, and from there on ppO_2 was gradually reduced to 21%. After reaching the surface, the divers stayed on the vessel for another 24 h for observation for decompression sickness, before they left the vessel. The saturation profile is shown in **Figure 1**.

Saliva Collection

Saliva samples were collected at four time-points for each diver, as shown in **Figure 1**. D0 is at surface before saturation, D2 and D5 are on days 2 and 5 during bottom phase, and D28 is the day decompression was completed and the divers were back to surface. The divers themselves collected the saliva and were instructed not to brush their teeth before sampling. All sampling was undertaken ≥2 h after the last meal and collected in an all-in-one kit for stabilization of microbial nucleic acids (OMNIgene ORAL OM-505, DNA Genotek, Ottawa, Canada). Samples obtained on days 2 and 5, while the divers were pressurized, were decompressed back to surface pressure through an airlock shortly after collection. During the collection period, samples were stored in a fridge in the vessel hospital. At the end of the operation, all samples were transported collectively to the Norwegian University of Science and Technology (NTNU) for analysis. The bottom phase time-points for sample collection were chosen based on earlier reports on hematology in saturation

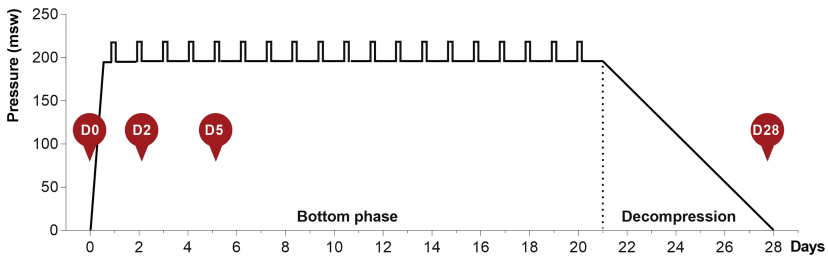

FIGURE 1 | The 28-day heliox saturation profile. Hyperbaric saturation was done in a heliox atmosphere with 6 h compression, 21-day bottom phase, and 7-day decompression. Daily underwater work excursions (bell-runs) in the bottom phase are indicated by vertical bars. Saliva collection time-points are shown as red circles: D0, before saturation; D2 and D5, days 2 and 5 of the bottom phase; D28, after decompression.

diving, and are in agreement with recent data on mice lung and gut microbiota in response to hyperoxia (Hofso et al., 2005; Ashley et al., 2020). At the time of sample collection, the divers had already performed at least on round of heliox saturation during the same diving campaign. The baseline (day 0) samples, therefore, also represent the status of the diver's microbiota 4–6 weeks after a similar exposure.

16S Library Preparation for Metagenome Sequencing

16S metagenomic sequencing libraries were prepared according to the QIAseq 16S Region Panel protocol (Qiagen, Hilden, Germany). In brief, 4 nanograms (ng) of genomic DNA extracted from the saliva samples (n = 92) were used as a template for PCR of the 16S V2–V3 and V4–V5 regions, in separate reactions using Illumina primers/reagents. The resulting 16S amplicons were purified using AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, United States). Purified PCR products from each sample were pooled and thereafter subjected to a second PCR amplification step, where dual samples indices and Illumina sequencing adaptors were added, according to the manufacturer's instructions. A second PCR clean-up step was performed using AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, United States), before the validation of the sequencing library using an Agilent High Sensitivity DNA Kit on a BioAnalyzer (Agilent Technologies, Santa Clara, CA, United States). Individual sample libraries were pooled and normalized to 10 pM, prior to sequencing of 2 × 300 cycles with a MiSeq V3 flowcell on a MiSeq instrument, according to the manufacturer's instructions (Illumina, Inc. San Diego, CA, United States). Sequence reads were demultiplexed and converted from BCL to fastq file format using bcl2fastq2 conversion software V2.20.0422 (Illumina, Inc. San Diego, CA, United States).

Bioinformatics

The sequencing data were merged and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2, version 2019.10) pipeline (Caporaso et al., 2010). All following procedures in this section were conducted in the QIIME2 environment using QIIME2 plugins. Demultiplexed paired-end reads from MiSeq (2 × 300 bp) were trimmed to remove primers and poor quality bases with fastp (version 0.20.0; Chen et al., 2018). The trimmed sequences were denoised and clustered with DADA2 (Callahan et al., 2016). The generated amplicon sequence variants (ASVs) were assigned to taxonomy using a targeted classifier. Briefly, we extracted sequences from the SILVA database (version 132) at a similarity threshold of 99% with locus-specific sequences from V2–V3 and V4–V5 QIAseq 16S primers and a targeted Naive Bayes trained on the extracted sequences. The QIIME2 phylogeny plugin was applied to construct the rooted phylogenetic tree by employing the FastTree program (Price et al., 2009). Based on the taxonomy generated, we filtered our feature-table to include only assigned reads of the taxonomic kingdom Bacteria and exclude reads assigned to mitochondria or chloroplasts. The generated BIOM file and phylogenetic trees were further imported into Phyloseq for comparison and visualization (McMurdie and Holmes, 2013).

Statistical Analysis

To estimate the diversity of the oral microbiota within individuals, i.e., the richness and evenness of the bacterial community (Kim et al., 2017), different indexes of the alpha diversity within samples such as the Shannon index were calculated by the function estimate_richness and the beta diversity between samples was calculated on a Bray-Curtis distance measure by the function ordinate using the Phyloseq package on R studio. Significance between groups in the PCoA subspace of the first two components was estimated by permuted Anova (permANOVA) from the vegan package. Statistical analysis on the Shannon index and the relative abundance at the phylum and genus level was done in IBM SPSS Statistics software Version 26.0. Normal distribution of the data was confirmed by visual inspection of Q-Q plots and Shapiro-Wilk's test for normality ($p > 0.05$), either directly or after the data were transformed. If the data were still non-normal after transformation, a non-parametric analysis was applied. A one-way repeated measures ANOVA, with Bonferroni *post hoc* adjustment for multiple comparisons, was used to assess within diver differences in Shannon index and relative abundance of phyla and genera between the four time-points. Differences were considered significant at $p < 0.05$. If the

assumption of sphericity was violated, as assessed by Mauchly's test which assumes homogeneity for $p > 0.05$, a Greenhouse-Geisser correction was applied. In the case of non-normal data, Friedman's test was applied and pairwise comparisons were performed with a Bonferroni correction for multiple comparisons.

Data Repository

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB40804.[1]

RESULTS

Ten different bacterial phyla were detected by taxonomic analysis of the 16S rRNA sequencing data from saliva collected from 23 divers before, during, and at the end of 4 weeks of commercial heliox saturation diving. The analysis of alpha diversity within samples, i.e. species richness and evenness using the Shannon index, showed a significant drop during the bottom phase (4.028 ± 0.47 for day 2, and 4.089 ± 0.48 for day 5, $p < 0.001$), compared to the baseline (day 0; 4.398 ± 0.33). However, the alpha diversity returned to baseline after the decompression (**Figure 2A**). The beta diversity is shown in **Figure 2B**.

Taken altogether, the total abundance of the 10 detected phyla did not significantly change during heliox saturation (**Figure 3**).

In general, five phyla were dominant in all the samples: *Firmicutes* (34%), *Proteobacteria* (27%), *Bacteroidetes* (17%), *Actinobacteria* (11%), and *Fusobacteria* (9%). Five other phyla were detected at lower levels (<1%), including *Patescibacteria*, *Epsilonbacteræota*, *Tenericutes*, *Spirochaetes*, and *Synergistetes*. Moving down the taxonomic tree, the most abundant significantly altered genera belonged to the phyla *Firmicutes* (genus *Veillonella* and *Oribacterium*), *Proteobacteria* (genus *Neisseria*), *Actinobacteria* (genus *Rothia* and *Actinomyces*), and *Fusobacteria* (genus *Fusobacterium* and *Leptotrichia*) (**Figure 4**).

The relative abundance of *Firmicutes*, *Fusobacteria*, and *Bacteroidetes* decreased during the first 5 days of the bottom phase, but only significantly so for *Fusobacteria*. Within the phylum *Firmicutes*, one of the dominant genera, *Veillonella*, decreased at day 2 ($p = 0.047$) and day 5 ($p = 0.004$), as well as the genus *Oribacterium*, that also decreased but only at day 2 of the bottom phase ($p = 0.040$; **Figure 4A**). In the phylum *Fusobacteria*, decreases in abundance were observed for the genus *Fusobacterium* at day 2 ($p < 0.0005$) and day 5 ($p = 0.027$), and in genus *Leptotrichia* at day 2 ($p = 0.0022$). We also observed a decrease in the genus *Prevotella* from *Bacteroidetes* but non-significant (**Figures 4B,C**). Conversely, during the bottom phase, at day 2, the abundance of *Actinobacteria* and *Proteobacteria* increased. In these phyla, genus *Rothia* (*Actinobacteria*) increased at day 2 ($p = 0.009$; **Figure 4D**), while genus *Neisseria* (*Proteobacteria*) increased at day 2 ($p = 0.010$) and day 5 ($p = 0.022$; **Figure 4E**). However, all changes observed during the bottom phase were abolished at the end of the dive.

DISCUSSION

In this study, we surveyed the oral bacterial microbiota of divers during 4 weeks of commercial heliox saturation diving. Using a taxonomic analysis through 16S rRNA sequencing, we identified 10 abundant bacterial phyla in saliva collected before, during, and immediately after saturation, and this composition is coherent with other studies that describe the human oral microbiota (Palmer Jr., 2014). We found that the microbial diversity was reduced during saturation and that the relative abundance of *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* changed significantly. In each phylum, these changes were mostly due to their most abundant genera, including a shift from obligate anaerobes (*Fusobacterium*, *Leptotrichia*, *Veillonella*, and *Oribacterium*) to aerobic/facultative anaerobic bacteria (*Neisseria* and *Rothia*). To our knowledge, this is the first study to address the relationship between hyperbaric environments and oral microbiota.

