UNIVERSITY OF CALIFORNIA,
IRVINE

The Effects of Recreational Water Exposure on Human Skin: Toxin Penetration and Microbiome Alteration

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Environmental Health Sciences

by

Marisa Chattman Nielsen

Dissertation Committee:
Professor Sunny Jiang, Chair
Professor Scott Bartell,
Professor Ulrike Luderer
Professor Oladele Ogunseitan

2020

DEDICATION

To my parents, Joann and Martin Chattman, for their unconditional love and support.

To my husband, Barton Nielsen, I could not have done this without you. Thank you for your encouragement.

To my son, Bradford Nielsen, thank you for helping me realize that if I can write a dissertation with a 2 year old on my lap, I can do anything.

To my brother, Jacob Chattman, for your guidance. Unfortunately, there was no opportunity for your creative input in this dissertation.

To Michael Johnson, thank you for believing in me and reminding me every Thanksgiving for the past 10 years that I needed to do this.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

It is an honor to express my deepest appreciation to my committee chair, Professor Sunny Jiang, who is not only an incredible mentor and advisor, but she is a wonderful friend. She has been so supportive and understanding throughout my time in her lab. She truly cares about her students and I am proud to represent her lab as I continue forward in my career.

I would like to thank my committee members, Professor Scott Bartell, Professor Ulrike Luderer and Professor Oladele Ogunseitan. Dr. Bartell taught me the basics of biostatistics and gave me my first glimpse into the computer program, R, which I have used for countless hours since. Dr. Luderer helped shape my public speaking and presentation skills, so that I am now a confident public speaker, unafraid to present my research to a room full of scholars. Dr. Ogunseitan gave me such a warm welcome when I first visited UCI during interview day and his support continued throughout my time at UCI. I am grateful for all the guidance and mentorship I have received from the entire Environmental Health Sciences program.

Financial support was provided by the University of California, Irvine, NSF CBET 1806066, 1804166 and UCI Oceans Fellowship.

CURRICULUM VITAE

Marisa Chattman Nielsen

EDUCATION

2020	Ph.D. in Environmental Health Sciences. University of California. Irvine, CA.
2006	M.S. in Soil, Water and Environmental Science. University of Arizona. Tucson, AZ.
2004	B.S. in Microbiology. University of Arizona. Tucson, AZ

PROFESSIONAL EXPERIENCE

2015-2020	Clinical Laboratory Scientist in Microbiology. Hoag Hospital. Newport Beach, CA.
2013-2015	Supervisor of the Clinical Microbiology Laboratory. Tufts Medical Center. Boston, MA.
2012-2013	Assistant Supervisor of the Clinical Microbiology Laboratory. Boston Medical Center. Boston, MA.
2008-2012	Medical Technologist II in the Clinical Microbiology Laboratory. Boston Medical Center. Boston, MA

PUBLICATIONS

Nielsen Chattman M. and Jiang S. Alterations of the human skin microbiome after ocean water exposure. Mar Pollut Bull 2019, 145:595–603.

Chattman M., Maxwell S. L., Gerba C. P. Occurrence of heterotrophic and coliform bacteria in liquid hand soaps from bulk refillable dispensers in public facilities. J. Environ. Health 2011, 73:26–29.

ABSTRACT OF THE DISSERTATION

The Effects of Recreational Water Exposure on Human Skin: Toxin Penetration and Microbiome Alteration

By

Marisa Chattman Nielsen

Doctor of Philosophy in Environmental Health Sciences

University of California, Irvine, 2020

Professor Sunny Jiang, Chair

Skin is the body's first line of defense against the external environment and exposure to recreational water can compromise the skin's protective functions. Recreational water often contains harmful algal blooms, cyanotoxins, pathogenic bacteria, antibiotics and antibiotic resistance genes. This research investigated the following effects of recreational water exposure on human skin: cyanotoxin skin penetration potential, changes in the human skin microbiome and acquisition of exogenous antibiotic resistance genes (ARGs), antibiotic biosynthesis genes (ABSGs) and virulence factor genes (VFGs).

Cyanotoxin penetration potential was investigated in an in-depth examination of the state of knowledge on cyanotoxins and their potential to cause negative health effects through dermal permeation. Epidemiological and toxicological studies of the health effects from algal toxin exposure are summarized to highlight the importance of better understanding of the effects on human skin. This research identified a disparity between the human health effects described in

epidemiology case studies and toxicological dermal exposure data, indicating potential dermal penetration. The penetrative abilities of specific cyanotoxins were predicted by their physiochemical properties indicating the potential for skin penetration. These predictions can be used to better evaluate human health risks.

Another component of the skin's protective role is the microbiome, which has been shown to provide immunity against exogenous bacterial colonization. This study explored the link between ocean water exposure and the human skin microbiome, and demonstrated that there are post-exposure alterations. Skin microbiome samples were collected from human participants' calves before and after they swam in the ocean, and at 6 hours and 24 hours post-swim, and were analyzed using 16s rRNA gene and metagenomic sequencing. Beta diversity analysis revealed that the skin microbial communities on all participants before swimming were different from one another, but immediately after swimming, all participants' microbial communities were tightly clustered, indicating that the communities were no longer different. Taxonomic analysis showed that ocean bacteria, including potential pathogens, replaced the native skin bacteria and remained on the skin for at least 24 hours post-swim. Metagenomic analysis and functional gene predictions showed that ARGs, ABSGs and VFGs present on the skin increased in diversity and abundance after participants swam in the ocean and persisted for at least 6 hours post-swim. This research provides insight into the relationship between human health, the skin microbiome and the environment.

INTRODUCTION

The skin is a complex organ responsible for protecting the body from physical, chemical and biological insults. Its extensive structure is organized into the epidermis (top layer) and the dermis (bottom layer), which are separated by the basement membrane [1]. Hair follicles, sebaceous glands, sweat ducts and in some body sites apocrine glands, span the epidermal and dermal layers [2]. These layers and structures create a complex environment that not only serves as a protective barrier but also sustains a variety of commensal and pathogenic bacteria that can either help maintain skin health or contribute to disease [3]. The protective functions of the skin can be compromised by environmental exposures and this research focuses specifically on the dermal effects of recreational water exposure.

Each year, approximately 41% of the U.S. population swim in oceans, lakes, rivers or streams (*National Survey on Recreation and the Environment (NSRE) 2000–2002*.). Even though exercise and water recreational activities have numerous health benefits such as improved aerobic fitness and cardiovascular health [5], recreational waters represent significant environmental exposures because they often contain pathogenic organisms which can be deposited onto the skin and toxins which may penetrate the skin. In addition, normal protective commensal bacteria are washed off, leaving the host susceptible to infection and intoxication. This research specifically focuses on several different effects of exposure: dermal penetration of algal toxins, alterations in the human skin microbiome, and acquisition of exogenous genes.

Algal toxins (Chapter 1)

Harmful algal blooms (HAB) have increased in both frequency and severity worldwide as a result of climate change, population growth, and rapid urbanization [6,7]. Toxic algal blooms in marine environments pose a significant threat to human health through ingestion and recreational water exposure. When the water is rich with nutrients, and the environmental conditions are favorable, algae can flourish into toxic blooms [8,9]. Exposure, ingestion and inhalation of contaminated water can be dangerous to humans and animals. Many species of algae produce a variety of toxins that can have negative health effects on humans and marine animals including death upon ingestion, but skin exposure has not been well studied.

In May 2019, United States Environmental Protection Agency (US EPA) issued recommendations for water quality criteria and swimming advisory values for two cyanotoxins based on the latest scientific information. However, the oral exposure route is the only route considered [10]. Chapter 1 contains an in-depth examination of the state of knowledge on cyanotoxins in recreational water and their potential to cause negative health effects through dermal exposure. The chapter examines human skin as an effective barrier for the prevention of cyanotoxin absorption and investigates the likelihood of negative health effects through skin penetration. Epidemiological studies of health effects from recreational exposure to algal blooms and toxins are summarized to highlight the importance of understanding the toxicological effects of dermal exposure. The ability of a specific cyanotoxin to penetrate human skin is inferred by its physiochemical properties according to transdermal drug studies on dermal diffusion rate. This chapter identifies a disparity between the human health effects described in HAB exposure case studies and the toxicological skin exposure data and investigates the skin penetration capabilities of algal toxins to better evaluate human health risks from HABs.

Microbiome changes (Chapter 2)

Recreational waters are often contaminated by wastewater and storm-water runoff [11]. The presence of a variety of pathogens, such as: *Salmonella spp.*, *Shigella spp.*, *Campylobacter spp.*, *Vibrio spp.*, *Staphylococcus aureus*, intestinal parasites, viruses and other organisms in sewage and storm-water runoff can cause illness in humans that contact the water. In addition to sewage-associated pathogens, naturally occurring bacteria, such as *Vibrio* species and *Mycobacterium* species, are found in marine environments and can cause human disease [12,13]. Exposure to these organisms can cause illnesses in those who spend time on beaches, rivers, lakes and oceans.

Recent studies have shown that the human skin microbiome plays an important role in immune system function against localized and systemic diseases, and infection [14]. A healthy microbiome protects the host from colonization and infection by opportunistic and pathogenic microbes [14] and alterations in the microbiome can leave the host susceptible to infection [15][16]. While direct exposure to pathogens can cause infection, the role of the human microbiome in immunity and infectious disease development has become increasingly recognized. Characterizing the changes in the resident skin microbiota associated with recreational water exposure provides insight into the complex balance between healthy skin and skin infection.

Antibiotic resistance and ARG and VFG acquisition (Chapter 3)

The changes in the human microbiome, resulting from exposure to exogenous bacteria, are not limited to alterations in species diversity and abundance, but also include the acquisition

of genetic information. In order to understand the factors contributing to the distribution of ARGs and mitigate the risk of acquisition from the environment, current research is being devoted to investigation of environmental reservoirs of resistance genes such as soil samples [17–19], glaciers [20], animal agriculture, wastewater and oceans [21]. The ARGs and VFGs present on human skin have not been investigated despite the importance of understanding human skin infection and mitigating the risk of acquisition from the environment.

This study specifically focuses on the diversity and abundance of ARGs and VFGs present on human skin and the changes in the genomic profile associated with ocean water exposure. We make comparative investigations using predicted profiles from 16s rRNA results and metagenomic sequencing data to help understand the role of marine environments in the distribution and incorporation of exogenous genes.

Significance of the research

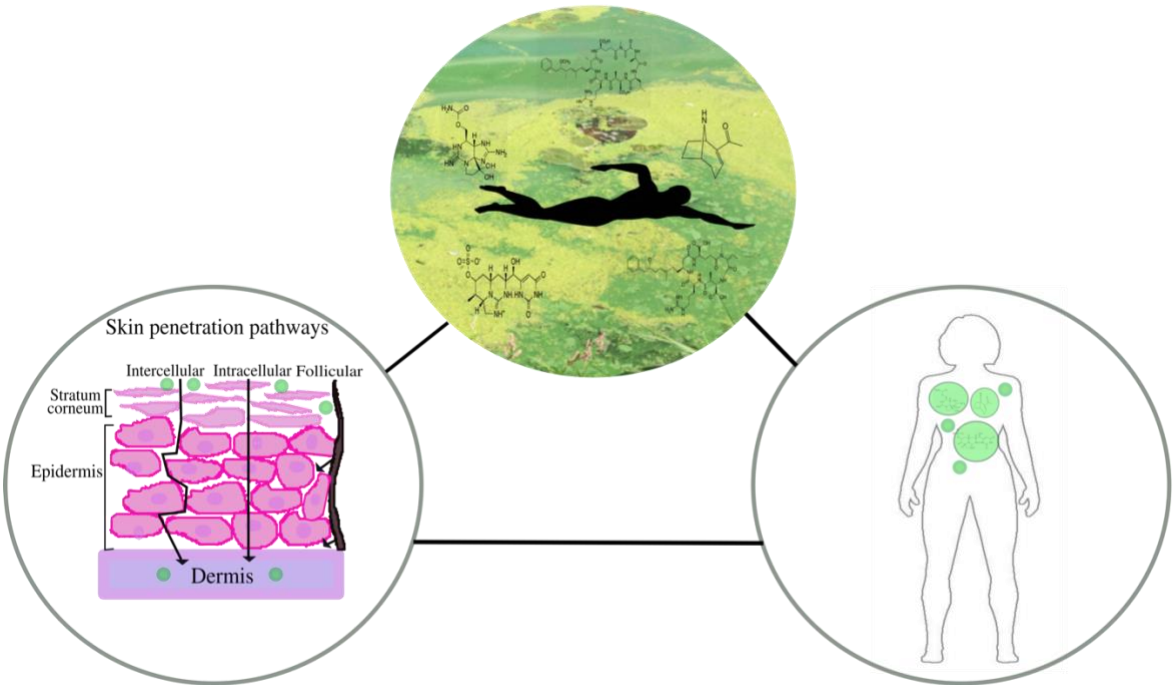
This research elucidates the effects of recreational ocean water exposure on human skin and provides greater insight into how environmental exposures affect human health. This information can be used to help determine the roles that normal flora and skin barrier functionality serve in protecting our skin from invading pathogens. Current recreational water guidelines are dependent upon the risk of infection or intoxication after a single exposure and do not consider toxin penetration, microbiome perturbation or exogenous gene distribution. Dermal penetration of algal toxins, pathogen persistence on the skin, and acquisition of genes after recreational water exposure have not been previously investigated. This research provides further insight into the effects of environmental contaminants on human health and a better

understanding of the skin microbiome and overall protective functions of the skin in response recreational water exposure.