In our study, we observed that the alpha diversity of the oral microbiota was reduced during hyperbaric heliox saturation, indicating that either the richness and/or evenness of the microbial communities changed. The relative abundance of *Fusobacteria* decreased during saturation, whereas *Proteobacteria* and *Actinobacteria* increased. The most probable explanation is that the evenness of the oral microbiota was impacted by the drop of *Fusobacteria* during the bottom phase together with the raise of *Proteobacteria* abundance, in particular the *Neisseria* genus – of which this phylum alone made up more than 30% of the total abundance during the bottom phase. Furthermore, the absence of change in the total abundance during saturation (**Figure 2B**) indicates that the environmental conditions inherent to the dive merely created a shift in the microbial balance between the different genera and therefore phyla. In apparent contrast with our results, a recent study on commercial saturation divers' gut microbiota reported no change in alpha diversity, but a decreased abundance of the genus *Bifidobacterium*, which are in fact *Actinobacteria* (Yuan et al., 2019).

This discrepancy may be explained by the fact that the oral microbiome is directly in contact with the breathing mixture during the dive and thus is more reactive to the steep changes in pressure and oxygen availability. Oral bacteria can be classified according to their oxygen needs and their ability to metabolize it in different environments regarding each site of the mouth from tooth surfaces to supragingival and subgingival regions. Obligate aerobes are found to grow optimally at atmospheric concentrations of oxygen (~20%), whereas microaerophiles prosper best well-below normal atmospheric concentrations. Unlike obligate anaerobes that cannot tolerate oxygen and thrive only under anoxic conditions; facultative anaerobes can grow by fermentation, use other terminal electron acceptors for anaerobic respiration, or their ability to breathe aerobically. Aerotolerant anaerobes as the name suggests can tolerate the

[1]https://www.ebi.ac.uk/ena/browser/view/PRJEB40804

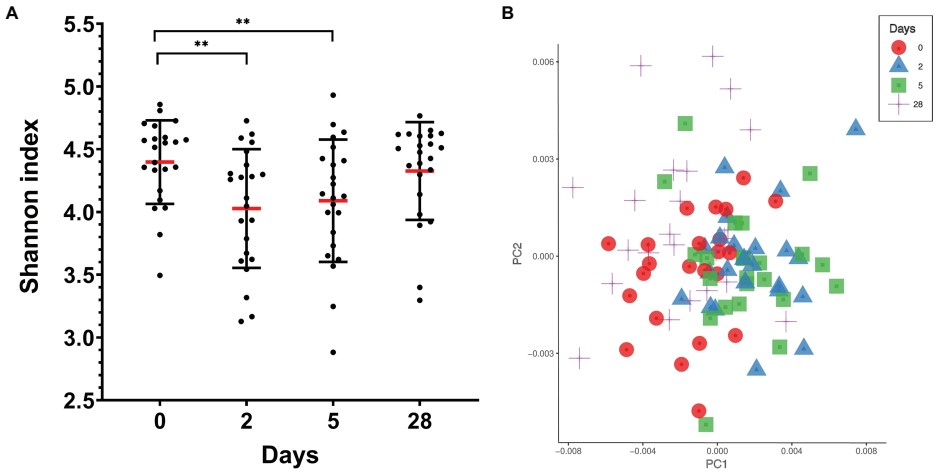

FIGURE 2 | Oral microbiota diversity in saliva from commercial saturation divers (n = 23) before, during, and after 28 days of heliox saturation. **(A)** The alpha diversity within samples, i.e., species richness and evenness, is given by the Shannon index (**p < 0.001). Means (red line) and individual values are shown. Error bars are ±1 SD. **(B)** Principal coordinate analysis (PCoA) plot of beta-diversity for individual samples showing the first two principal components (PC1 and PC2) using Bray-Curtis distances (p = 0.001 using permANOVA). Colored symbols indicate times of sample collection. In both panels, day 0, before saturation; days 2 and 5, during the bottom phase; day 28, after decompression.

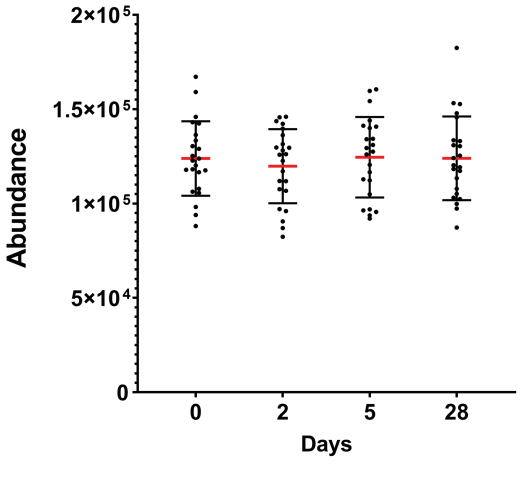

FIGURE 3 | Total abundance of the oral bacterial communities in saliva from commercial saturation divers (n = 23) before, during, and after 28 days of heliox saturation, displayed as the number of reads for all detected OTU after 16S sequencing. For each time-point, means (red line) and individual values are shown. Error bars are ±1 SD. Day 0, before saturation; days 2 and 5, during the bottom phase; day 28, after decompression.

presence of oxygen, but do not benefit from aerobic respiration and thrive optimally without oxygen (Morris and Schmidt, 2013). Many facultative anaerobes rapidly become anaerobic as the oral biofilm develops, which explains the predominance of obligate anaerobic bacteria in the mouth (Wade, 2013).

Hyperbaric hyperoxia in the divers' breathing gas is a likely contributing factor to the bacterial shift we observed. It has been noted that hyperoxia gave a selective relative growth advantage to oxygen-tolerant respiratory microbial species (e.g., *Staphylococcus aureus*) in patients with respiratory failure who received high concentrations of therapeutic oxygen. In parallel, using neonatal and adult mouse models, the same authors demonstrated that lung and gut bacterial communities were altered within 24 and 72 h, respectively, in mice exposed to hyperoxia (Ashley et al., 2020). To overcome the toxic effects of oxygen, both aerobic and facultative anaerobic organisms contain a highly regulated complex of antioxidant defense enzymes such as catalase or superoxide dismutase as well as other enzymatic and non-enzymatic defense mechanisms against the toxic effects of reactive oxygen species (ROS; Brioukhanov and Netrusov, 2007; Henry et al., 2014). These have been particularly well-described in the two pathogens in the species *Neisseria* (*Neisseria gonorrhoeae* and *Neisseria meningitidis*), when even within the same genus, Neisserial species appeared to have different contents of antioxidant enzymes (Archibald and Duong, 1986; Seib et al., 2004, 2006). Our divers were exposed to hyperoxia during the bottom phase, with ppO_2 of 40 kPa in the pressurized living chambers and up to 60–80 kPa during the bell-run excursions, which is double to triple the amount of oxygen in normobaric air.

An increase in oxygen availability has been reported to induce gut dysbiosis, thus driving an uncontrolled luminal expansion of the family *Enterobacteriaceae*, which are facultative anaerobic (Rivera-Chavez et al., 2017). Nevertheless, it is difficult to draw comparisons between the oral and the gut microbiome in relation to the normoxic and hyperoxic conditions because

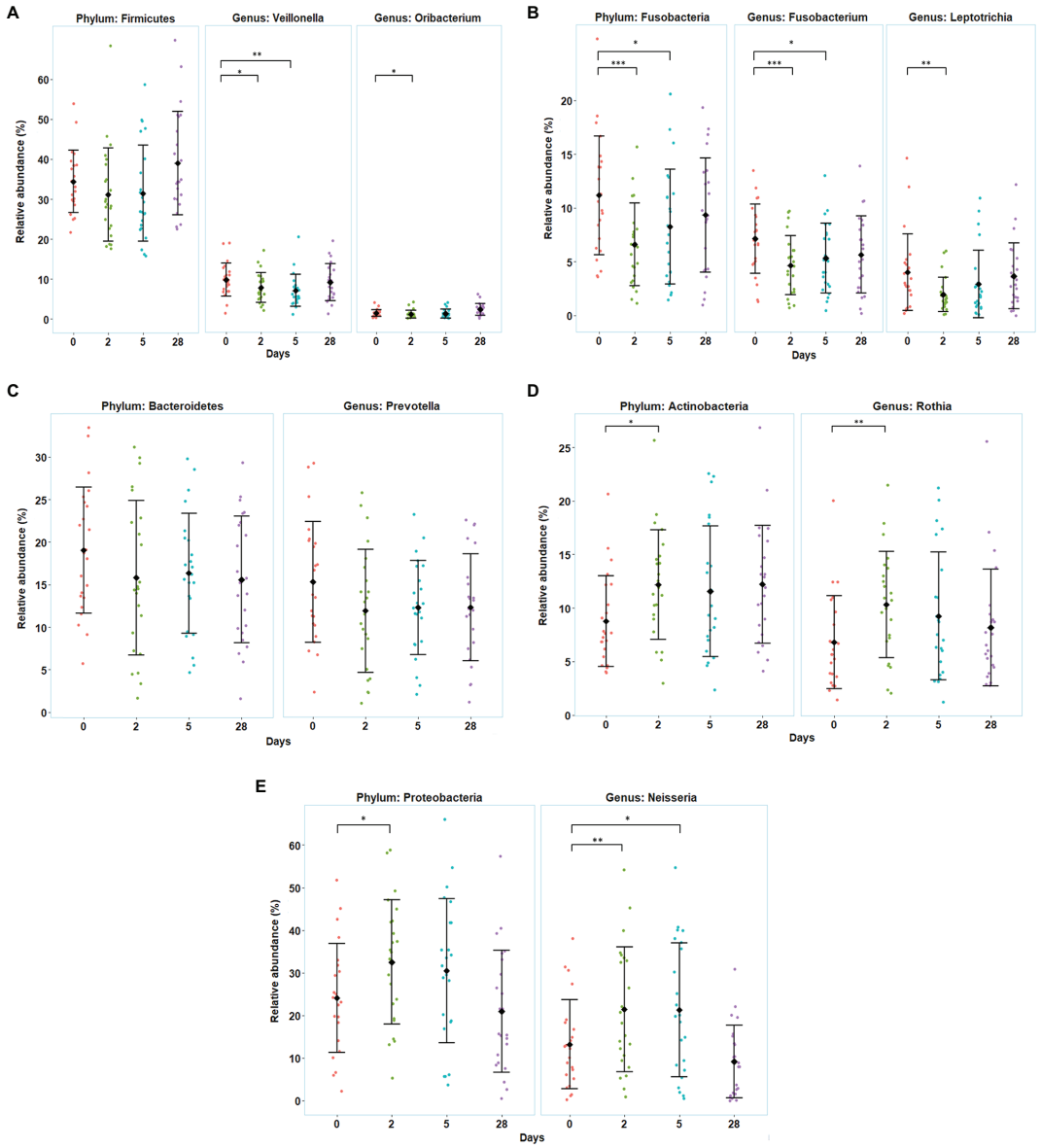

FIGURE 4 | Variation in the divers' oral microbiota before (day 0), during the bottom phase (days 2 and 5), and after decompression (day 28) from heliox saturation diving. **(A–E)** Observed shifts in the relative abundance of the five most abundant bacterial phyla, along with their significantly altered genera. For each time-point: mean (♦) and individual values are shown. Error bars are ±1 SD (*$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$). The relative abundance is given in percentage of total bacterial sequences.

the gut microbiome is usually more stable than the oral microbiome in terms of environmental conditions. However, we can compare the gut microbiome and the neonatal gut microbiome. The latter being more abundant in oxygen, it is frequently dominated by facultative anaerobes such as *Proteobacteria* species (Guaraldi and Salvatori, 2012). By consuming the oxygen in the 1st week of life, these facultative anaerobes create a more suitable environment for obligate

anaerobes such as *Fusobacteria* (Kelly et al., 2018). The hyperoxic conditions experienced by the divers during the bottom phase may thus be inadequate for *Fusobacteria*, which might in turn leave space for *Proteobacteria*.

The gut microbiota also seems to be related to the host response to hypobaric hypoxia exposure associated with increased inflammation and risk of infection (Hartmann et al., 2000; Khanna et al., 2018). High altitude tends to be positively correlated with obligate anaerobes (Maity et al., 2013; Suzuki et al., 2019) and is associated to relative abundance of *Prevotella* (Karl et al., 2018). It has recently been described that intermittent hypoxia can induce alterations in the gut microbiota (Moreno-Indias et al., 2015; Ramos-Romero et al., 2020). Together, these results suggest that obligate anaerobes may have a competitive advantage under hypoxic conditions over aerobes that require oxygen.

What might the consequences be of a shift in diversity and abundance of bacteria during saturation diving? Most studies on the oral microbiome established associations between oral health conditions and the bacterial composition in saliva. Indeed, healthy periodontal conditions were mainly related to the genus *Neisseria*, while the predominance of the genera *Prevotella* and *Veillonella* was associated with periodontal diseases (Yamashita and Takeshita, 2017). In addition, many studies also associate the gut microbiome to human health. A raise of *Proteobacteria* abundance in the gut microbiome has been linked to pathologies such as obesity (Zhu et al., 2013) and Type 2 diabetes (Larsen et al., 2010; Zhang et al., 2013). *Proteobacteria* also appear to be associated with extraintestinal diseases such as asthma and chronic obstructive pulmonary disease (COPD), making it a common microbial signature of states linked to various degrees with inflammation (Rizzatti et al., 2017). However, it is still unclear whether a transient bacterial shift from *Fusobacteria* to *Proteobacteria* in the oral microbiota may have an impact on the divers' health. Additional studies should be conducted to investigate the bacterial metabolic pathways altered during saturation diving.

LIMITATIONS

This study imposed no restrictions on the divers' routines or diet. Since they worked overlapping shifts over the 24-h day, the time for saliva collections varied accordingly. We can, therefore, not rule out possible effects of circadian variation which may have a functional impact on bacterial activity (Takayasu et al., 2017). The divers were also free to choose individually from daily selections of meals from the vessel galley. A modest association between diet and oral microbiota has been reported (Kato et al., 2017). However, these limitations are not expected to cause false-positive results.

CONCLUSION

We identified changes in the abundance of three bacterial phyla in the oral microbiota during commercial heliox saturation diving: *Fusobacteria* decreased, whereas *Proteobacteria* and *Actinobacteria* increased during the bottom phase. At the genus level, there was a decrease in the relative abundance of *Fusobacterium*, *Leptotrichia*, *Oribacterium*, and *Veillonella*, and an increase of *Neisseria* and *Rothia*. However, no changes persisted at the end of the decompression. The transient nature of the change could imply that hyperbaric heliox saturation has no lasting effect on the oral microbiota, but it is unknown whether and how the bacterial shift during saturation may impact the divers' health or well-being.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena/browser/view/PRJEB40804.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Norwegian Regional Committee for Medical and Health Research Ethics (REK). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, KH, and IE designed the study. IE collected the material. RM, JL, AH, and AF conducted the analyses. RM initiated the manuscript. All authors contributed in the writing and approval of the final version.

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Conflict of Interest: TechnipFMC sponsored helicopter transfers and boarding on the DSV Deep Arctic for IE.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

frontiers in Physiology

ORIGINAL RESEARCH
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Functional Profiling Reveals Altered Metabolic Activity in Divers' Oral Microbiota During Commercial Heliox Saturation Diving

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Background: The extreme environment in saturation diving affects all life forms, including the bacteria that reside on human skin and mucosa. The oral cavity alone is home to hundreds of different bacteria. In this study, we examined the metabolic activity of oral bacteria from healthy males during commercial heliox saturation diving. We focused on environmentally induced changes that might affect the divers' health and fitness.

Methods: We performed pathway abundance analysis using PICRUSt2, a bioinformatics software package that uses marker gene data to compute the metabolic activity of microbial communities. The analysis is based on 16S rRNA metagenomic data generated from the oral microbiota of 23 male divers before, during, and after 4 weeks of commercial heliox saturation diving. Environmentally induced changes in bacterial metabolism were computed from differences in predicted pathway abundances at baseline before, versus during, and immediately after saturation diving.

Results and Conclusion: The analysis predicted transient changes that were primarily associated with the survival and growth of bacteria in oxygenated environments. There was a relative increase in the abundance of aerobic metabolic pathways and a concomitant decrease in anaerobic metabolic pathways, primarily comprising of energy metabolism, oxidative stress responses, and adenosylcobalamin biosynthesis. Adenosylcobalamin is a bioactive form of vitamin B_{12} (vitB_{12}), and a reduction in vitB_{12} biosynthesis may hypothetically affect the divers' physiology. While host effects of oral bacterial vitamin metabolism are uncertain, this is a finding that concurs with the existing recommendations for vitB_{12} supplements as part of the divers' diet, whether to boost antioxidant defenses in bacteria or their host or to improve oxygen transport during saturation diving.

Keywords: 16S rRNA sequencing, hyperbaric stress, hyperbaric hyperoxia, oral microbiome, oxidative stress, vitamin B_{12}, energy metabolism

INTRODUCTION

During commercial saturation diving, divers may reside for weeks in a pressurized environment while breathing a mix of oxygen and helium (heliox). They must acclimatize to the extreme environment, including elevated partial pressures of oxygen (ppO_2). Hyperoxia is a powerful driver of biological processes (Kiboub et al., 2018a,b; Luczynski et al., 2019). In saturation diving, the divers are exposed to hyperbaric hyperoxia, and oxygen also affects the composition of the microbiota: the microbial communities that reside on human skin and mucosa (Loesche et al., 1983; Rivera-Chávez et al., 2017). These microbiota have co-evolved with their human hosts and contribute symbiotically to maintain ecological and physiological balance (Cornejo Ulloa et al., 2019).

More than 700 bacterial species have been detected in the human oral cavity, with any one single individual's mouth harboring up to 200 distinct bacteria (Dewhirst et al., 2010). This makes it the second most complex of the human microbiota after that of the gut (Kilian et al., 2016). The constant contact between the oral cavity and the external environment contributes to a large inter-individual diversity in microbial composition (Griffen et al., 2012; Sato et al., 2015). While they are primarily recognized for their role in digestion and oral diseases, oral bacteria can also modulate human physiology and pathology (Sampaio-Maia et al., 2016). Commercial divers are generally healthy and must satisfy specific requirements for fitness but it is unknown whether and how environmentally induced changes to their oral microbiota may challenge their physiology.

We have previously reported a transient shift in the composition of the oral microbiota during commercial saturation diving (Monnoyer et al., 2021). The shift reflected changes in oxygen availability and consisted primarily of an increase in aerobic and oxygen tolerant bacteria at the expense of obligate anaerobes. In the current study, we aimed to predict environmentally induced changes in bacterial metabolism. We used metagenomic data to model metabolic pathways and processes before, during, and after saturation diving, hypothesizing that there would be a relative increase in oxidative respiration during the bottom phase and a concomitant decrease in anaerobic fermentation. The resultant predictions may be examined as targets for modulation through interventions such as nutrition.