CHAPTER 1

Understanding the Risk of Cyanotoxin Skin Penetration during Recreational Water Exposure

Graphical Abstract



Introduction

As part of the U.S. Environmental Protection Agency (EPA)'s efforts to better protect Americans' health during water recreation, the EPA issued new recommendations for water quality criteria and swimming advisory values for two cyanotoxins in May 2019. Based on the latest scientific information, EPA recommended 8 µg/L microcystins (MC) and 15 µg/L cylindrospermopsin as the maximum recreational water concentrations that are protective of public health. These recommendations are based on peer-reviewed and published science and are supposed to be protective of all age groups. However, the oral exposure route is the only route considered. The EPA acknowledged that dermal exposure occurs during swimming but commented that significant dermal absorption of MC and cylindrospermopsin is not expected due to the large size and charged nature of these molecules [10]. The EPA also estimated that exposure from inhalation is likely negligible compared to incidental ingestion while recreating.

The goal of this review is to examine the state of knowledge on cyanotoxins in recreational water and their potential to cause negative health effects through dermal exposure. The ecology of cyanobacteria blooms and potential mitigation strategies have been presented in previous reviews [6,10,22,23], and will not be replicated here. The main focus of this review is to examine human skin as the effective barrier in the prevention of cyanotoxin absorption and the likelihood of any negative health effects through dermal exposure during water recreation. Analyses of molecular size, charge and structure of diverse cyanotoxins are presented to estimate the penetration potential. Future research directions are suggested for achieving a quantitative risk assessment of dermal exposure to cyanotoxins during recreational water activities.

Cyanobacteria blooms in recreational water

Cyanobacteria are found in bodies of water all over the world. When the water is rich with nutrients and the environmental conditions are favorable, the cyanobacteria can flourish into toxic algal blooms. The nutrient runoff associated with agriculture to sustain the growing population has been shown to facilitate the dominance of harmful cyanobacteria over existing microalgae assemblages in freshwater ecosystems [9]. In addition to nutrient availability, temperature, light intensity, pH and other environmental factors are known to influence algal blooms. These blooms have increased in both frequency and severity worldwide as a result of climate change, population growth, and rapid urbanization [7,24]. Cyanobacteria blooms in freshwater lakes pose a significant threat to human health through drinking and recreational water exposure.

Many cyanobacteria are responsible for the production and release of toxins that are harmful to humans and ecosystems [25,26]. They occupy many different niches and can be found in all terrestrial and aquatic marine ecosystems [7,22]. The most common freshwater genera are

Anabaena, *Nostoc*, *Oscillatoria*, *Planktothrix*, and *Microcystis*, which produce a suite of biotoxins, including MC, nodularin, anatoxin, saxitoxin, and cylindrospermopsin [25,27–29]. The concentration of toxins is highest during warmer months, which coincides with the busiest times for recreational water activities.

In 2007, the US EPA surveyed 1,161 lakes in the continental United States for the presence of 3 cyanotoxins: MC, cylindrospermopsin and saxitoxin (Fig 1.1A). Over the 6-month sample-collection period, MC (Fig 1.1B), cylindrospermopsin (Fig 1.1C), and saxitoxin (Fig 1.1D) were detected in 32, 4.0, and 7.7% of samples, respectively. However, cyanobacteria that potentially produce cylindrospermopsin, MC, saxitoxin and anatoxin were detected in a much higher proportion of samples. They were present in 67, 95, 79 and 81% of the samples, respectively [28]. There are currently no available comprehensive surveillance data available for nodularin and anatoxin in US lakes.

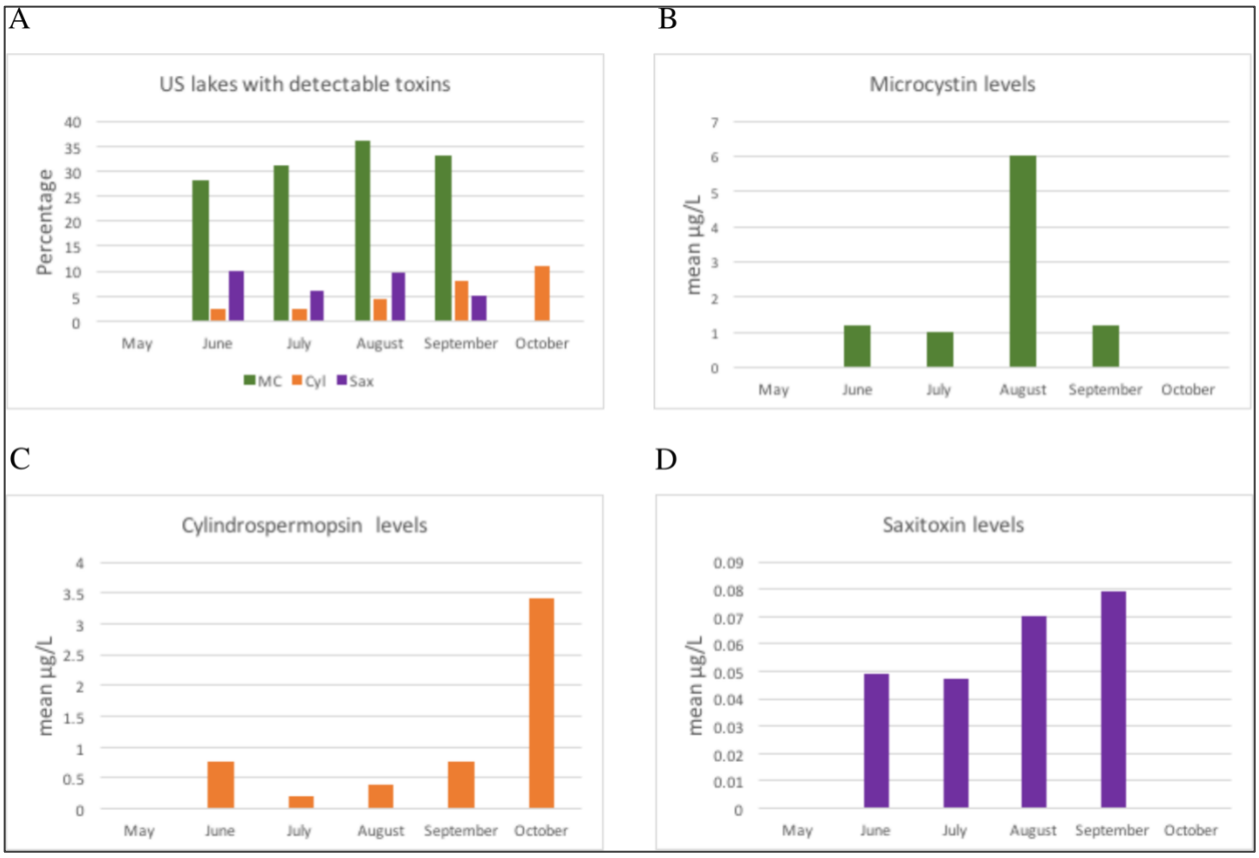


Figure 1.1. Mean levels of cyanotoxins in US lakes by month. Graphs are compiled from EPA reported data collected in 2007 [28].

Common cyanotoxins

Microcystin

MC is the most common, arguably the most toxic cyanotoxin found worldwide, and has been reported in surface waters in all the states in the United States [30]. MC includes over 100 structural congeners, which are formed by seven unique amino acids and classified as heptapeptides [31]. The names of structural congeners also reflect the amino acid composition. MC-LR has leucine (L) and arginine(R) attached to variable sites in the cyclic peptide. MC-LR ($C_{49}H_{74}N_{10}O_{12}$, molecular mass 995 g/mol) is by far the most common and the most studied [32]. MC-LR is often used as a surrogate for other congeners although they may differ significantly in toxicity and environmental persistence.

MC-LR has been identified as a potent hepatotoxin that inhibits the hepatocyte protein phosphatase 1 and 2A, leading to acute damage to the structure and function of liver cells, tumor formation, and liver cancer development in mammals [33,34]. MC-LR has also been shown to interact with mitochondria which results in dysfunction of the organelle, induction of reactive oxygen species (ROS) and cell apoptosis. MC activity leads to the differential expression/activity of transcriptional factors and protein kinases involved in the pathways of cellular differentiation, proliferation and tumor promotion activity [35].

The primary investigation of MC toxicity has focused on drinking water exposure, although water recreation during bloom events and consumption of seafood (fish or shellfish) from freshwater environments have demonstrated high risks for intoxication. The chief pathway of MC entry into cells is through the bile acid carrier, which is found in liver cells and to a lesser extent, in intestinal epithelia [36]. Evidence for the permeability of other cell membranes to MC is controversial. However, Fitzgeorge et al. published evidence for disruption of nasal tissues by MC-LR [37]. The authors indicated the intranasal application in these experiments was as toxic as intraperitoneal injection, which is at least an order of magnitude greater than toxicity by oral uptake. There is limited data on the toxic effects of dermal MC exposure.

Nodularin

The toxic effects of nodularin ($C_{41}H_{60}N_8O_{10}$, molecular mass 825 g/mol) have not been well studied. Nodularin has a similar chemical structure and assumed toxic mechanism to MC. Therefore, much of what we know about the toxicity of nodularin has been inferred from MC. There are 10 known variants, but nodularin-R is the most studied. Like MC, nodularin is a potent hepatotoxin with tumor promotion and carcinogenic effects [38]. Both nodularin and MC induce

inflammatory responses upon exposure but the specific cytokines induced differ between the toxins [39,40]. There is no data available on dermal toxic effects from exposure to nodularin.

Anatoxin-a

Different from the mechanism of toxicity of MC and nodularin, anatoxin-a is a potent neurotoxin that blocks pre and post-synaptic depolarization by efficiently competing with acetylcholine by binding to nicotinic receptors [41]. With a molecular mass of 165 g/mol, anatoxin-a ($C_{10}H_{15}NO$) can pass through the cell membrane of its producer [42] and therefore has the potential to pass through the cell membranes of those exposed. Although anatoxin-a causes suffocation due to respiratory failure, there have not been reports of human lethality from the toxin associated with recreational water use. Freshwater concentrations have measured as high as 1170 µg/L in the US [43]. The majority of research on anatoxin-a are in-vitro experimental studies on its mode of neurotoxic action [43]. The mechanisms of toxicity and penetration other than via the ingestion route have not been reported and there is no data available to evaluate the carcinogenicity or skin permeability of anatoxin-a in humans [43].

Saxitoxin

Saxitoxins ($C_{10}H_{17}N_7O_4$, molecular mass 299 g/mol) are part of a group of structurally related neurotoxins known as paralytic shellfish toxins. The most well studied saxitoxins are those produced by marine organisms known as dinoflagellates, but freshwater cyanobacteria produce them as well. They have been detected in freshwater bodies worldwide and concentrations have been measured as high as 193 µg/L in the U.S. [44]. These toxins are potent neurotoxins that act by blocking voltage gated sodium channels and therefore inhibiting the generation of action potentials [45]. While there have not been any reported human saxitoxin

poisonings due to recreational water exposure, many animal poisonings have been reported [46]. These toxins can persist in water for several months [46] and are so potent that they have been shown to inhibit proper neurite outgrowth at concentrations well below guideline levels [45]. To date there has been limited research on the reproductive, teratogenic, genotoxic or carcinogenic effects of paralytic shellfish toxins despite extended low dose exposure being a possibility [27,28]. Currently, there is no data available specific to skin exposure.

Cylindrospermopsin

Previously, blooms associated with cylindrospermopsin were restricted to tropical climates, but have recently appeared in more temperate climates throughout the U.S. [23]. Cylindrospermopsin ($C_{15}H_{21}N_5O_7S$, molecular mass 415 g/mol) has been identified as a hepatotoxic, genotoxic, cytotoxic, developmentally toxic, and possibly carcinogenic substance [47–49]. Concentrations have been measured up to 800 µg/L in bodies of freshwater [50]. A limited passive diffusion through biological membranes has been observed and this is most likely because of the small molecule size [51]. Moderate skin irritation and sensitization has been associated with extracts from cylindrospermopsin producing cyanobacteria [52]. It is unclear whether these effects are from the toxin or other components of the cells. Other studies have shown that whole cell suspension elicits more skin irritation than purified cylindrospermopsin [53,54]. Drinking water contamination is common because cylindrospermopsin mainly exists in the dissolved form instead of the intracellular form [55]. Short term exposure to cylindrospermopsin in drinking water can lead to liver, kidney, gastrointestinal, thymus, and heart damage by interfering in several metabolic pathways including the inhibition of glutathione, protein synthesis, and cytochrome P450 [48,56–59]. Although the dissolved toxin

concentration can be reduced by dilution, mixing from wind, adsorption to the sediment and biodegradation, cylindrospermopsin can persist in the environment for longer than a month [60].

Unknown toxins

While known toxins are an important environmental health concern, other cell components can also cause injury to the lungs, adrenals, intestines and skin, indicating unknown toxicology or toxins in the organisms. Sensitization to cyanobacteria through inhalation and oral exposure in humans has not been explored because of the potential for combined exotoxin and endotoxin adverse effects [54]. It is important to understand how mixtures of organisms and toxins affect humans and animals because toxic blooms vary in species, toxins and concentrations.

Current recreational water exposure guidelines

The recent release of the drinking water health advisory and the recreational ambient water criteria for MC and cylindrospermopsin by the US EPA have highlighted the progress towards human health protection from harmful algal blooms. However, the recreational ambient water criteria only address the risk associated with accidental water ingestion because less is known regarding health risks through dermal exposure during water recreation.