MATERIALS AND METHODS

Ethics

This study was conducted on metagenome data from the oral microbiota of divers who participated in a commercial diving campaign in the Mediterranean Sea in 2018. The protocol was approved in advance by the Norwegian Regional Committee for Medical and Health Research Ethics (REK), number 2018/1184. The participants were informed of the aims and scope of the study and their right to withdraw without repercussions and provided signed consent prior to inclusion. All experimental procedures were conducted according to the Declaration of Helsinki principles for ethical human experimentation. The divers' characteristics and criteria for eligibility are presented in Monnoyer et al. (2021).

Study Material

The study material was generated from saliva samples from 23 divers before, during, and after a 28-day saturation dive. The diving protocol has been presented previously by Luczynski et al. (2019). In brief, the divers were compressed over 6 h to 182–200 meters of sea water (msw). During the bottom phase, they worked in teams of three, with shifts lasting 12 h. Each team did one underwater work excursion (bell run) per day to 192–210 m, 7 days per week. After the bottom phase, the divers were decompressed to atmospheric pressure over 8 days. During the bottom phase, the ppO_2 was kept at 40 kPa in the living chamber and 60–80 kPa during work. During the decompression, ppO_2 was increased to 50 kPa until 13 msw and then gradually reduced to 21 kPa until surface. Saliva samples were collected into OMNIgene ORAL OM-505-tubes (DNA Genotek, Ottawa, Canada) and sequenced on a Illumina NextSeq flowcell generating 16S rRNA metagenomic data, as described earlier (Monnoyer et al., 2021). The saturation diving profile and sample collection time points for this study are shown in **Figure 1**. The 16S rRNA fastq files are available at the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB40804.[1]

Bioinformatics

The fastq files were quality checked and trimmed before subjected to denoising by DADA2 (Callahan et al., 2016) to generate a table of Amplicon Sequence Variants (ASVs) counts for each sample. The taxonomic identity of each ASV was determined by region-specific classifiers trained on the SILVA database (v132; Quast et al., 2013). The ASV count table and taxonomic classifications were subjected to pathway abundance analysis by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Douglas et al., 2020). Quality filters, denoising, taxonomic classification, and pathway abundance results were all generated using the Quantitative Insights Into Microbial Ecology (QIIME2, version 2019.10) framework (Bolyen et al., 2019). Significant differentially activated pathways were identified by paired *t* tests as implemented in the voom method in the limma R package (Law et al., 2014; Ritchie et al., 2015) using a Benjamini-Hochberg correction adjusted value of $p < 0.05$. Outputs from the voom method can be found in **Supplementary Data**. The different time points were compared to the reference value using the individual dives as a blocking factor. Pathway's abundances were summarized using the MetaCyc Metabolic Pathway database: https://metacyc.org/ (Caspi et al., 2020).

RESULTS

The PICRUSt2 analysis predicted several changes in biological pathways and processes during commercial saturation diving.

[1]https://www.ebi.ac.uk/ena/browser/view/PRJEB40804

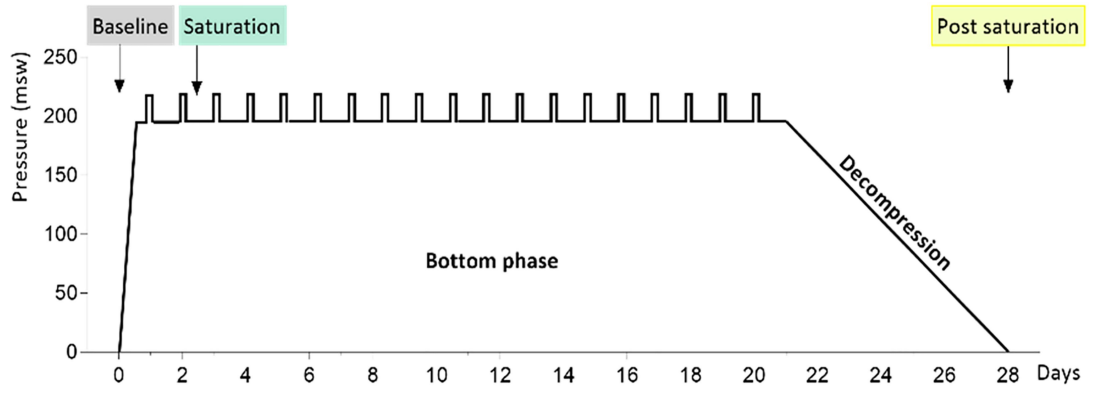

FIGURE 1 | The 4-week hyperbaric saturation profile. Pressure is shown as meters of sea water (msw). The saturation was done in a heliox atmosphere, with 6-h compression, app. 21-day bottom phase at or near 200 msw, and app. 8-day decompression. Daily bell runs are indicated by vertical bars on the bottom phase profile. Colored labels point to saliva collection times before, during, and after the saturation.

All changes were transient: They were observed only during the bottom phase and disappeared after the decompression. **Figure 2** summarizes the main results during the bottom phase (green bars) and after decompression (yellow bars).

The analysis estimated changes in pathways involved in bacterial energy metabolism. There was a decrease in anaerobic pathways, including anaerobic respiration, pyruvate fermentation, and glycolysis. In aerobic pathways, the pentose phosphate pathway and tricarboxylic acid (TCA) cycle increased. In the electron transport chain of the aerobic respiration, the analysis predicted changes in electron carrier biosynthesis following the TCA cycle: The biosynthesis of ubiquinol increased, while the biosynthesis of demethyl/menaquinone decreased (**Figure 3**).

Bacterial oxidative stress responses increased (**Figure 4**), with elevated activity of the pentose phosphate pathway, and pp(G)pp (guanosine tetraphosphate), and mycothiol biosynthesis.

Biosynthesis of bioactive vitamin B_{12} (vitB_{12}) decreased through changes of the adenosylcobalamin biosynthesis pathways I (aerobic) and II (anaerobic), including adenosylcobinamide-GDP, a compound that can be used to synthesize different forms of the vitamin (**Figure 5**). There was also a decrease in the cobinamide salvage pathway.

The analysis also predicted changes in the biosynthesis and breakdown of universally present biomolecules, including a rise in lipid biosynthesis and fall in fatty acid biosynthesis. Furthermore, changes in the metabolism of some amino acids were predicted, with an increase in L-arginine biosynthesis and L-tyrosine degradation, and a decreased L-tryptophane degradation. Nitrogen compound metabolism was represented by a decrease in urea cycle activity (**Figure 2**). The other pathways that changed during the bottom phase included nucleoside and nucleotide metabolism, carbohydrate metabolism, and aromatic compound degradation (see **Supplementary Data**).

DISCUSSION

This study applied an *in silico* approach to predict changes in oral bacterial metabolism during commercial saturation diving. In light of emerging awareness of the intimate interactions between the human body and its microbiota, the results may contribute to a better understanding of physiological responses to the extreme environments in saturation diving. We used PICRUSt2 for functional abundance analysis on 16S rRNA sequence data from a previous study (Monnoyer et al., 2021) and confirmed our hypothesis of a relative increase in the abundance of aerobic pathways and a concomitant decrease in anaerobic pathways. Interestingly, the analysis also predicted decreases in several components of vitB_{12} biosynthesis.

The predicted changes in oxygen-dependent metabolism are consistent with the results from our prior taxonomic analysis, where the composition of bacterial communities was found to shift during the bottom phase in favor of aerobes (*Proteobacteria* and *Actinobacteria*) over anaerobes (*Fusobacteria*) (Monnoyer et al., 2021). An increase in pathways involved in the TCA cycle and electron carrier biosynthesis is consistent with communities in which aerobes predominate. On the contrary, the decrease in pathways of metabolites resulting from the fermentation of pyruvate may represent the decrease in relative abundance of anaerobes. There was a predicted increase in ubiquinol biosynthesis and a decrease in menaquinol and demethylmenaquinol biosynthesis. Ubiquinol, menaquinol, and demethylmenaquinol are the reduced forms of ubiquinone (coenzyme Q), menaquinone (vitamin K), and demethylmenaquinone, respectively. Ubiquinone, menaquinone, and demethylmenaquinone are all quinones (Aussel et al., 2014), which are essential components of the electron transfer chain in respiratory processes (Pandya and King, 1966; Unden, 1988). Quinones differ by their redox potential: Ubiquinone has high midpoint