Current recreational water guidelines differ for each state in the U.S. (Fig 1.2) and only include 2 specific cyanotoxins: MC and cylindrospermopsin (Table 1.2). For example, in California, the recreational water advisory level for MC is at a concentration 0.8 μg/L but other states have advisory levels at >20 μg/L. In comparison, most states do not have guidelines for individual toxins and tend to rely on warning swimmers only if there is a visible algal bloom, or specific density of algal cells regardless of toxin concentration. However, the link between toxin

concentration in water and visible algal bloom is not always straight forward. The only way to determine if an algal bloom is toxic, is to test for the toxins.

These U.S. state advisories also differ when compared to The World Health Organization's recreational water guideline. WHO guideline is based on the relative probability of acute effects to MC concentration (Table 1.1). None of the guidelines have included dermal exposure as a potential risk factor. However, an in-depth examination of skin as the barrier to cyanotoxin absorption is needed to rule out the negative health effects from recreational exposure.

The limited skin exposure data indicates that these toxins can cause irritation and allergic reaction, but the mechanisms are not well understood. There have been many case reports regarding recreational water exposure to cyanobacterial toxins and their health effects. However, there is a disparity between the health effects described in epidemiological reports when compared to toxicology studies on dermal exposure to cyanobacteria and their toxins. This may be due to the absorption of the algal toxins through the skin during water recreation, causing systemic health consequences.

Table 1.1 EPA and WHO algal toxin guidelines.

Toxin	EPA Drinking water guidelines	EPA Recreational water guidelines	WHO Recreational water guidelines
Microcystin	0.7 μg/L (infants and pre-school children) 3 μg/L (school-aged children and adults	Currently in development. Several states have very different guidelines.	<10 μg/L = Low risk 10-20 μg/L = Moderate risk 20-2,000 μg/L = High risk >2,000 μg/L = Very high risk
Nodularin	nd	nd	nd
Anatoxin	nd	nd	nd
Saxitoxin	nd	nd	nd
Cylindrospermopsin	0.7 μg/L (Infants and Pre-school children) 3 μg/L (School-aged children and Adults)	Currently in development. Several states have very different guidelines.	nd

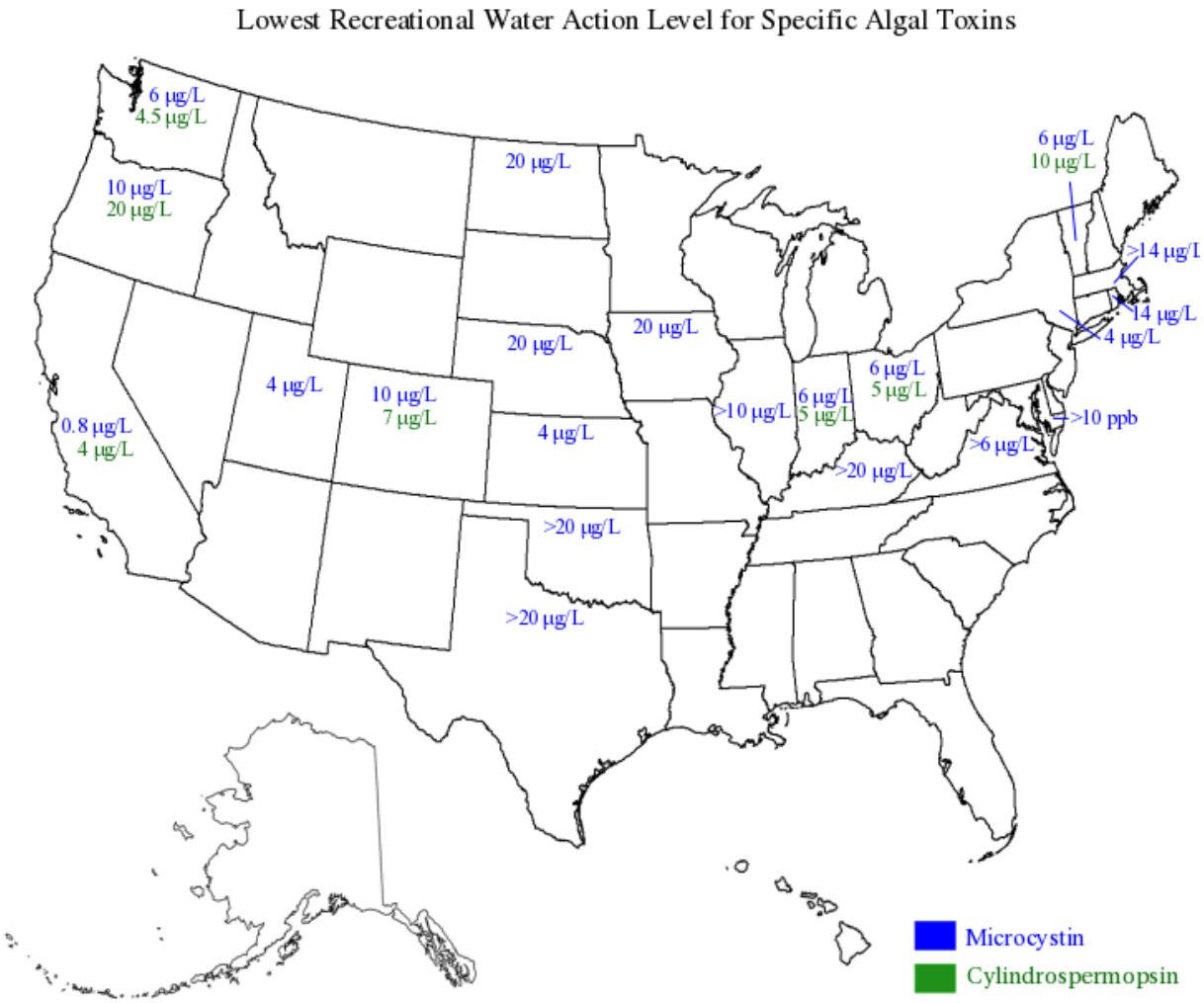


Figure 1.2. Lowest Recreational Water Action Level for specific cyanotoxins by individual State in the U.S. based on EPA guidelines.

Dermal exposure toxicology studies

Since the skin is the first barrier of defense against the outside environment, the function of skin cells and mucous membranes have direct effects on toxin penetration during recreation in water with algal blooms. The skin stratum corneum may be able to block toxins from entering the systemic circulation or metabolize them. Even so, the penetrative capabilities of cyanotoxins have not been studied among the limited toxicological investigation of the effects of cyanotoxins on skin cells. Skin cells are also challenged with high exposure levels during water recreation

because a large surface area is submersed for extended periods of time. Such exposure time and level have not been accounted for in published toxicological experiments.

Some cyanobacterial toxin experiments have been done with traditional cell culture, but these have primarily focused on the effect of MC on hepatocytes. To the best of our knowledge, only one study was published on the exposure of human keratinocytes to MC-LR. The authors investigated the effects on cell viability, migration and actin cytoskeleton organization after exposure to MC-LR at several concentrations and exposure times. Furthermore, they demonstrated that toxicity is dependent on both exposure time and concentration in a dose-dependent manner and concluded that the observed effects could cause considerable health effects in humans [61].

A study performed by Stewart et al., (2006a) assessed the dermal toxic effect of cylindrospermopsin using the Mouse Ear Swelling Test (MEST). They used 3 species of cyanobacteria in suspension (*C. raciborskii, M. aeruginosa* and *A. circinalis)* and purified cylindrospermopsin. The *M. aeruginosa* suspension had 13.6 mg/L MC-LR, the *C. raciborskii* had 73 mg/L of cylindrospermopsin and the *A. circinalis* had 6 mg/L of saxitoxin. They also prepared 100 μg/ml and 50 μg/ml solutions of purified cylindrospermopsin. The mice that were dosed with either *M. aeruginosa* or *A. circinalis*, had no skin reactions. All the mice that were dosed with *C. raciborskii* and purified cylindrospermopsin had skin reactions with various degrees of severity. This study demonstrated that there was a dermal effect on mice exposed to high concentrations of cylindrospermopsin and *C. raciborskii* cells [53]. These concentrations are unlikely to be encountered in nature and whether or not this is indicative of human response remains unclear. Further investigations, specifically involving dose-dependent responses and penetration potential on human skin is warranted.

Other studies attempted to use cyanobacterial extracts to assess a dose- response relationship. And while there appeared to be strong sensitization effects in some animal species, it did not seem to be related to the concentration of individual toxins, but more on other components of the cyanobacterial cells. Using intradermal injections of cyanobacterial cell extract, Torokne et al. (2001) demonstrated a significant dose dependent sensitization effect on guinea pigs but there was minimal to no effect seen in rabbits [52]. This response was not dose dependent on the concentration of MC but was dependent on the cell concentration. These animal models may be inappropriate for assessment of human dermal exposure effects.

Pilotto et al. (2004) performed two dermal exposure skin patch experiments on a total of 114 volunteers [62] . In the first trial (64 volunteers), each volunteer was exposed to *M. aeruginosa* (non-toxic strain), *A. circinalis* (toxic) and *N. spumigena* (toxic) on separate skin patches. In the second trial (50 volunteers), each volunteer was exposed to *M. aeruginosa* (toxic strain), *Apanocapsa incerta* (non-toxic) and *Cylindrospermopsis raciborskii* (toxic). Each volunteer was exposed to three different cyanobacterial species at six cell concentrations per species with both whole and lysed (to release intracellular toxins and components) cell solution. 20% to 24% of the volunteers had a skin reaction to at least one of the three species. When the volunteers that reacted to the non-toxic strains were removed from the analysis, 11%-15% of the volunteers had a significant skin reaction. Unfortunately, the study did not determine a dose-response relationship nor was there a difference between whole and lysed cell application.

A similar experiment was performed on 20 human volunteers using a series of cyanobacterial suspension skin patches [63]. These suspensions were comprised of whole, non-lysed cells and only one individual developed a clinically detectable skin reaction [63]. In some cases, the cyanobacterial toxin existed mostly intracellularly, and would not have an effect if not

released from the cell. In addition, the cells were washed before being applied to the test subjects, so the extracellular toxin that might have existed in the suspension, was most likely washed away.

Overall, the dermal exposure studies indicate that algal toxins cause mild to moderate skin irritation, but additional cytotoxic, carcinogenic and penetrative effects have not been thoroughly investigated. There is no dermal exposure literature on other algal toxins such as saxitoxin or anatoxin-a, most likely due to the neurotoxic nature of the compounds. However, the possibility that cyanobacterial toxins penetrate the skin and/or cause health effects that are not indicated by visible skin responses cannot be overlooked. More research should be done in this area before concluding that the effects are mild, especially since the epidemiological data seems to indicate otherwise.

Epidemiological studies

The incidence of outbreaks associated with freshwater harmful algal blooms (FHAB) has increased over the last three decades. In the United States, there were three FHAB-associated outbreaks from 1978 to 2008 compared to 11 outbreaks from 2009 to 2010 reported to the Waterborne Disease Outbreak Surveillance System (WBDOSS) and the Harmful Algal Bloom-Related Illness Surveillance System (HABISS) [64]. An outbreak must meet the following two criteria: 1) two or more people linked epidemiologically, and 2) the epidemiologic evidence must implicate recreational water as the probable source of illness [64]. The main source of human health effects data for cyanotoxins is from acute recreational exposure to cyanobacteria blooms. Symptoms include: headache, sore throat, vomiting and nausea, stomach pain, dry cough, diarrhea, blistering around the mouth, and pneumonia [65]. Rashes, eye, nose, mouth or throat

irritation, allergic reactions (including urticarial rash), malaise, and even respiratory failure, seizure and death are also reported [66]. These reported health endpoints could be related to other biological or biochemical mechanisms that are not yet understood since they are dramatically different from studies in mice and rats exposed to purified toxins, where liver and kidney toxicity are the frequent observed endpoints following acute oral exposure [67–69] and mild skin irritation following skin exposure.

Deaths in domestic animals, livestock and waterfowl that were exposed to water containing cyanotoxins from cyanobacteria blooms have been reported [70]. The signs of toxicity have been mostly neurologic, with deaths resulting from respiratory paralysis [70]. In the majority of cases, the cause of illness or death was suspected to be due to ingestion of cyanobacterial toxins, but not always confirmed [70]. Absence of a visible bloom at the time symptoms occurred, failure or delay in collection of appropriate specimens for analysis, and a lack of awareness of cyanobacteria toxins all contributed to the lack of certainty of the cause of illness and death, especially in animals [70]. Specific diagnoses are also hindered by the inability to detect and identify the various toxins produced by cyanobacteria in both the water and tissue samples [70]. Serum and blood analyses of algal toxins are difficult to perform because the toxins either breakdown quickly, are transformed, or rapidly accumulate within organs [71]. In addition, algal blooms may contain many different species of algae and many different and unknown mixtures of toxins.

Heise (1949) described one of the earliest recorded cases of human recreational exposure to toxic algal blooms [72]. The man had recurring episodes of asthma, eye irritation and discharge, and swelling of his nasal passages after recreating in the same lake every summer [70]. The patient swam in other lakes without incident. Some of the bloom, that was shown to be

comprised of mainly cyanobacteria, was collected from the lake and inoculated onto the patient's skin, initiating an immediate skin reaction [70]. In this case, the specific toxins present in the bloom were not identified. This research highlighted the need for additional cyanotoxin studies in the interest of public health.