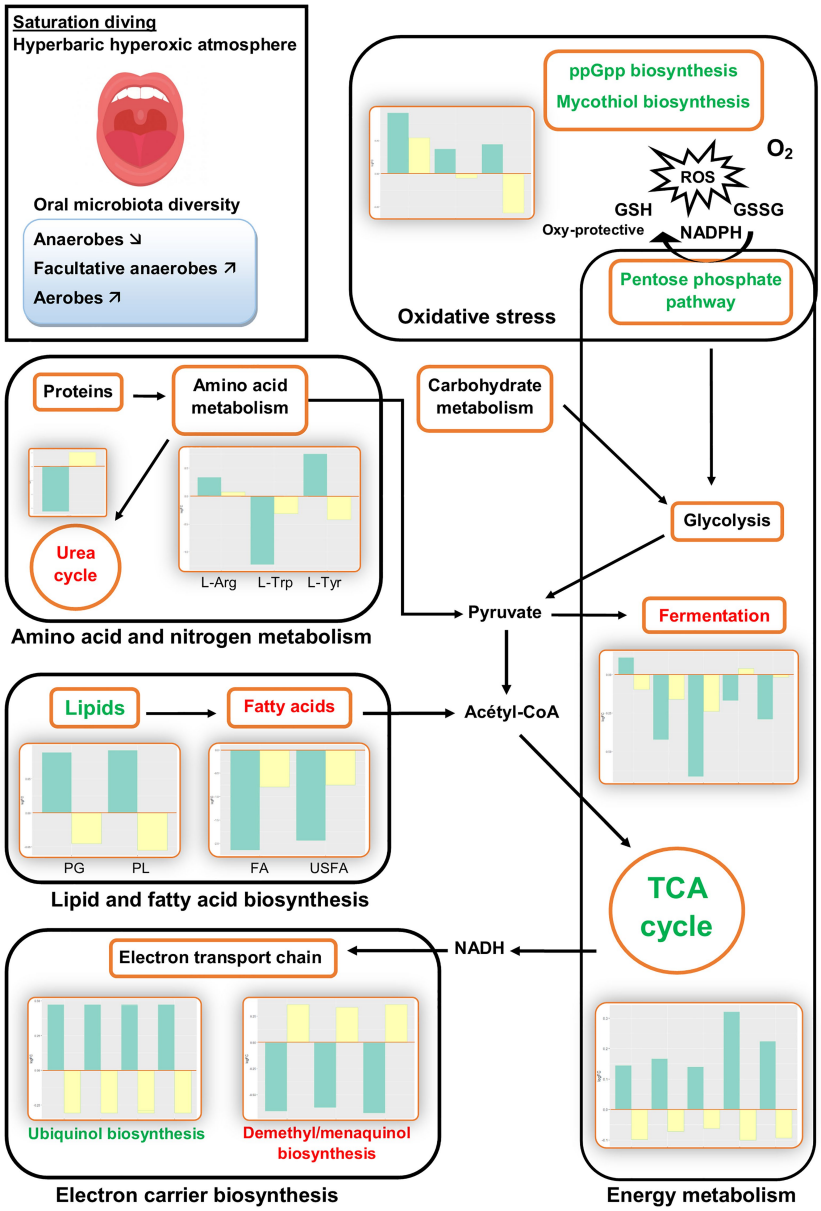

FIGURE 2 | Illustrative overview of inferred changes in oral bacterial metabolic pathways during hyperbaric heliox saturation. The bar plots show log2 fold change from paired *t* tests (means relative to baseline, $n = 23$): green = after 2 days of heliox saturation; yellow = after decompression. For each pathway, the overall direction of change is indicated by green (more active) or red (less active) text. L-Arg, L-arginine biosynthesis ($p = 0.0205$); L-Trp, L-tryptophane degradation ($p = 0.0066$); L-Tyr, L-tyrosine degradation ($p = 0.0337$); PG, phosphatidylglycerol ($p = 0.0066$); PL, phospholipid ($p = 0.0066$); FA, fatty acids ($p = 0.0233$); and USFA, unsaturated fatty acids ($p = 0.0209$).

potential, and the midpoint potentials of menaquinone and demethylmenaquinone are low. This result is in agreement with our prior report of shifts in the bacterial composition: Whereas aerobic bacteria use ubiquinone, anaerobes use menaquinone or demethylmenaquinone (Aussel et al., 2014).

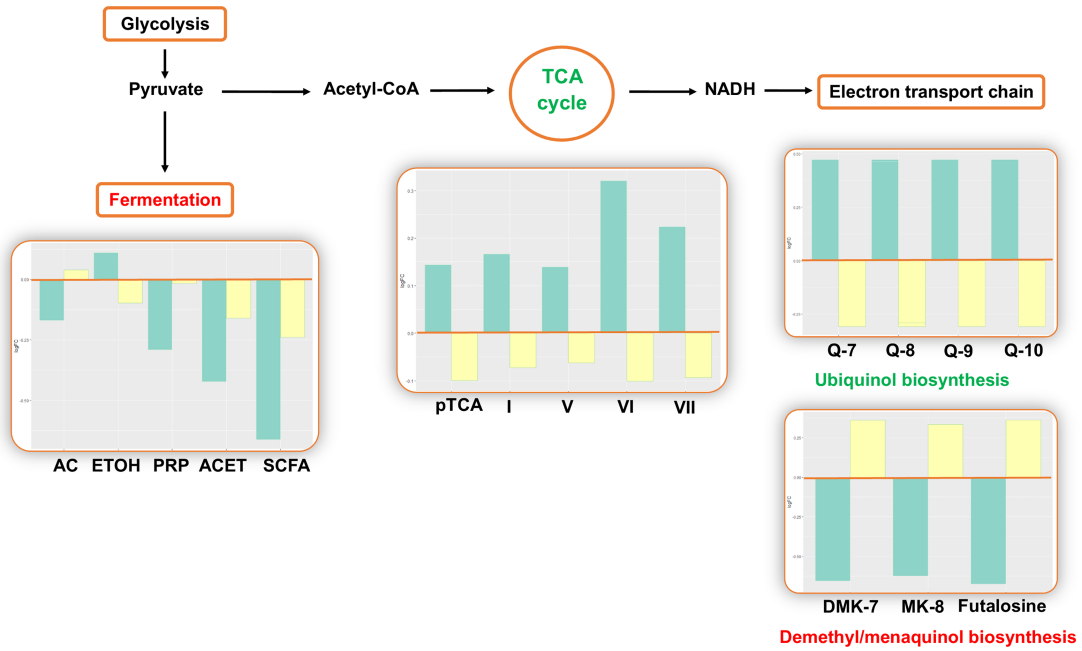

FIGURE 3 | Inferred changes in bacterial energy metabolism. The bar plots show log2 fold change from paired *t* tests (means relative to baseline, $n = 23$): green = after 2 days of heliox saturation; yellow = after decompression. AC, acetate ($p = 0.0354$); ETOH, ethanol ($p = 0.0155$); PRP, propanoate ($p = 0.0234$); ACET, acetone ($p = 0.0047$); SCFA, short-chain fatty acids ($p = 0.0014$); pTCA, partial tricarboxylic acid (TCA) cycle (obligate autotrophs, $p = 0.0240$); I, TCA cycle I (prokaryotic, $p = 0.0234$); V, TCA cycle V (2-oxoglutarate synthase, $p = 0.0360$); VI, TCA cycle VI (Helicobacter, $p = 0.0213$); VII, TCA cycle VII (acetate-producers, $p = 0.0368$); Q-7, Ubiquinol-7 ($p = 0.0368$); Q-8, Ubiquinol-8 ($p = 0.0368$); Q-9, Ubiquinol-9 ($p = 0.0368$); Q-10, Ubiquinol-10 ($p = 0.0368$); DMK-7, demethylmenaquinol-7 ($p = 0.0266$); and MK-8, menaquinol-8 ($p = 0.0266$).

We observed an increase during hyperbaric heliox saturation of certain defense mechanisms against oxidative stress involved in bacterial survival: ppGpp biosynthesis, pentose phosphate pathway, and mycothiol biosynthesis. ppGpp (guanosine tetraphosphate) is a stress signal molecule that is usually produced under conditions of nutrient starvation (Fritsch et al., 2020). Thiols play a major role in the detoxification of stress-inducing factors, one of these thiols being glutathione (GSH). The role of the pentose phosphate pathway aside from its involvement in energy metabolism is to provide NADPH which is a cofactor of GSH reductase. ppGpp and reduced GSH are essential for maintaining the cell's redox state and fighting oxidative stress and reactive oxygen derivatives. Diving is associated with oxidative stress from excess production of reactive oxygen species (ROS) due to hyperoxia exposure (Brubakk et al., 2014). Excess ROS promotes inflammation and can cause direct damage to cells and tissues, specifically proteins, lipids, and nucleic acids. Cells that are exposed to oxygen have evolved defensive mechanisms to limit damage from oxidative stress, and it has been shown that saturation diving causes upregulation of endogenous antioxidant defenses in human cells (Kiboub et al., 2018b). Bacteria that can use oxygen for respiration, both aerobes and facultative anaerobes, also contain a highly regulated complex of antioxidant defense enzymes. These include catalase or superoxide dismutase, as well as other non-enzymatic detoxifying mechanisms such as alkyl hydroperoxide reductase and the GSH-cycling system to overcome the toxic effects of ROS (Brioukhanov and Netrusov, 2007; Henry et al., 2014).

While the changes discussed above are reasonable in light of an oxygen-driven shift in the oral microbiota, we have no information that suggests that they affect the divers' health and fitness. But the analyses also predicted changes in pathways associated with adenosylcobalamin biosynthesis. Adenosylcobalamin, known as coenzyme B_{12}, is an active form of vitB_{12}. Adenosylcobalamin is synthesized *via* the *de novo* or salvage pathways. Microbial *de novo* biosynthesis of vitB_{12} happens through two different routes: one aerobic and one anaerobic. In our analysis, both aerobic or anaerobic routes were predicted to change.

B vitamins are essential in several metabolic pathways. In saturation diving, the role of vitB_{12} in erythropoietin activity and the production of red blood cells (erythrocytes) is particularly interesting, as a decrease in biosynthesis and salvage of adenosylcobalamin in the oral microbiota may have an impact on the bioavailability of vitB_{12}. Vitamin B_{12} deficiency can occur

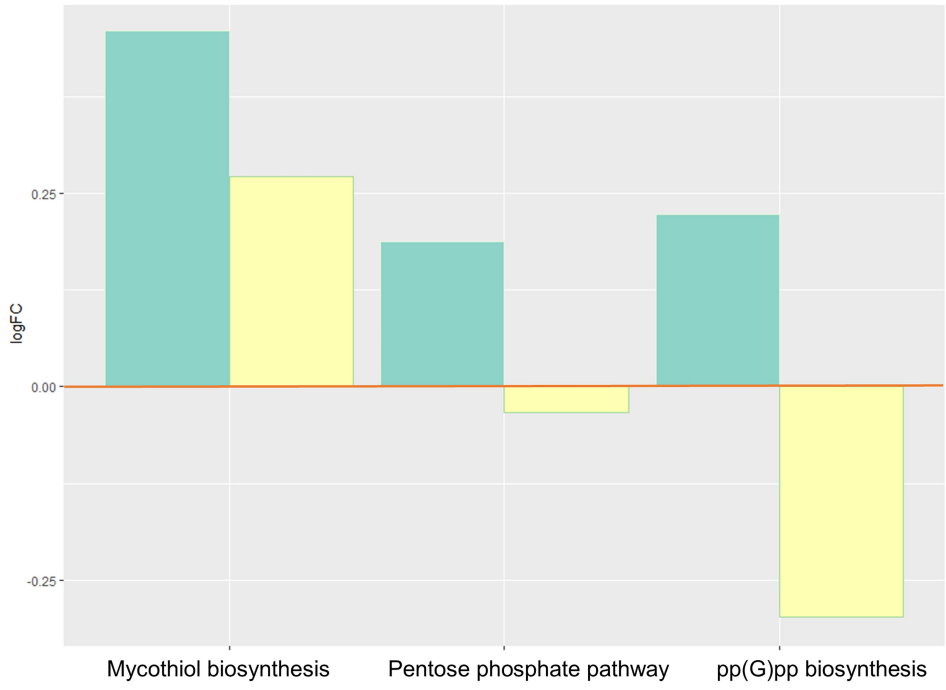

FIGURE 4 | Inferred changes in bacterial oxidative stress responses: mycothiol biosynthesis ($p = 0.0274$), pentose phosphate pathway ($p = 0.0047$), and pp(G)pp biosynthesis ($p = 0.0429$). The bar plots show log2 fold change from paired *t* tests (means relative to baseline, $n = 23$): green = after 2 days of heliox saturation; yellow = after decompression.

through malabsorption, dietary insufficiency, and autoimmune conditions (e.g., pernicious anemia) (Ankar and Kumar, 2021). Mild anemia is common after saturation diving since hyperbaric environment causes a depression of erythropoiesis (Kiboub et al., 2018a) and hemoglobin (Luczynski et al., 2019). Anemia is hypothesized to be partly responsible for the fatigue that saturation divers experience up to 1 week after decompression (Imbert et al., 2018), and vitB_{12} is among the nutritional factors that should be monitored (Deb et al., 2016). Correlations have been observed between nutrient intake (e.g., vitamins) and its impact on the microbial abundance, diversity, and richness of the oral microbiome (Kato et al., 2017). Moreover, data from gut microbiota indicate that bacteria contribute to the supply of vitB (vitB_{12} included) to their human hosts (Magnúsdóttir et al., 2015). Daily requirements for vitB_{12} are typically achieved through a diverse diet containing meat, fish, and fortified foods; however, in some cases, supplementation may be required, if availability is insufficient through food intake. Our findings are consistent with recommendations for vitB_{12} supplements as part of the divers' diet.

Limitations

The analytic approach in this study comes with some caveats. First, as the PICRUSt2 software uses metagenomic data for functional predictions, the results are a direct reflection of the relative abundance of the bacteria that harbor these functions. By extension, the analysis does not determine changes in metabolic activity within individual bacteria. Second, this analysis can only identify functions in bacteria for which metagenomic data were present in the reference. To this second point, our analysis predicted changes in the metabolism of cellular biomolecules that are universally present in bacteria: nucleosides, nucleotides, lipids, amino acids, and carbohydrates. These results may be artifacts caused by the absence of data from bacteria for which 16S rRNA sequences were not annotated in KEGG at the time of the analysis (Langille, 2018). Therefore, the conclusions must be tempered to whether they are reasonably linked by biology to the environmental stressors in saturation diving and whether they are likely to be of consequence for the divers' health and fitness. To confirm our findings, future studies should include direct measurements of metabolites.

CONCLUSION

This study is the first to examine the metabolic activity of oral microbiota during commercial saturation diving. Whereas

FIGURE 5 | Inferred changes in bacterial vitB$_{12}$ metabolism. The bar plots show log2 fold change from paired *t* tests (means relative to baseline, $n = 23$): green = after 2 days of heliox saturation; yellow = after decompression. AdoCbi-GDP, adenosylcobalamin biosynthesis from adenosylcobinamide-GDP I ($p = 0.0011$); AdoCbl AE, adenosylcobalamin biosynthesis II (aerobic, $p = 0.0488$); AdoCbl AN, adenosylcobalamin biosynthesis I (anaerobic, $p = 0.0338$); CbiI, adenosylcobalamin salvage from cobinamide I ($p = 0.0011$); and CbiII, adenosylcobalamin salvage from cobinamide II ($p = 0.0011$).

the predicted changes were transient and appeared primarily to be associated with the survival and growth of bacteria in hyperoxic environments, a reduction in bacterial vitB$_{12}$ biosynthesis during the bottom phase may hypothetically disturb the divers' physiology. While there is limited knowledge of how oral bacterial metabolism affects human physiology, the results from this study are in line with published recommendations for vitB$_{12}$ supplements as part of the divers' diet.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Norwegian Regional Committee for Medical and Health Research Ethics (REK). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, IE, and JL designed the study. IE collected the material. RM, JL, and AH conducted the analyses. RM, IE, SD, KH, and JL contributed to the interpretation of the results. All authors contributed to the article and approved the submitted version.

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NTNU Genomics Core Facility (GCF) were involved in discussions during the design phase of the study, and contributed in DNA extraction, sequencing, and data sorting and quality control. The GCF was funded by the Faculty of Medicine at NTNU and Central Norway Regional Health Authority.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.702634/full#supplementary-material

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Conflict of Interest: TechnipFMC sponsored helicopter transfers and boarding on the DSV Deep Arctic for IE.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper III

frontiers in Physiology

ORIGINAL RESEARCH
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Using Salivary Biomarkers for Stress Assessment in Offshore Saturation Diving: A Pilot Study

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Health monitoring during offshore saturation diving is complicated due to restricted access to the divers, the desire to keep invasive procedures to a minimum, and limited opportunity for laboratory work onboard dive support vessels (DSV). In this pilot study, we examined whether measuring salivary biomarkrers in samples collected by the divers themselves might be a feasible approach to environmental stress assessment. Nine saturation divers were trained in the passive drool method for saliva collection and proceeded to collect samples at nine time points before, during, and after an offshore commercial saturation diving campaign. Samples collected within the hyperbaric living chambers were decompressed and stored frozen at −20°C onboard the DSV until they were shipped to land for analysis. Passive drool samples were collected without loss and assayed for a selection of salivary biomarkers: secretory immunoglobulin A (SIgA), C-reactive protein (CRP), tumor necrosis factor (TNF)-α, interleukins IL-6, IL-8, IL-1β, as well as cortisol and alpha-amylase. During the bottom phase of the hyperbaric saturation, SIgA, CRP, TNF-α, IL-8 and IL-1β increased significantly, whereas IL-6, cortisol and alpha-amylase were unchanged. All markers returned to pre-dive levels after the divers were decompressed back to surface pressure. We conclude that salivary biomarker analysis may be a feasible approach to stress assessment in offshore saturation diving. The results of our pilot test are consonant with an activation of the sympathetic nervous system related to systemic inflammation during hyperbaric and hyperoxic saturation.

Keywords: saliva, biomarkers, cytokine, inflammation, hyperbaric heliox saturation, physiological stress, hyperbaric hyperoxia

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INTRODUCTION

Saturation diving is used for underwater work that requires direct human intervention. During saturation diving campaigns, the divers work and live in hyperbaric (pressurized) environments where they are constantly exposed to environmental stressors to which they must acclimatize (Brubakk et al., 2014). The human capacity to tolerate environmentally induced stress depends on a complex network of biological processes that serve to uphold homeostasis, some of which may be quantified *via* molecular biomarkers: biomolecules that correlate in abundance or state with the process in question (Godoy et al., 2018). Since biomarkers can also be used for risk

prediction and to facilitate the diagnosis of environmentally induced disorders, they are frequently used in occupational health monitoring. But for biomarkers to be useful tools in offshore saturation diving, they must be safe and easy to obtain, robust to decompression, and not require advanced on-site laboratory processing.

Blood biomarkers are extensively used in clinical care. Blood biomarkers also reflect the oxidant-antioxidant balance involved in local and systemic pathologies, e.g., diabetes, cancer, inflammatory disorders, cardiovascular, and neurological conditions (Ho et al., 2013; Marseglia et al., 2014; Singh et al., 2019). With advancements in genomics and molecular biology, blood biomarkers of oxidative stress and inflammation have been studied in different types of diving, including saturation diving, to deepen the knowledge on the interactions between the immune system and the divers' physiology (Eftedal et al., 2013; Sureda et al., 2014; Lautridou et al., 2016, 2017; Kiboub et al., 2018). However, phlebotomy is not an ideal procedure in saturation diving: it is difficult to perform under pressure, there is a small risk of infection, and a centrifuge is needed to prepare serum or plasma. Biomarkers are also present in other body fluids that are less challenging to obtain and prepare for analysis. One such fluid is saliva. Saliva is a readily available source of stress biomarkers, comprising of diverse chemical compounds, such as hormones (e.g., cortisol), enzymes (e.g., alpha-amylase), cytokines (e.g., TNF-α, IL-1β, IL-6, and IL-8), and immunoglobulins (e.g., secretory IgA) (Soo-Quee Koh and Choon-Huat Koh, 2007). Many studies have shown the interaction of the immune system and the central nervous system in stress responses (Hellhammer et al., 2009; Usui et al., 2011; Bozovic et al., 2013; Nater et al., 2013; Slavish et al., 2015; Dhama et al., 2019). This study aimed to examine whether salivary biomarker analysis might be a feasible approach for the assessment of environmental stress in offshore saturation diving. A panel of eight biomarkers was chosen to reflect stress responses in salivary glands and tissues, with emphasis on oxidative stress and inflammation which we hypothesized would increase due to the divers' hyperbaric and hyperoxic work environment.