While adult humans and animals are at risk for intoxication from toxic algal species, children are especially sensitive because of their lower body weight, behavior in the water, and toxic effects on development [71]. In 1979, there was an outbreak involving 12 teenagers and one adult at a lake-shore community in Pennsylvania. Upon contact exposure to the water, these individuals developed gastrointestinal illness and hay-fever allergy like symptoms [73]. During the investigation, it was discovered that there was a high concentration of *Anabaena spp.* in the water. Because of the lapse in time between the outbreak and the investigation, it was never proven if the algal bloom was the cause of the outbreak [73].

In July, 2002 in Dane County, Wisconsin, 5 previously healthy teenage boys all became ill after swimming in a golf course pond with visible algal blooms [71]. All of the boys had some symptoms, but those who were submerged under water were most affected. One boy suffered a seizure and died of heart failure 48 hours after exposure [71]. After nearly a year of investigation, the coroner concluded that the most likely cause of death was from anatoxin-a [71].

Night swimming is riskier than day swimming because scums are not as visible at night and swimmers may not notice that the water looks visibly contaminated. In 2008, several teens went for a late night swim in Lake Mendota, WI [71]. After exposure, one of the teens developed severe joint pain, rash, headache, fatigue, and gastrointestinal distress. The specific algal species

and toxins involved was unclear, but it was suggested that the symptoms were consistent with MC poisoning [71].

In 2011, the Kansas Department of Health and Environment received 25 reports of human illnesses associated recreational water activity in Milford Lake. Seven cases were confirmed to be due to the algal blooms, and 2 patients were hospitalized. The most common primary route of exposure was direct skin contact (all cases), followed by possible accidental ingestion (3 of 7 cases), and one case included possible inhalation [64]. Symptoms included: eye and upper respiratory tract irritation, sore throat, rash, gastrointestinal distress, cough, malaise, headache and fever. Both hospitalized cases occurred during periods of high cyanobacterial cell densities and MC toxin levels as confirmed by the water samples analyses (110 and 1600 μg/L maximum concentrations by ELISA) [64].

In Argentina, a young boy was jet skiing, ended up in a contaminated portion of the bay, and remained there, immersed in the water for 2 hours. Within a few hours after exposure, he developed nausea, vomiting, abdominal pain and muscle weakness. His condition worsened over the following 4 days and he was admitted to the hospital. He suffered from fever, respiratory distress, liver damage and pneumonia [74] He had to be mechanically ventilated for 3 days, was in the intensive care unit for 8 days, and made a full recovery after 20 days [74]. The water had obvious visible blooms and a measured MC-LR concentration of 48.6 μg/L. This intoxication likely occurred through multiple exposure routes: dermal contact, accidental ingestion and inhalation.

The effects of inhalation of algal toxins are complicated by the bioavailability of toxins, and presence of other cell components and debris in aerosols. In California, 81 people were exposed to aerosols from lakes contaminated with MC. MC was detected in the nasal swabs of

the individuals, but not in the plasma [75]. This was probably due to the difficulty detecting MC in the plasma and was influenced by the wide range of variability. Their findings indicated that recreational activities in contaminated water could generate aerosols that contain algal toxins. In addition to contaminated water, there is a necessity for further investigations with regards to dried scums that could be inhaled by humans and animals.

Algal blooms consist of a variety of cyanobacteria species and toxins. It can be difficult to attribute health effects to specific toxins when a mixture of toxins is present. The co-occurrence of numerous cyanotoxins complicates the association between specific toxin and subsequent health effects. Furthermore, epidemiological and dermal exposure data is insufficient for many algal toxins. There have been severe health effects described in case studies (Table 1.2) and the effects of dermal exposure to HABs in recreational water needs to be investigated more thoroughly in order to create appropriate safety guidelines to protect the public.

Table 1.2. Summary of human health effects described in case studies of exposure to contaminated recreational water.

Toxin	Human health effects from recreational exposure
saxitoxin	Fever, eye irritation, abdominal pains, and skin rash [76]
anatoxin-a	Seizure, heart failure, death [63,66,71]
microcystin	Joint pain, rash, gastrointestinal illness, pneumonia, fever, liver damage, sore throat, cough, headache, nausea vomiting [71,74]
cylindrospermopsin	No data specific to cylindrospermopsin.
Nodularin	No data specific to nodularin.
Toxin(s) not identified	Headache, sore throat, vomiting and nausea, stomach pain, dry cough, diarrhea, blistering around the mouth, pneumonia, rashes, eye, nose, mouth or throat irritation, allergic reactions, malaise, respiratory failure, seizure and death [64,70]

Permeation through the skin

While the skin does provide a barrier against chemicals, toxins and microorganisms found in recreational water, it can be permeated. For example, transdermal drug relies on skin's permeability to deliver effective dose. The skin is made up of three layers: the stratum corneum (SC), the epidermis and the dermis. In order for a molecule to gain entry to the bloodstream, it would need to pass through all three layers, however, the SC is considered the rate-determining layer for most chemicals [1]. Molecules can permeate this layer through 3 routes: 1) through or between the cells of the intact SC layer, 2) entry into the hair follicles through the space between the hair shaft and the follicular wall, and 3) entry into the sweat gland ducts [77].The intact SC layer is made up of mostly dead cells packed in a lipid matrix. Therefore, some lipophilic substances can pass with relative ease [78] while hydrophilic molecules can penetrate through sweat ducts and hair follicles [79]. Biphasic substances (soluble in water and lipids) have the greatest propensity for skin penetration [80]. Even though some compounds can be retained in the dermis, it is believed that once an exogenous substance has passed through the SC, further passage into the epidermis, dermis and capillaries is likely [77].

Closely related to the permeability of a specific toxin is the partition coefficient (P) between octanol and water (reported as Log P) of a given compound. Log P coefficients are used as a parameter for characterizing lipophilicity and can be predicted using computational algorithms such as XlogP3-AA. This algorithm predicts log P values of a query compound using a log P value of a similar reference compound as a starting point [81]. Log P values are predicated by an additive model using a multivariate linear regression [81]. Log P values around 0 indicate that the compound is equally partitioned between lipid and aqueous phases. Low log P

values indicated that the compound is more hydrophilic, while high log P values indicate high lipophilicity. Transdermal drug research has demonstrated that an optimal log P value for best skin absorption is between -1.0 and 4.0 [82].

The molecular weight of a given compound can help predict skin permeation as well. Smaller molecules, less than 500 Daltons (Da), can penetrate the skin more easily than larger molecules [79,80]. This has been derived from studies that show that nearly all contact allergens and almost all topical and transdermal drugs are under 500 Da [79]. However, compounds with molecular weights of 800 Daltons can penetrate broken skin and compounds up to 1200 Daltons have been shown to penetrate mucous membranes [79]. Algal toxin studies have shown that contact irritation and skin reaction are common after skin exposure and it should be noted that in order for a compound to elicit an immune response, it has to penetrate the SC. This may indicate that algal toxins and other cell components can penetrate the SC.

Skin permeation of algal toxins has not been well-characterized, but based on transdermal drug absorption research, certain molecular properties can be used to predict absorption (Fig1.3). It also important to note that these characteristics predict skin permeation as if the drug were applied to intact, healthy skin. Algal toxin contaminated recreational water is a unique exposure because most of the body is submersed in the water for extended periods of time. This gives the toxins more skin surface exposure for long durations, which could lead to increased absorption and toxic effects.

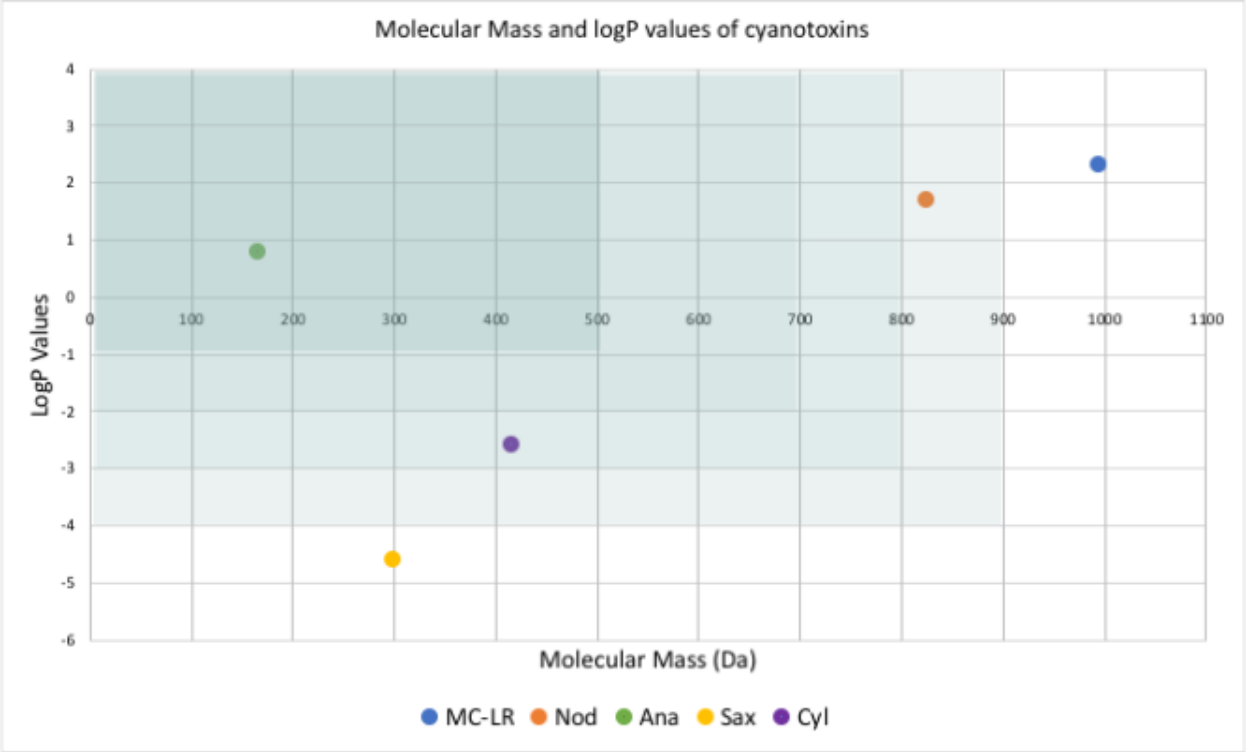


Figure 1.3. The plot of five cyanotoxins (MC-LR, nodularin, anatoxin-a, saxitoxin and cylindrospermopsin) according to their logP values and molecular mass for prediction of skin permeation. The likelihood for skin permeation decreases from darker shaded region (top left) to lighter shaded region (bottom right). Graph is based on data acquired from published literature [79,82].

As shown in Fig 1.3, based on the physiochemical characteristics, there should be some concern that all five toxins discussed in this review have the capability to be absorbed through the skin. Anatoxin-a has an ideal log P value and small molecular weight indicating that passive diffusion through intact skin is likely. MC and nodularin have larger molecular weights, which would make intact skin permeation more difficult, but they have ideal log P values. Saxitoxin and cylindrospermopsin are smaller molecules but their predicted log P values are outside the range indicated for optimal skin absorption. However, these toxins may be easily absorbed through mucus membranes and small breaks in the skin that remain in contact with water for extended periods of time during recreation. The values in Figure 1.3 represent the most well studied toxins but many of the known algal toxins have numerous congeners and variants that

may have different physiochemical properties. In addition, there are numerous lesser studied and unknown toxins that may be easily absorbed through the skin and/or may provide a synergistic effect on not only skin absorption, but systemic toxicity.

Skin characteristics affecting permeation

An intact SC layer is one of the most protective characteristics of human skin [77]. Damaged skin is more easily penetrated than intact, healthy skin [83]. The damaged skin contributes to water loss and increased permeation of exogenous substances through small breaks [77]. Many people exposed to contaminated water have suboptimal skin integrity due to cuts, irritation, psoriasis, eczema, and even dry skin. Nielsen et al. discovered that slightly damaged skin significantly increased the rate of chemical absorption, even with chemicals that normally have a low penetration rate on intact skin [78].

Skin hydration status influences transdermal absorption. As the skin is soaked in water, exposed to high humidity, and/or well moisturized, the cells in the SC begin to swell, enabling molecules to permeate more easily [80]. In fact, ethanol/water co-solvent has been used to increase the transdermal delivery of certain pharmaceuticals [84].

Penetration varies with each body site. The biological factors influencing this variation are number of hair follicles, thickness of the SC, distance between capillaries and sebum composition [78]. A study by Feldman and Maibach (1967) assessed the absorption of hydrocortisone for different anatomic locations on human skin and showed that there is a large difference between body sites. Scrotal skin had the largest measured absorption which was a 42-fold increase as compared to forearm skin. Plantar skin was the most resistant to hydrocortisone absorption as compared to the forearm. Back, scalp, axilla, forehead and jaw angle skin all

showed increasing rates of absorption, respectively, as compared to the forearm [85]. In addition, mucous membranes often come in contact with the water during recreational activities. This involves not only the incidental splash to the face and eyes, but the constant contact between genital mucosal epithelia and the water. When fully submersed in contaminated water, toxins have access to all body sites, increasingly the possibility of skin absorption.

Age is an important characteristic when assessing skin penetration as substances more readily penetrate the skin of younger individuals [78]. Aging skin has a lower moisture content [86] which lessens transdermal absorption of molecules [80]. Children and young adults may be more likely to stay in the water longer and fully submerse their entire bodies. This could explain the increased algal toxin rate and severity that we see in young people in the epidemiological studies.