MATERIALS AND METHODS

Ethics

The study material was collected during a commercial offshore saturation diving campaign in the Norwegian North Sea, in the fall of 2019. The protocol was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REK), approval number 2018/1184. The participants provided their informed consent prior to inclusion, and all procedures were conducted according to the Declaration of Helsinki principles for ethical human experimentation.

Study Participants

Nine certified and experienced saturation divers, all male, were considered eligible and recruited as participants after passing a mandatory pre-saturation medical examination. The medical examination was performed in accordance with NORSOK U-100 Standards (Standards Norway, 2014) and ensured that the divers were currently in good health and fit to dive and committed to saturation onboard TechnipFMC's DSV Deep Arctic. Anthropometric data for the participants are shown in **Table 1**.

Saturation Diving

Heliox saturation diving was conducted according to NORSOK U-100 Standards (Standards Norway, 2014). The saturation system was compressed to an initial depth of 66 meters of seawater (msw), later increasing to 85 msw, for the divers to work at water depths of 74 – 93 msw. In addition to pressure control, the saturation chamber environment was continuously monitored and controlled by a life support crew to ensure that the divers' living conditions adhered to NORSOK standards for gas mixture: partial pressure of O_2 (ppO_2) $\leq$ 40 kPa, $pCO_2 \leq$ 1 kPa, pCO < 0.5 Pa, relative humidity 40–60%, and temperature 22–33°C. The divers kept regular meals throughout the saturation, choosing their individual diets from daily selections from the vessel galley.

To facilitate continuous underwater activity, the divers were organized into four teams of three divers working overlapping 12-h shifts, with consecutive teams starting their shift every 6 h. Each diver worked the same shift for the duration of his saturation. A pressurized diving bell was used to transport the dive team from the living chambers on the DSV to work on the ocean floor. During work excursions (bell-runs), the divers' environment was monitored and controlled by diving supervisors *via* umbilicals from the DSV to maintain a breathing gas ppO2 of 60–80 kPa, and hot water supply to the divers' suits to preserve their body temperature. Daily bell-runs lasted for up to 8 h, during which the divers spent a maximum of 6 h in the water interspersed by breaks inside the bell for restitution and hydration. Their work consisted of installation of blind flange plugs, pipe support installation, seal replacements, bell mouth installation, and inspection work. The divers described the workload as relatively light compared to deeper and longer saturations, with most of their work performed standing on the ocean floor. At the end of their work assignment, the decompression back to ambient surface pressure lasted approximately 4 days 4 h, during which the ppO_2 in the living chambers were kept at 50 kPa until 13 msw, and gradually reduced to reach 21 kPa at the end of the decompression.

TABLE 1 | Study subject anthropometrics prior to saturation (n = 9).

	Mean ± SD
Age (years)	47 ± 8.4
Height (cm)	180.4 ± 7.4
Weight (kg)	89.0 ± 10.2
BMI (kg/m^2)	27.3 ± 1.9

BMI = body mass index. The table is reprinted with permission from Deb et al. (2021).

Saliva Collection

The participants were trained in the passive drool method and supplied with instructions for later reference before they proceeded to collect saliva into 2 ml SalivaBio cryovials (Salimetrics, Carlsbad, CA); first during the pre-saturation medical examination (Day 0), then seven times during hyperbaric heliox saturation: three during the bottom phase (Days 1, 2, and 4) and four during decompression (Days 11, 12, 13, and 14), and finally post-saturation when the divers were back at surface pressure (Day 15). Samples in the pressure chambers were taken before bell-runs at the beginning of the divers' work shifts, 1–2 h after breakfast without brushing their teeth prior to the collection. Due to the continuous rotation with a new team starting their shift every 6 h, each individual participant of the same diving team collected all his samples at the same time of day, whereas participants on successive teams did their sample collections at 6 h intervals. The saliva samples were transferred out to the vessel hospital *via* a decompression lock, frozen at −20°C onboard the DSV and shipped frozen to NTNU at the end of the diving campaign. There the samples were stored at −80°C until they were shipped to Salimetrics' SalivaLab (Carlsbad, CA) on dry ice for analysis.

Salivary Biomarker Analysis

Samples were assayed in duplicate at the Salimetrics' SalivaLab using the Salimetrics Salivary Assay Kits for C-Reactive Protein (CRP; Cat. No. 1-2,102), Secretory Immunoglobin A (SIgA; Cat. No. 1-1,602), Interleukins 1β (IL-1β), 6 (IL-6), 8 (IL-8), and Tumor necrosis factor alpha (TNF-α) (Salimetrics Cytokine Panel), Cortisol (Cat. No. 1-3,002), and Alpha-amylase (Cat. No. 1-1902), according to the manufacturer's protocol. The assay data, with volumes, range, and sensitivity for each analyte, are shown in **Supplementary Material**.

Statistics

Prior to the statistical analysis, data from saliva samples obtained during saturation were split into two groups: one for the bottom phase and one for the decompression. Means were calculated at baseline (I in **Figures 1, 2**), bottom phase (II in **Figures 1, 2**), decompression (III in **Figures 1, 2**), and post-saturation (IV in **Figures 1, 2**). Statistical analysis was done in IBM SPSS Statistics software Version 26.0. After visual inspection for data distribution using Q-Q plots, the Shapiro–Wilk's test for normality was calculated ($p > 0.05$), either directly or after the data were transformed. A non-parametric analysis was applied if the data were still non-normal after transformation. For each biomarker, the differences within divers between the three time points were assessed by a one-way repeated measures ANOVA, with post-hoc Bonferroni adjustment for multiple comparisons. Differences were considered significant at $p < 0.05$. Homogeneity of the data was assessed by the Mauchly test with $p > 0.05$. If the assumption of sphericity was violated, a Greenhouse–Geisser correction was applied. Friedman's test was applied for non-normal data, and pairwise comparisons were made with Bonferroni's correction for multiple comparisons.

RESULTS

Salivary biomarkers were assayed before, during, and after offshore saturation diving. The saliva samples were collected by the divers themselves during a commercial diving campaign and transferred from the DSV to shore for analysis. The biomarkers were chosen to cover pro- and anti-inflammatory responses and oxidative stress: CRP, TNF-α, IL-6, IL-8, IL-1β, and SIgA as well as generalized stress responses: cortisol and alpha-amylase (Slavish et al., 2015; Dhama et al., 2019). The saliva collection proceeded without sample loss or technical issues, and the analysis yielded data that lay within the expected range for every biomarker analyzed.

During the bottom phase of the saturation dive, SIgA ($p = 0.005$), CRP ($p = 0.005$), TNF-α ($p < 0.0005$), IL-8 ($p = 0.002$), and IL-1β ($p < 0.0005$) increased, whereas IL-6 was unchanged (**Figure 1**). There were no significant changes at any time in cortisol or alpha-amylase (**Figure 2**). All biomarkers returned to pre-dive baseline levels during or after the decompression.

DISCUSSION

In this study, we examined whether salivary biomarkers analysis would be a feasible approach for the assessment of environmentally induced stress in offshore saturation diving. In summary, we found passive drool collection performed by the divers themselves within the pressurized living chambers to be practical and to provide material that was suitable for analysis. Salivary levels of CRP, IL-1β, IL-8, SIgA, and TNF-α were elevated during the bottom phase of the hyperbaric saturation, whereas IL-6, cortisol, and alpha-amylase were unchanged. All changes observed during saturation were abolished at the end of the decompression.

The hyperbaric and hyperoxic environment in saturation diving induces pro-inflammatory responses which help the body adapt to the inherent oxidative stress in the maintenance of homeostasis. Prior work has shown that genes involved with the divers' endogenous antioxidant defenses along with immune activity and inflammatory signaling pathways are upregulated at the time they complete their decompression from saturation (Kiboub et al., 2018). However, this knowledge was obtained from blood, and phlebotomy is not an ideal procedure to perform in hyperbaric chambers. Other body fluids, such as saliva, contain bioactive molecules that regulate inflammation and immune defenses (Cavalla et al., 2014) which have been recently used to evaluate the effects of saturation diving on measures of oxidative stress, including production of reactive oxygen species and total antioxidant capacity (Mrakic-Sposta et al., 2020). Saliva collection is non-invasive and requires no professional skills although passive drool collection requires prior training of the study subjects. Samples can be stored without pre-processing in a standard freezer (Wilde et al., 2013) or at room temperature with analyte stabilization (Bonne and Wong, 2012). Furthermore, saliva can be collected frequently, which makes it possible to semi-continuously monitor the effects