Chemical penetration enhancers (CPEs) are commonly used in the pharmaceutical industry to help drugs penetrate the skin. Some components of sunblock, such as octyl salicylate are used as CPEs. People often apply sunblock during recreational water activities, and this may facilitate penetration of toxins present in the water.

The studies of the effects of algal toxins on skin have not investigated the relationship between algal toxins and skin absorption. Most experiments have been performed with single toxins applied to intact skin of humans and animals. Animals have different skin matrices and absorption rates than humans [87]. Algal toxin studies performed on humans apply toxin only to one anatomic location, such as the forearm. These studies do not take into account the possibility of algal toxin skin permeation and reveal conservative results based on localized application of toxin to intact skin which do not adequately represent exposure to contaminated recreational water.

Summary and future directions

Exposure to cyanobacterial cells and toxins in recreational water can cause severe health effects in humans and animals. Current data are limited regarding dose-response of human keratinocytes to MC and other algal toxins. The recommendations and guidelines regarding algal toxin skin exposure limits are mainly based on ingestion/intravenous exposure of animals. While this is helpful regarding those particular routes of exposure, the literature does not assess the effects of dermal exposure, including mucous membrane and eye exposure, and the possibility of skin absorption. For example, while MC have been well-studied, the mechanisms of cellular toxicity are not limited to the liver cells and may not only cause irritation/allergic reactions but may be cytotoxic and carcinogenic to skin cells. An ability to induce irritation and inflammation is a common property of tumor promoters, and the importance of irritation in tumor promotion is consistent with production of inflammatory cytokines [88]. Adequate and specific dose-dependent studies are required for skin exposure risk-assessment and recreational water safety guidelines.

The results of the dermal exposure studies have concluded that there is a mild irritation effect, but this is very different from the outcomes observed in the epidemiological data. Many of these studies involve single toxins but there are often numerous toxins, some of which are undiscovered with unknown effects, in HABs. Of the known toxins, many of them have different mechanisms of action. Exposure could result in a synergistic or penetrative effect and could potentially be responsible for the deleterious health consequences we see in the epidemiological studies. The health impact involved with exposure to multiple toxins at one time is poorly characterized and cannot be underestimated. While accidental ingestion does play a role in

symptom presentation, that does not seem a likely explanation for some of the health effects seen in recreational exposure to HABs. Based on the discrepancies between dermal exposure studies and epidemiological evidence of severe toxicity, we have to wonder if prolonged submersion in contaminated water and subsequent large skin surface area contact with a wide variety and often unknown mixture of cyanobacterial cells and toxins is responsible for the disparity.

Moreover, not only do the skin SC cells serve as a barrier, but the skin microbiome may be involved in metabolism of xenobiotics encountered in the environment. Numerous microbiome studies have determined that our resident flora metabolize various chemicals ([89][90][91]. To the best of our knowledge, there are no studies investigating the role of the skin microbiome on xenobiotic metabolism, but one study has demonstrated that the skin microbiome was perturbed during recreational water exposure [92], so it is unclear if the metabolic potential of resident microbes is also affected. This further justifies the need for studies assessing the toxic effects on human skin since the barrier functions may be compromised during recreational water exposure.

Future research should focus on assessing skin penetration and toxicity of single toxins, mixtures of toxins and cell components. The recent development and commercialization of human 3D tissue models presents new opportunities to expand cyanotoxin toxicity research. They have advantages beyond the humane and sociopolitical benefits. They are faster, cost-effective, and yield more reliable and relevant results compared to the traditional assays and 2D in vitro systems. These human tissue models are more physiologically and metabolically relevant to humans than animal models. Their high reproducibility allows comparison of different cyanotoxins using the same testing protocols. Many commercial companies are now using 3D tissue models to test for the toxicity of compounds and have been accepted by regulatory

authorities (UN GHS) for evaluation of skin and eye irritation and damage. These models can be analyzed for additional endpoints such as total cell viability, oxidative stress, localized cytokine levels, and penetration of toxin. An advantage to using 3D cell models is the unique opportunity to analyze for skin penetration by the toxin, which cannot be easily assessed using traditional cell culture or animal models.

Ideally, toxin measurements should be included in routine water quality monitoring in future toxic algal bloom outbreaks. This data should include comprehensive measurements of toxin and cyanobacterial species and concentration in order to potentially correlate individual toxins and mixtures of toxins to health effects. Most epidemiologic data that is currently available does not include information on specific toxin or cyanobacterial species. Currently, toxins are not routinely monitored in recreational water and are only assessed when there appears to be a visible algal bloom [93]. Traditional recreational water monitoring involves discrete sampling which is often an underestimation of toxin presence and fails to account for the temporal and spatial variability of toxins and cyanobacteria in the water [93]. There may be long term health effects associated with chronic low-level exposure. Therefore, we should be aware of toxin levels at all times, not just during toxic blooms. Newer technologies, such as Solid Phase Adsorption Toxin Tracking (SPATT) and remotely deployed biosensors are being implemented in order to passively and quickly assess toxin levels [93,94]. It is important to not only actively monitor HABs, but also to passively monitor water quality to measure algal toxin levels to help predict and possibly prevent HABs.

There is a disparity between the human health effects described in HAB exposure case studies and the toxicological skin exposure data. The symptoms described in the case studies are more severe and include systemic effects such as fatigue, organ damage, paralysis, and even

death. It is difficult to discern whether or not skin exposure to algal toxins is completely responsible for these symptoms as incidental ingestion and inhalation of aerosols also contribute. However, it is imperative that we acknowledge the need for appropriate water quality monitoring and research designed to address the current knowledge gaps in order to prevent future outbreaks. We need to investigate the skin penetration capabilities of algal toxins and assess if toxin mixtures may synergistically compound toxicity and subsequent health effects. This data will help provide preliminary information for water quality management authorities to accurately and rapidly evaluate human health risks from harmful algal blooms.

CHAPTER 2

Alterations in the human skin microbiome after ocean water exposure

Abstract

Skin is the body's first line of defense against invading microorganisms. The skin microbiome has been shown to provide immunity against exogenous bacterial colonization. Recreational water exposures may alter the skin microbiome and potentially induce skin infections. This study explored the link between ocean water exposures and the human skin microbiome. Skin microbiome samples were collected, using swabs, from human participants' calves before and after they swam in the ocean, and at 6 hours and 24 hours post-swim. Genomic analysis showed that skin microbiomes were different among individuals before swimming. But after swimming, microbial communities were no longer different, which was demonstrated by a decrease in inter-sample diversity. Taxonomic analysis showed that ocean bacteria, including potential pathogens, replaced the native skin bacteria and remained on the skin for at least 24 hours post-swim. This research provides insight into the relationship between the human skin microbiome and the environment.

Introduction

It is estimated that 41% of the U.S. population swim in oceans, lakes, rivers or streams each year (*National Survey on Recreation and the Environment (NSRE) 2000–2002.*). Even though exercise and recreational activities have numerous health benefits such as improved aerobic fitness and cardiovascular health [5], poor water quality and reports of recreational water related illness (RWRI) can significantly impact the value of beaches. Exposure to these waters can cause negative health effects including: gastrointestinal and respiratory illness, ear infections, and skin rashes [95]. In fact, 16.3% of all ocean beachgoers reported a new health issue after going to the beach [95]. Similarly, windsurfers were 2.9 times more likely to get one

or more of the following symptoms after windsurfing in contaminated water: gastroenteritis, conjunctivitis, otitis and skin infection. The relative risk of the symptoms increased with reported numbers of times the windsurfers fell into the water [96]. Arnold et al., (2017) reported that surfers in San Diego, CA were three times more likely to get a skin infection during dry weather months and nearly five times more likely during wet weather months than those with no water exposure. Among various RWRI, skin irritation or infections are frequently reported by those that engage in recreational water activities but are less studied than gastrointestinal illnesses.

Recreational beach waters are often contaminated by wastewater and storm-water runoff [11]. The presence of a variety of pathogens, such as: *Salmonella spp., Shigella spp., Campylobacter spp., Vibrio spp., Staphylococcus aureus,* intestinal parasites, viruses and other organisms in sewage and storm-water runoff can cause illness in humans that contact the water. The 2009-2010 Waterborne Disease and Outbreak Surveillance System (the most recent report) reports 24 disease outbreaks associated with natural (untreated water including rivers, lakes, streams, oceans) recreational waters [97]. Southern California coastal regions are among the most urbanized in the world [11] and most of the rainfall occurs during the wet weather season (November to May) [98]. Human fecal contamination, which includes potentially pathogenic bacteria, of ocean water is significantly higher in the wet weather months due to the increase of storm-water run-off during rainfall events [98].

Wastewater and storm-water runoff are not the only sources of potential pathogens in ocean water. Naturally occurring bacteria, such as *Vibrio* species and *Mycobacterium* species, are found in marine environments all over the world and can cause human disease [12,13]. Environmental parameters (e.g. temperature, turbidity, salinity, sea level height and climate change) can contribute to the virulence and abundance of *Vibrio* species [12]. Several *Vibrio*

species such as *Vibrio cholerae* and *Vibrio parahemolyticus* are well known human pathogens. And *Vibrio vulnificus* is considered one of the most dangerous waterborne pathogens, causing severe wound infections and septicemia [99]. While predominately found in warm waters such as the U.S. Gulf Coast, these pathogenic *Vibrio* species have all been detected in the coastal waters of Southern California [100], and have been implicated in non-foodborne infections in all coastal regions of the U.S. and other parts of the world [101]. Furthermore, the extensive use of antibiotics has affected environmental bacteria, including *Vibrio* species, rendering them more resistant to antibiotics which makes treating these skin infections especially difficult [102]. Naturally occurring atypical *Mycobacterium species*, such as *Mycobacterium marinum* and *Mycobacterium scrofulaceum*, have been associated with skin infections directly related to aquatic exposure [13,103]. These organisms can cause self-limiting, slowly-healing ulcers as well as more invasive health effects (e.g. joint and bone infections) in up to 29% of the cases [104]. As the climate changes and ocean temperatures rise, *Vibrio vulnificus* and other organisms that prefer warmer temperatures may increase in abundance in locations that are not currently suitable [105]. This could result in increased water contamination and more frequent infections.

Skin is the body's first line of defense, both physically and immunologically, during exposure to contaminated water. Recent studies have shown that the human skin microbiome plays an important role in immune system function against localized and systemic diseases, and infection [14]. The human skin microbiome refers to the microorganisms that inhabit human skin. The microbial composition differs among individuals and skin sites but individuals are more similar to themselves than they are to others [106][107]. The topographical difference in microbial composition is associated with the skin types. For example, sebaceous sites (i.e. face, back), moist sites (i.e. axilla, groin, toe webs) and dry sites (i.e. forearm, buttocks, calf) all have

different microbial communities, even in the same individual [14]. Even though there is much variability among individuals and body sites, there is no significant temporal variation among individuals. Most bacteria detected on normal human skin belong to the following phyla: Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%) [106]. The dominant genera are also quite stable and include: *Staphylococcus, Corynebacterium, Propionibacterium, Lactobacillus and Streptococcus* [106,108].

A healthy microbiome protects the host from colonization and infection by opportunistic and pathogenic microbes [14]. Recent research has demonstrated that changes in the microbiome can leave the host susceptible to infection and influence disease states [15][16]. For example, Naik et al. (2015) showed that *Staphylococcus epidermidis,* a normal human commensal, activated skin-resident dendritic cells and specific T cells that helped protect the skin from invading pathogens [15]. Nakatsuji et al. (2017) demonstrated that human skin commensal bacteria produced antimicrobials to prevent *S. aureus* infection. They also showed that patients with cutaneous disorders were deficient in these protective organisms [16].

The skin microbiome not only protects hosts from pathogen colonization but also may modulate the pathogenesis of a variety of cutaneous disorders [109]. Alterations in the microbial communities on the skin have been linked to psoriasis, atopic dermatitis, acne and chronic wound infections [110–112]. Environmental factors that alter microbiome diversity [113] are often associated with disease conditions [111]. For example, Chang et al. showed a healthy microbiome differed significantly from the microbiome associated with psoriasis. Normal skin bacterial species, such as *Staphylococcus epidermidis* and *Propionibacterium acnes,* were less abundant but opportunistic pathogens like *Staphylococcus aureus* were more abundant in psoriatic patients than in healthy patients [114]. This was attributed to a decrease in

immunoregulatory bacteria such a *S. epidermidis* and *P. acnes*, that subsequently led to increased colonization by *S. aureus* [114]. Similar characteristics have been observed in patients with atopic dermatitis. Skin microbiome dysbiosis in the affected individuals results in decreased normal commensal bacteria and increased colonization by *S. aureus* [115].

Much of the current research on the skin microbiome parallels that on the gut microbiome and human health. Alterations in the gut microbiome are not only physically associated with the gastrointestinal illnesses, like irritable bowel syndrome, inflammatory bowel disease and colon cancer, but also other diseases such as rheumatoid arthritis, obesity and Parkinson's disease. This past research demonstrates that the effects of change in the normal microbiome may have a greater and more far reaching impact than previously thought [116]. While direct exposure to pathogens can cause infection, the role of the human microbiome in immunity and infectious disease development has become increasingly recognized. Characterizing the changes in the resident skin microbiota associated with recreational water exposure may provide insight into the complex and fragile balance between healthy skin and skin infection.