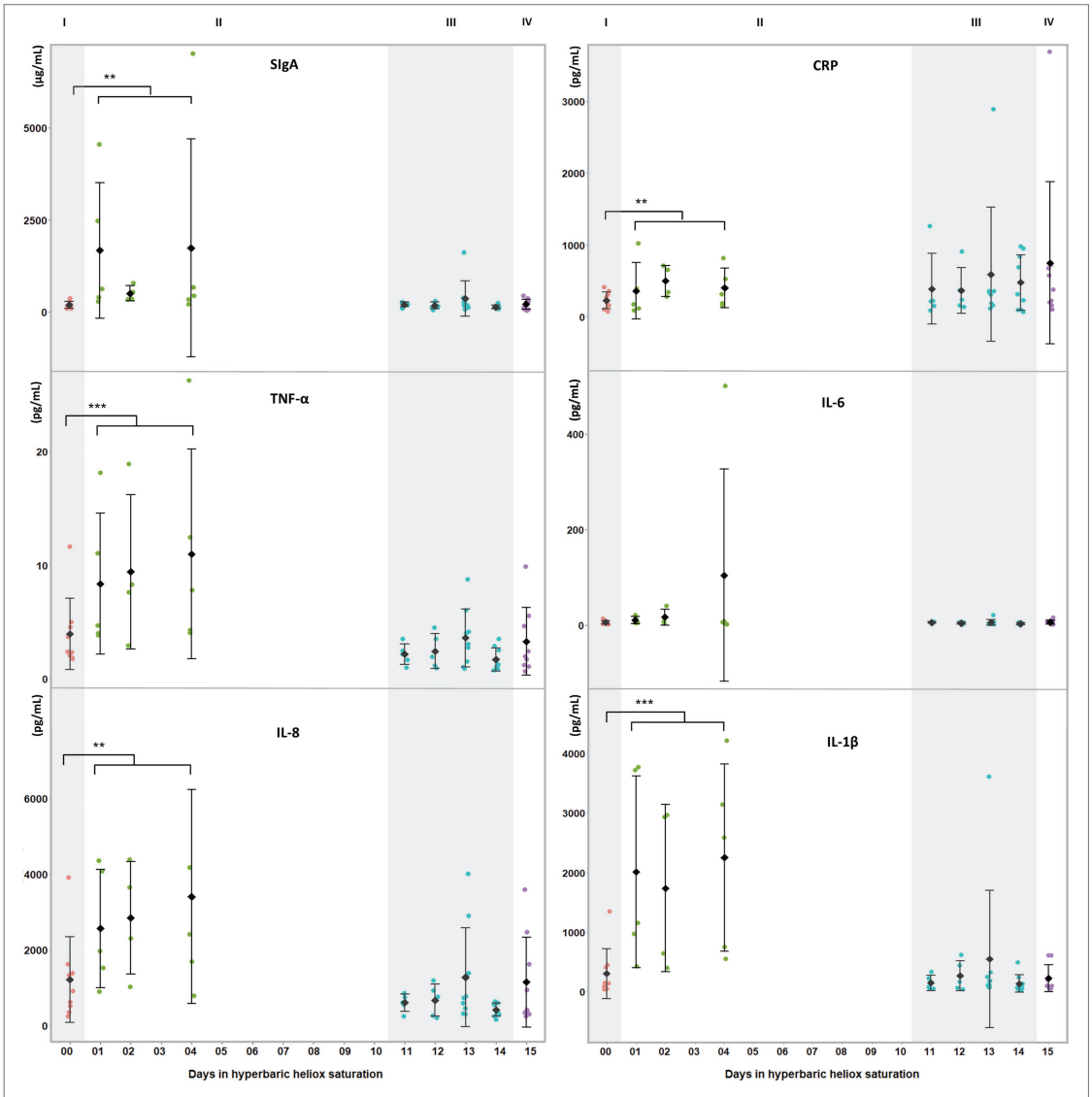

FIGURE 1 | Salivary biomarkers of inflammatory and oxidative stress during a heliox saturation dive. For each time-point: mean and individual values are shown ($n = 9$). Error bars are ±1 SD. **, $p \leq 0.01$ and ***, $p \leq 0.001$. ANOVA was calculated on mean of days 1, 2, and 4 during the bottom phase and mean of day 11 to 14 during decompression. I (baseline: red dots); II (bottom phase: green dots); III (decompression: blue dots); and IV (post-saturation: purple dots). SIgA = secretory Immunoglobulin A; CRP = C-reactive protein; TNF-α = tumor necrosis factor alpha; IL-6 = interleukin-6; IL-8 = interleukin-8; and IL-1β = interleukin-1β.

of environmental exposure. Development of rapid tests may also facilitate real-time monitoring for some biomarkers, such as is already possible for salivary cortisol (Shirtcliff et al., 2015).

Saliva contains proteins and hormones from systemic sources *via* infiltration through the salivary tissues, and from local sources in the oral cavity *via* secretion by the acini and ducts of the salivary glands. The acini are responsible for the volume and flow of saliva, while the duct cells determine its composition (de Paula et al., 2017). The autonomic nervous system controls the function of both glands: Activation of the sympathetic nervous system triggers the production of protein-rich saliva at a low flow, whereas the parasympathetic nervous system causes the flow of liquids to increase, but results in saliva with lower protein content

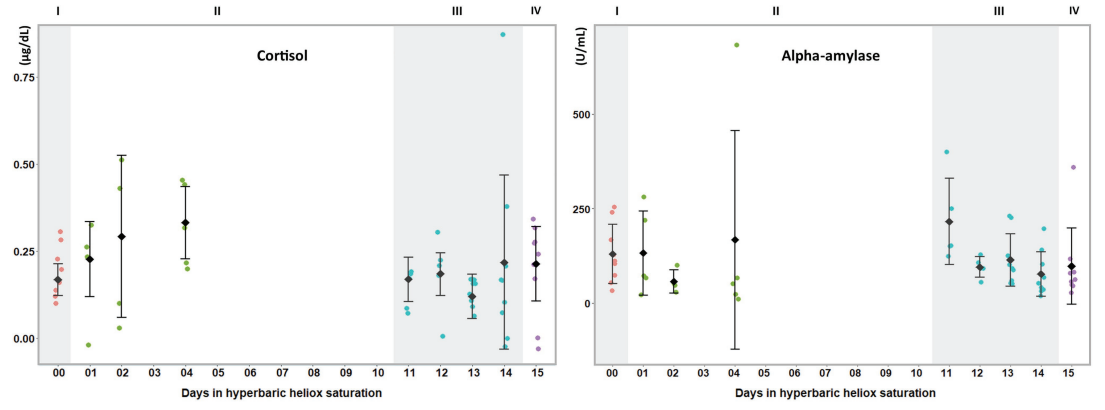

FIGURE 2 | Salivary biomarkers of stress during a heliox saturation dive. Cortisol and alpha-amylase are reliable markers of HPA and ANS activity, respectively, in stress responses. For each time-point: mean and individual values are shown ($n = 9$). Error bars are ±1 SD. ANOVA was calculated on mean of days 1, 2, and 4 during the bottom phase and mean of day 11 to 14 during decompression. I (baseline: red dots); II (bottom phase: green dots); III (decompression: blue dots); and IV (post-saturation: purple dots).

(Punj, 2018). SIgA is mainly secreted in the stroma of salivary glands by local plasma cells and stocked in the secretory epithelium (Brandtzaeg, 2013). Its secretion is governed by both parasympathetic and sympathetic afferences, with the latter having a stronger impact (Carpenter et al., 1998). IL-1β and TNF-α, most commonly known to be produced by activated macrophages, are also secreted by the salivary glands (Fernandez-Solari et al., 2010; Idris et al., 2015), whereas CRP and IL-8 have been hypothesized to enter the saliva *via* the gingival crevicular fluid (Out et al., 2012). The elevated levels of CRP and IL-8 in our study may be indicative of a systemic inflammation, especially since CRP does not originate from local tissues in the oral cavity (Megson et al., 2010). The activation of the sympathetic-adreno-medullar system in response to acute stress is measured by salivary alpha-amylase, whereas long-term or chronic stress is measured by salivary cortisol produced by the hypothalamic–pituitary–adrenal axis (Nater et al., 2013; Ali and Nater, 2020). Salivary alpha-amylase is also used as a marker of local inflammation in oral diseases (Gutierrez-Corrales et al., 2017).

Saturation diving can be mentally and physically stressful. In our study, inflammatory biomarkers IL-1β, TNF-α, SIgA, CRP, and IL-8 increased during the bottom phase of saturation diving. Conversely, there were no changes in IL-6, cortisol, and alpha-amylase. Although saturation diving is demanding, the physical workload in this campaign was reported to be light (Deb et al., 2021), and perceptions of stress and panic are unlikely to be prominent in experienced divers. IL-6 is known to increase in plasma in response to physical exercise, but this does not appear to be reflected in saliva (Cullen et al., 2015). Taken together, our results imply that the divers experienced an activation of the sympathetic nervous system secondary to systemic inflammation, rather than a generalized stress response. This is consistent with prior reports of oxidative stress and concomitant inflammatory responses in commercial saturation diving (Kiboub et al., 2018; Mrakic-Sposta et al., 2020).

Limitations

The basal levels of salivary cortisol, SIgA, and alpha-amylase exhibit circadian variation (Stefaniak and Kaczmarek, 2013; Engeland et al., 2019), but due to the divers' shift patterns and the limited number of participants, our study was not powered to account for this. However, circadian variation would cause inter-sample variation to increase, which is not expected to produce false positive results. Also, due to the low number of participants, we chose to merge samples taken during the bottom and decompressions phase, respectively, in the statistical analysis, thus forfeiting the option to monitor temporal development in biomarker expression.

Conclusion

In conclusion, salivary biomarker analysis appears to be feasible as a non-invasive approach to environmental stress assessment in commercial saturation diving. The results of our pilot study are consonant with an activation of the sympathetic nervous system associated with systemic inflammation during the bottom phase, which was abolished by the time the divers completed the decompression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Norwegian Regional Committee for Medical and Health Research Ethics (REK). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, AH, SD, and IE designed the study. IE collected the material. RM and AH performed the statistical analysis. All authors collaborated on the interpretation of results and writing and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.791525/full#supplementary-material

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