High throughput sequencing technologies, like next-generation sequencing (NGS), have revolutionized microbiome research. NGS utilizes sequencing parallelization that results in millions of reads originating from specific amplified DNA sequences. The 16S rRNA gene is highly conserved among bacteria, but also has numerous variable regions that facilitate bacterial identification. The taxonomic composition of the microbiome has proven to be an important feature for distinguishing healthy individuals from those with disease states in numerous studies [116].

Understanding the changes of the skin microbiome during recreational water exposure and the role of the human microbiome against pathogen invasion and infection can offer new

strategies in protecting humans against RWRI. This research provides the foundation for the investigation of the potential link between alterations in the human skin microbiome and increased risk of infections. This research may aid in the revision of safety guidelines for exposure and the development of diagnostic and therapeutic tools that can help correct alterations in the skin microbiome for treatment or prevention of infections.

Methods

Sample collection

This study was approved by the University of California, Irvine Institutional Review Board (IRB #2017-3751). Sample collection occurred in April 2018 at Huntington Dog Beach in Huntington Beach, CA. A large poster summarizing the study and asking for volunteers was displayed at the collection site. Interested participants inquired and were given a detailed study description if they met the participant criteria. Only those who were 18 years of age or older, could speak and read English, and could swim were allowed to enroll. Participants gave verbal consent to enroll in this study. We obtained skin microbiome samples from nine participants including three males and six females, age ranges from 24-39, with no sunscreen application, infrequent exposure to the ocean and beach (once per month or less), no shower/bath in the past 12 hours, no antibiotic usage in the past 6 months, and no active infections. No identifying information was collected from the participants; samples were assigned a number (1 through 9). Samples were collected from an 8cm x 8cm section of skin on the back of the participants' calves using rayon-tipped swabs moistened in sterile saline. The calf was selected as the body site of interest because it has a large flat surface area and sustains constant water exposure while wading/swimming without requiring the participant to be completely submerged in the water.

Samples were collected before the individuals swam in the ocean. They were then instructed to swim or wade in the ocean for 10 minutes and the second set of samples was collected after they completely air-dried, which took approximately 20-30 minutes. The before and after samples were collected from the same calf but on different sections of the skin to ensure the sample collection taken before swimming did not remove bacteria from the section of skin swabbed after swimming. The before swimming samples were collected from the right side of the right calf and the after swimming samples were collected from the left side of the right calf after the area had air-dried.

The participants were then instructed not to shower or to wash the leg area for 24 hours and were trained using the above-mentioned swabbing method to collect their own samples for the 6 hour and 24 hour post-swim collections. The 6 hour sample was collected from the right side of the left calf and the 24 hour sample was collected from the left side of the left calf. Participants were instructed to keep the samples on ice after collection and investigators met with participants to retrieve the samples; no samples were shipped to the laboratory. Ice packs and coolers were provided, if requested. All samples were received on ice and processed within 24 hours of collection. An ocean water sample (75ml) from the swim site, at the time of sample collection, was collected and analyzed in the same manner as the experimental samples.

DNA extraction, PCR amplification and 16s rRNA gene sequencing

All samples were kept on ice until centrifuged to concentrate bacteria. Cell pellets were frozen at -80°C within 24 hours of collection. DNA was extracted from the cell pellets, and a single-step 30 cycle PCR was performed for the 16S rRNA gene V4 variable region using PCR primers 515F/806R (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′; and 806R: 5′-

GGACTACVSGGGTATCTAAT-3′). PCR conditions were: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes. All samples underwent DNA extraction, PCR and analysis by NGS using the 16S rRNA gene V4 variable region on an Ion Torrent PGM at MR DNA Laboratory (Shallowater, TX.).

Analysis and interpretation of sequencing results

Sequence data were analyzed using QIIME [118] at The University of California, Irvine. After importing the raw sequencing data, sequences were demultiplexed; primers, barcodes, short sequences, sequences with ambiguous base calls, and sequences with homopolymers exceeding 6 bp were removed using QIIME default settings. After initial quality control, each sample had between 6,320 and 167,432 DNA sequences, with an average of 97,048 sequences per sample and a total of 3,299,632 sequences in the data set. Chloroplast sequences were removed from the data. Sequences were filtered using a cut-off quality score of 25, clustered (using Uclust at 97% sequence similarity) into an open reference operational taxonomic unit (OTU) table and taxonomically classified (using Uclust consensus taxonomy assigner).

Alpha and beta diversity analyses were created from the resulting OTU table with taxonomic assignments. The OTU alignment failures were removed. Simpson and Shannon indices were calculated in QIIME and the table outputs were uploaded into R Studio using R version 3.5.0 (R Studio Inc., Boston, MA) for boxplot generation and further statistical analyses. P values were calculated using the Wilcoxon Rank Sum Test in R Studio.

Results

Skin microbiome diversity

Alpha diversity: To investigate whether the skin microbiome changes after ocean water exposure, we first examined the alpha diversity (intra-sample diversity) metrics of the samples collected before swimming, after swimming, 6 hours post-swim and 24 hours post-swim. Results from all participants were pooled together by sample collection time. Community richness (chao1-abundance-based richness estimator and observed OTUs), evenness (Simpson index) and overall diversity (Shannon index) are shown in Figure 2.1a-2.1d and Table A.1. Overall, we observed statistically significant differences in microbial diversity before and after the subjects swam in the ocean (Figure 2.1c-2.1d). Microbial diversity was the highest immediately after swimming, followed by 6 hours post-swim and before swimming. Samples collected 24 hour post-swim had the lowest diversity. Over time, the bacterial communities decreased in diversity as they trended towards baseline (before swimming). These results indicate that skin microbiome is altered by exposure to ocean water and changes are evident for at least 24 hours post-swim.

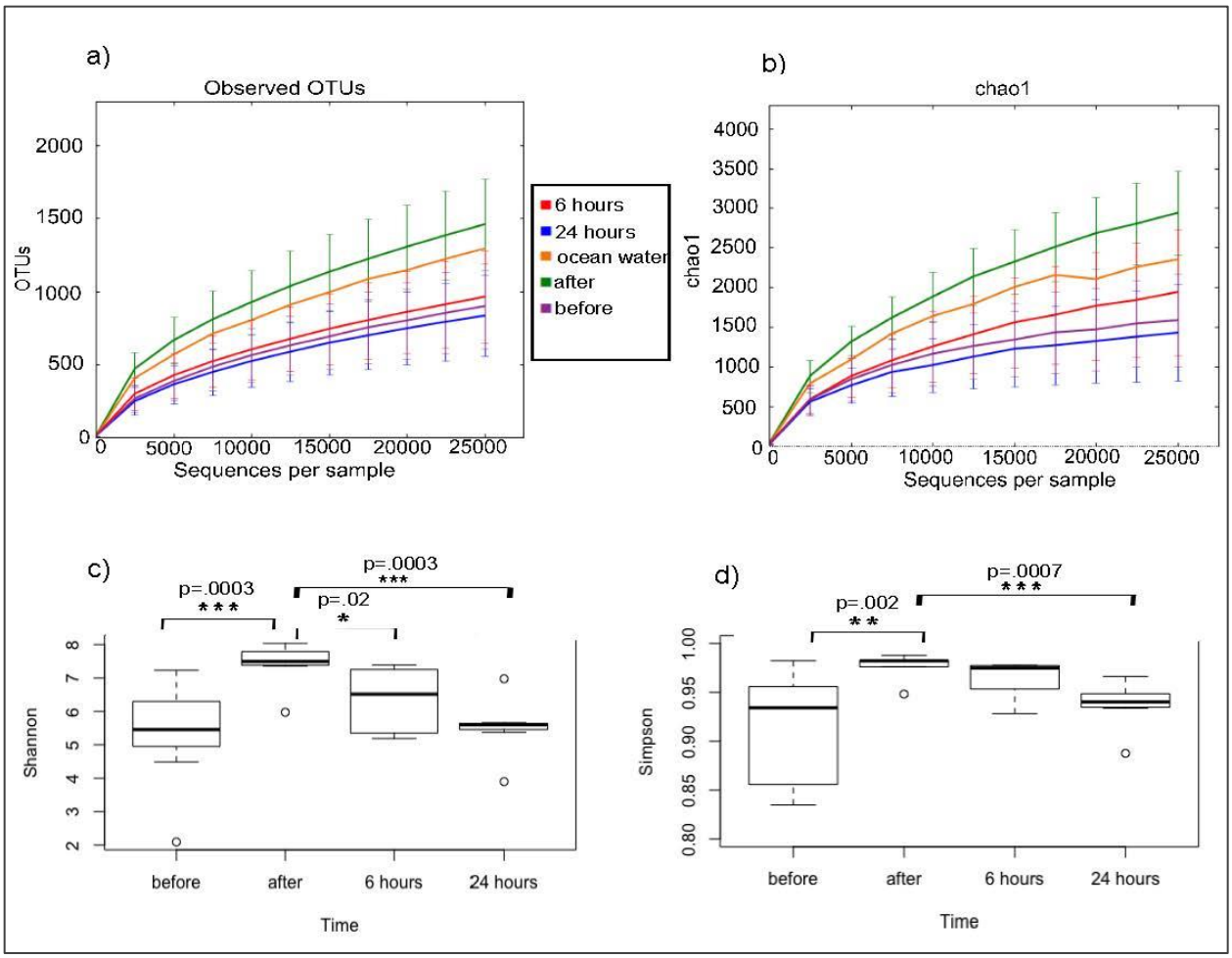


Figure 2.1. The bacterial community alpha diversity of skin microbiome samples before and after ocean swimming according to a) observed OTUs, b) Choa1 index c) Shannon index and d) Simpson index.

Beta diversity: We further explored the effects of ocean water exposure on the skin microbiome using beta diversity metrics (inter-sample diversity). We generated a Weighted Unifrac Distance Matrix, which is a qualitative representation of the difference in communities using phylogenetic branches that are weighted by the relative abundances of sequences. Using this matrix, Principle Coordinate Analysis (PCoA) plots were created (Figure 2.2). Principle Coordinate 1 (PC1) represents 54.52% variation, Principle Coordinate 2 (PC2) 8.22% and Principle Coordinate 3 (PC3) 6.84%. After ocean water exposure, we observed a distinct cluster indicating that even

though the alpha diversity (intra-sample diversity) was the highest, the samples from individual subjects were not distinctly different from one another. Each point on Figure 2.2 represents a sample from a human subject identified by the first number and followed by either B (before swimming), A (after swimming), 6 (6 hours post-swim) or 24 (24 hours post-swim). As time passed, the beta diversity measurements trended toward baseline (before swim) for each individual subject. As illustrated by the dash-lines in Figure 2.2a, the skin microbiome of subjects 1 and 2 slowly returned to the microbiome signature of the skin before swimming and at different rates. Similar patterns were also observed for other subjects, but the dash-lines were not included on the graph to avoid overcrowding of the lines. It should be noted, participant 3 and 4 did not have 6 hour or 24 hour samples collected, participant 8 and 9 did not have 6 hour samples collected and participant 6's before sample was below the sampling depth of 25,000 sequences for the analyses and was not included.

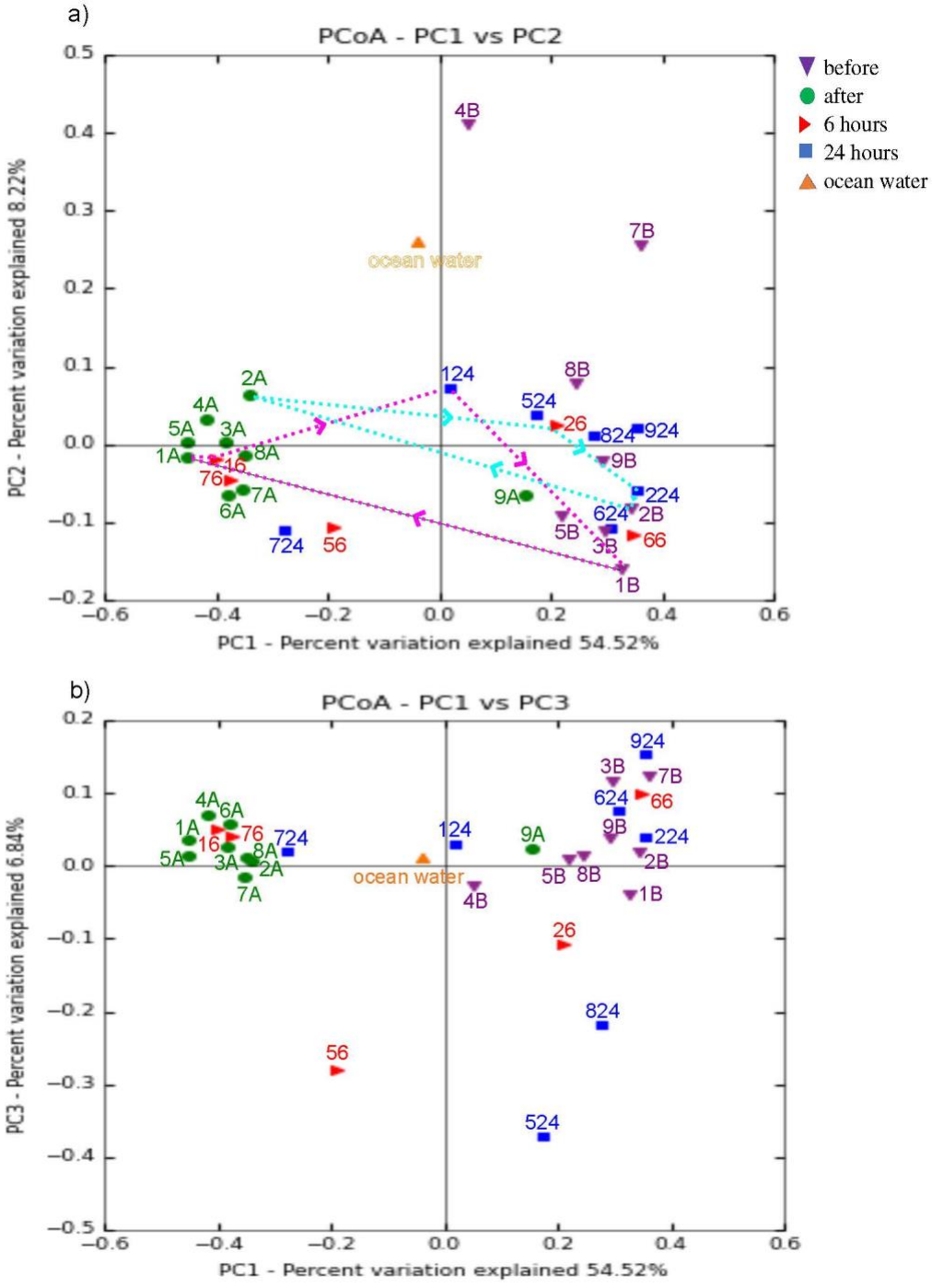


Figure 2.2 The bacterial community beta diversity of the skin microbiome before and after ocean swimming. Each point on the PCoA plot represents a skin microbiome sample where the first number indicates the human subject number. Beta diversity analysis was performed by weighted Unifrac PCoA where the 3 primary axes are shown a) PC1 vs PC2 and b) PC1 vs PC3. These coordinates represent 69.58% variation (PC1=54.52%, PC2=8.22%, and PC3=6.84%).

Taxonomy changes in the skin microbiome after exposure

The predominating phyla (Figure 2.3a) on the skin changed after swimming when compared to before swimming. *Actinobacteria* decreased from 34% to 6.7%, *Firmicutes* decreased from 32.3% to 9.4%, *Proteobacteria* slightly decreased from 24.8% to 24% and *Bacteroidetes* increased from 7% to 41.8%. As time passed, the bacterial community composition trended towards baseline. In comparison, the ocean water sample was comprised of 63% *Proteobacteria*, 17.2% *Bacteroidetes*, 8.4% *Cyanobacteria*, 7.4% *Actinobacteria*, 1.7% *Verrucomicrobia* and 0.7% *Firmicutes*.

A similar difference was seen at the familiae level (Figure 2.3b). Before swimming, the predominating familiae on the skin were *Micrococcaceae* (23.4%), *Staphylococcaceae* (17.7%), *Corynebacteriaceae* (6.7%), *Streptococcaceae* (5.4%) and *Lactobacillaceae* (3.4%). After swimming, the predominating familiae were *Flavobacteriaceae* (29.6%), *Puniceicoccaceae* (6.7%), *Cryomorphaceae* (5.9%), *Rhodobacteraceae* (4.2%) and *Corynebacteriaceae* (4%). At 24 hours post-swim, *Staphylococcacea*e began to establish dominance at 13.5% and *Corynebacteriaceae* increased to 9.6%. These changes were even more obvious at the genus level (Figure 2.3c). Before swimming, the skin is inhabited by indigenous bacteria such as *Staphylococcus*, *Streptococcus*, and *Corynebacteria*, as expected [106]. After swimming, however, those organisms were significantly reduced, and ocean-borne bacteria predominated. Even though a significant amount of normal skin flora was washed off and subsequently replaced by marine bacteria, the data demonstrate that as time passed, indigenous flora began to re-establish.

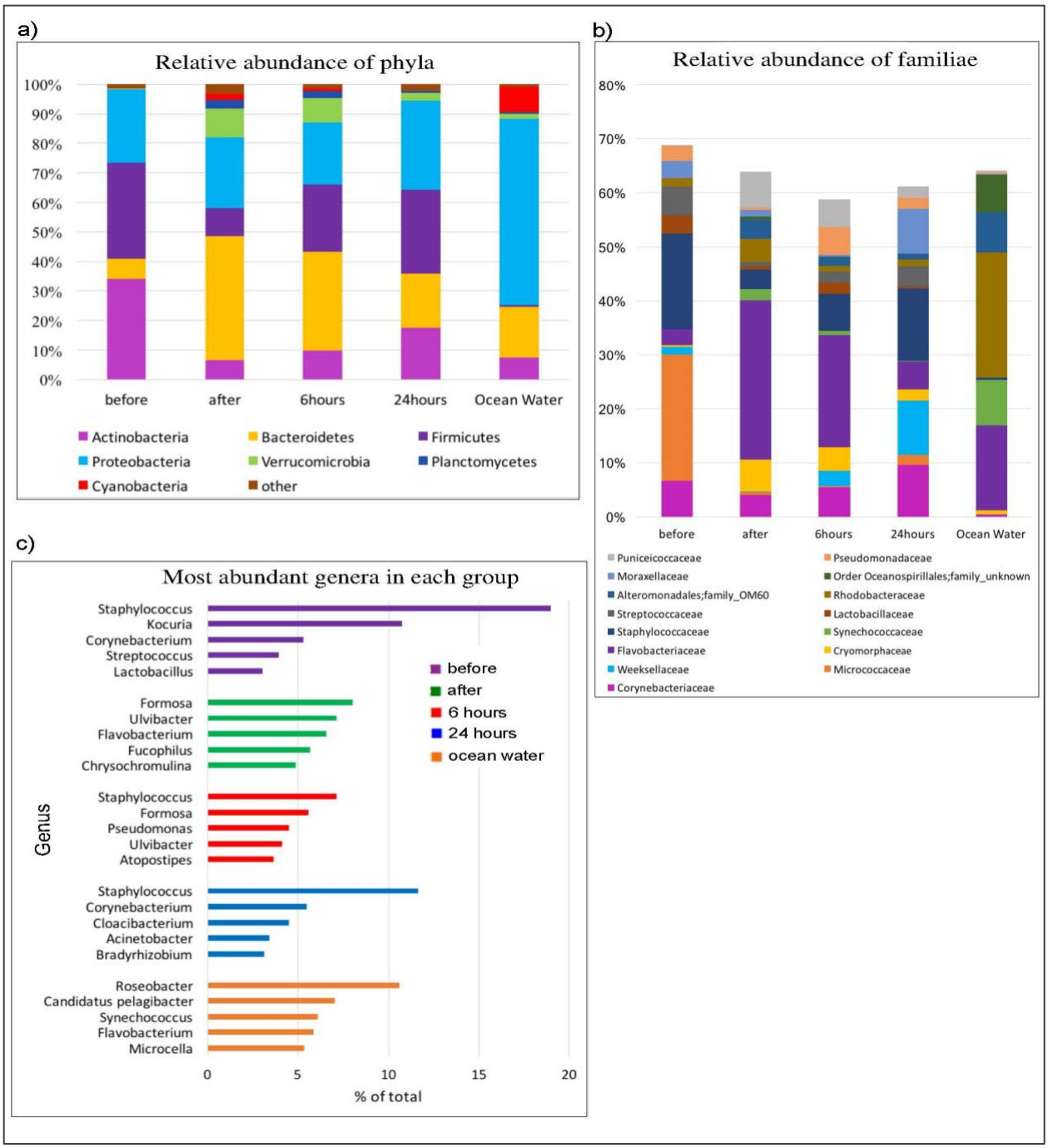


Figure 2.3. Microbial community composition of the skin microbiome by a) most abundant phyla, and b) most abundant familiae in each category collected before swimming (before), after swimming (after), 6 hours post-swim (6 hours) and 24 hours post-swim (24 hours). c) Most abundant genera in each category.

Changes in the skin microbiome of individual participants

Taxa alterations were also evident in individual subjects as time passed (Figure 2.4). The phyla and genus relative abundance was shown side by side for individuals 2, 5 and 7 to demonstrate that the individual participant results were similar to those seen in the pooled data shown in Figure 2.3. At the phyla level (Figure 2.4a), *Firmicutes* and *Actinobacteria* initially decreased in relative abundance after exposure to ocean water and slowly increased back to baseline levels. *Bacteroidetes* increased after swimming and slowly decreased as time passed. The ocean water appeared to simultaneously wash off resident skin bacteria and deposit ocean-borne bacteria onto the skin. This change may be dependent on the relative abundance of *Proteobacteria* (62.94%), *Bacteroidetes* (17.18%), *Actinobacteria* (7.45%) and *Firmicutes* (0.70%) present in the ocean water. These data are summarized for each individual participant (Table A.2). All of the participants acquired bacteria from the genus *Vibrio* after swimming (Figure 2.4b). This genus includes potential pathogens, although specific pathogenic species were not identified because organisms were only reported to the genus level. While this genus made up a very small percentage of total OTUs (0.37%) on the participants' skin, it still demonstrated that *Vibrio spp.* were present on the skin after swimming in the ocean. In some participants, these organisms persisted for 6 hours, and in one participant (7), for 24 hours. It is also worth mentioning that the fraction of *Vibrio spp.* detected on human skin was more than 10 times greater than the fraction of *Vibrio spp.* in the ocean water sample (only 0.032%), suggesting it has a specific affinity for attachment to human skin. Even though the human skin microbiome differed greatly between individuals, the effects of ocean water exposure on the skin microbiome were similar among individuals.

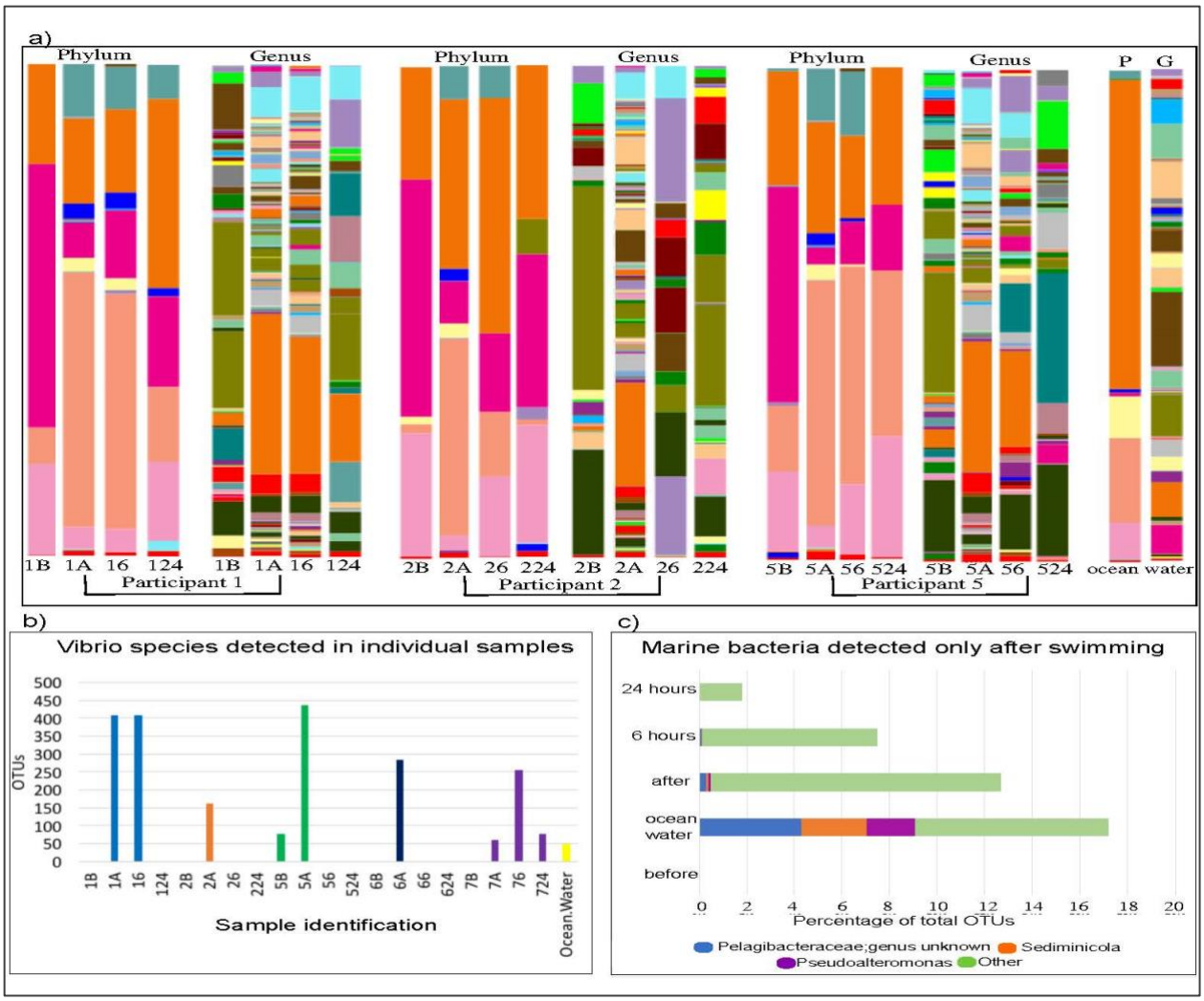


Figure 2.4. Changes in microbial composition before and after ocean exposure on individual human subjects. a) Changes at the phylum and genus levels. b) OTU counts representing members of the *Vibrio* genus detected in the samples. c) Bacteria that were detected on the skin only after swimming.

Approximately 17.2% of all bacteria detected on human skin after swimming were likely ocean bacteria because they were found in the ocean water but were not detected on the participants before they entered the water (Figure 2.4c). These ocean bacteria persisted on the skin for at least 24 hours and decreased in concentration over time. The top 3 most abundant ocean bacterial genera that were detected in the after swimming samples are shown in Figure

2.4c and Table A.3. The most abundant of these were members of the family *Pelagibacteraceae* (unknown genus), *Sediminicola spp.*, and *Pseudoalteromonas spp*. Again, Figure 2.4 data demonstrated that exogenous bacteria found on the skin after swimming, originated from the ocean, and persisted on the skin of individual human subjects for at least 24 hours.

Discussion

Linking skin microbiome change with skin health

Skin is the primary barrier protecting us from the external environment. The microbiome is currently believed to be an integral part of our immune system because of the association between host and microbial factors and the downstream effects on immune cells [119,120]. A healthy microbiome, which is largely stable over time [121], has been shown to defend our bodies from invading pathogens and protect us from disease. Alterations in the skin microbiome have been associated with skin diseases [122]. Our data demonstrate for the first time that ocean water exposure can alter the diversity and composition of human skin microbiota. A portion of the native skin microbiota was replaced by ocean bacteria, which was reflected in the increase in diversity and detection of ocean-borne bacteria in post-swim skin microbiome samples. This conclusion was further confirmed by the observation that although most individuals had different microbiomes when compared to one another pre-swim, they all had similar compositions after swimming, as shown in the beta-diversity analyses.

The dramatic changes in the skin microbiome from a normal indigenous microbiome signature to a completely different diversity signature after ocean water exposure signifies the importance of understanding the relationship between ocean exposure and skin health. Washing off native skin bacteria could weaken immunity against exogenous bacteria in the ocean. Ocean

bacteria, including *Vibrio spp.,* are clearly evident on the skin after exposure although *Vibrio* is not the predominant bacterial genus in the ocean water. This result implies that skin has the ability to attract exogenous bacteria from recreational water. *Vibrio vulnificus*, a naturally occurring pathogen in marine water, can cause necrotizing wound infections that can result in sepsis and death [123]. Although it is not the intention of this study to expose participants to water of poor quality, the results of the study imply that microbial pathogens would attach to human skin if they were present in the water.

Attachment and persistence of pathogens on the skin not only has implications for increased risk of skin infections, but harmful organisms found in recreational water of poor quality may infect humans through the fecal-oral route. It is possible that as pathogens persist on the skin, there is a chance for accidental transfer from the skin to the mouth, or even transfer to another individual. This could cause gastrointestinal illness not only at the beach, but in the hours or days post-exposure. This is especially dangerous for children and immunocompromised individuals whom are more susceptible to infection.

Study Limitations

There were several limitations in this study. The 'before swimming samples' were collected after most of the individuals had entered the beach area (with the exception of participant 1 and 2) but before they entered the water. In some cases, participants were walking and standing in the sand for 2 hours or more before samples were collected. Participants 1 and 2 did not have ocean bacteria detected on the skin before they entered the water, but all the other participants did. We believe that aerosols produced by the waves and skin contact with the sand may deposit a small number of ocean-borne bacteria onto the skin. Ideally, all 'before swimming

samples' should have been collected before participants entered the beach area but the phenomenon that ocean bacteria may be acquired on the skin without entering the water should be investigated.

The participant size in this study was small and conclusions from the study population may not apply to the general population. However, the microbiome sample size is sufficient for the study because even though only 9 participants were analyzed, 2-4 different time point samples were collected on each individual for a total of 34 samples. The samples averaged 97,048 bacterial sequences per sample. This sample size is similar to numerous highly influential human skin microbiome studies published in recent years. For example, Grice et al. investigated the human skin microbiome in healthy individuals and used samples from only 10 human participants [106]. Another study assessing the temporal stability of the human skin microbiome used samples from 12 healthy human participants for genomic analysis [121]. Studies on the association between certain disease states and the skin microbiome have also utilized small participant numbers. A 2018 study investigating the difference in the skin microbiome of healthy individuals and those with atopic dermatitis was based on 10 diseased individuals compared to 8 healthy individuals [122]. We had the opportunity to include additional participants but instead chose to adhere to strict study criteria designed to minimize confounding factors and additional variables.

Medical history was not collected from the participants and sex differences were not assessed due to the small number of participants. Chronic skin disorders and other characteristics may influence how the human skin microbiome responds to environmental exposures. In order to account for these variables, each person served as their own control to assess the effects of ocean water exposure and minimize confounding factors. Additional studies are necessary to

understand how unique skin characteristics influence the effects of ocean water exposure on the human skin microbiome.

Another potential limitation was the use of 16S rRNA gene hypervariable region 4 sequencing for our study. V4 region was selected for the greatest coverage of bacteria with different niches due to the large number of environmental and human commensal bacteria present in our samples. However, V4 has limitations in detection of some human skin commensals, particularly *Propionibacteria spp.* 16S rRNA gene sequencing of the V4 region is likely to identify as many bacteria as possible and to elucidate the effects of ocean water on the skin microbiome with the expectation that metagenomic analysis would be a useful tool for future research.

Lastly, this study only analyzed the skin microbiome on the calf. This body site was chosen because of its large, flat surface area and sustained water contact while wading. Different body sites are known to have different microbial community compositions. We anticipate these effects would be similar on other body sites and may even be accentuated in areas that have a lower abundance of commensals, have a higher abundance of more fastidious commensals, have the ability to trap water (inside the ears and nasal cavity), and/or maintain contact with the ocean sediment (toes).

Future research

There has been little research devoted to investigating the effects of environmental exposures on the skin microbiome. The microbiome can be altered in response to external substances, such as antibiotics and toxic chemicals, however, ocean water is unique in that it removes resident bacteria and simultaneously deposits foreign bacteria on the skin. A large

portion of the population is exposed to ocean water; therefore, a better understanding of ocean exposure and skin microbiome may protect public health during water recreational activities. This is especially of concern with increasing water temperatures and pollutant runoffs and a higher concentration of pathogens in natural waters [105]. Future work to connect changes in the skin microbiome with a prospective epidemiological study in poor quality water (i.e. post-storm condition) may offer a direct link between changes in the microbiome and skin infections.

Some participants encountered more drastic changes in the skin microbiome that persisted for a longer time as compared to the other participants. The physical characteristics of an individual's skin, such as: skin type, hydration level, skin product usage, sun exposure, hygiene, etc., may affect the changes seen. Some participants have a less diverse skin microbiome before swimming (as measured by species richness) and would therefore appear to have a larger increase in diversity after swimming. Research by Wang et al. (2016) has shown that differences in the human skin microbiome may be governed by differences in available carbon sources on the skin. They demonstrated that increasing sucrose on the skin promoted the fermentative capabilities of *S. epidermidis*, but not *P. acnes*. When *P. acnes* and *S. epidermidis* were co-cultured in the presence of sucrose, *P. acnes* growth was diminished [124]. Individuals have different levels of available sugars which support the growth of different indigenous bacteria [124]. Such differences may also support the attachment and persistence of exogenous bacteria on the skin. The skin microbiome and its responses to environmental exposure may also differ by sex. There is an unexplained observation reported in the literature that males are more likely to acquire *Vibrio vulnificus* [125] and *Aeromonas spp*. infections [126] after water exposure. Future research in this area may shed new light on wound and other necrotizing infections and the differences in the skin microbiome of males and females after exposure.

Wastewater, storm-water, discharges from animal agriculture, aquaculture and hospitals all contribute to the release of antibiotics and antibiotic resistant bacteria into the environment. Antibiotic resistant organisms have been found in ocean water [127–129], which present additional risk to recreational bathers for acquiring antibiotic resistant infections [128,130]. Future metagenomic research is needed to elucidate the connection between recreational water exposure and acquisition of antibiotic resistance organisms on human skin.

Providing evidence that ocean water exposures under certain circumstances (e.g., geographic, seasonal, exposure frequency) may increase health risks, will allow public health organizations to generate appropriate mitigation recommendations to help reduce the occurrence of RWRI. Potential strategies for reducing skin related health risks from exposure to ocean water could include protective recommendations (e.g., showering immediately post-swim, exposure time limits based on age, immune status and other characteristics) and therapeutic interventions targeted at re-population of normal skin commensals. Public and occupational health organizations will be better positioned to make recommendations that protect people while enabling them to continue to enjoy and work in marine environments.

CHAPTER 3

Changes in the antibiotic resistant gene profile of the skin microbiome in response to ocean water exposure

Introduction

Human skin, the largest organ in the human body, provides protection from diverse environmental insults, including xenobiotics, pathogens, particles, radiation and many others. The skin microbiome also helps improve this protection, especially with regards to exposure to pathogenic organisms. The resident microflora on the skin interact with skin cells to develop immunity to prevent invasion and infection from pathogens [15]. Perturbation of the skin microbiome can leave the host with an increased risk of microbial infection [16,110–112]. Early work has shown that swimming in the ocean not only removes normal commensal bacteria from the skin, but at the same time, deposits exogenous organisms onto the skin [92].

Antibiotic resistance genes (ARGs) are commonly found in diverse bacteria, including marine bacteria, and confer resistance to many different antibiotics through a wide range of mechanisms [131]. The ARGs can rapidly spread among microbial communities through horizontal gene transfer (HGT) such as conjugation, transformation and transduction. We hypothesize that the changes in the human microbiome, resulting from exposure to exogenous bacteria, are not limited to alterations in species diversity and abundance, but also may include the acquisition of genetic information. The bacteria that come in contact with our microbiome have the potential to transfer genetic information to our commensal flora. In fact, current research is recognizing that the human microbiome itself has become a reservoir of ARGs in response to the environment and antibiotic usage [18,132,133].

Recent studies have shown that ARGs are ubiquitous in the environment; they have been detected in soil samples [17–19], glaciers [20], animal agriculture, wastewater and oceans [21]. Some of these environments, for example, ocean waters, are influenced by human-driven contamination with antibiotics and exogenous ARG-harboring microorganisms. However, there

is also an ecological role of antibiotic biosynthesis in the environment, which slows the growth of competing organisms. Therefore, ARGs are also found in pristine environments with minimal anthropogenic impact. In fact, most of the ARGs acquired through HGT originated from environmental microbes [17]. ARGs have even been detected in ancient 30,000-year-old DNA from permafrost sediment, indicating that antibiotic resistance existed long before the clinical implementation of antibiotics [134].

Even though antibiotic biosynthesis and ARG transfer are naturally occurring phenomena, human activity has exacerbated the prevalence of antibiotic resistant organisms. Wastewater has been known to contain residual antibiotics and antibiotics resistant organisms due to clinical and agricultural antibiotic usage and disposable [135]. Sewage discharge to oceans, not only facilitates the potential to transfer ARGs from sewage bacteria to marine bacteria, but also contaminates the ocean with the residual antibiotics and poses selective pressure to promote the survival of resistant ocean bacteria [136]. Antibiotics naturally produced by marine microorganisms may further select for resistant populations in the ocean [21]. Therefore, ARGs have been associated with both marine and non-marine bacteria in the urban ocean [21].

Ocean bacteria in general are non-pathogenic to humans, however a minor proportion of the marine bacteria, such as some *Vibrio spp.* and *Mycobacterium spp.*, are opportunistic human pathogens. *Vibrio vulnificus*, an organism that is acquired solely from marine environments and known to cause severe disease in human, has gained widespread antibiotic resistance [137]. This demonstrates the importance for investigating the role of ocean swimming in the spread of antibiotic resistant organisms.

ARGs conferring resistance to several important sub-types of antibiotics were investigated in this study. These included: beta-lactams, glycopeptides, tetracyclines, fluoroquinolone-quinolone-florfenicol-chloramphenicol-amphenicol **(**FCA), aminoglycosides, and macrolide-lincosamide-streptogramin b (MLSb). These antibiotics are used to treat and prevent infections in both humans and animals and most of them have environmental origins.

Beta-lactam antibiotics have been used to treat infections since the discovery of penicillin. They represent a significant majority of the world's antibiotic usage (>65%) [138]. New classes of beta-lactam antibiotics have been developed to battle the constantly evolving resistant organisms. Each new class targets new resistance mechanisms and/or increases the spectrum of activity to incorporate additional bacterial species [139]. Bacterial production of beta-lactamases confers resistance to beta-lactam antibiotics; there are nearly 2800 known beta-lactamases and new variants continue to arise [140]. These enzymes have been isolated in many remote geographical locations and have ancient environmental origins [140].

Glycopeptides are a class of antibiotics that are active against many gram-positive bacteria and are used to treat serious infections caused by antibiotic resistant organisms. In fact, they are often used as a last resort for disseminated Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections [141]. Resistance to glycopeptides can often result in limited or no treatment options for patients with these infections. Vancomycin Resistant *Staphylococcus aureus* (VRSA), Vancomycin Intermediate (VISA) *Staphylococcus aureus*, and Vancomycin Resistant Enterococcus (VRE) are important human pathogens that have gained resistance through acquisition of various Van genes [141]. Genes that confer glycopeptide resistance most likely

originated in glycopeptide-producing Actinomycetes since they need survival mechanisms to resist self-produced antibiotics [141].

Tetracyclines are a class of broad-spectrum antibiotics that are often used in human and animal medicine for treatment and prophylactic prevention of a variety of infections. This class includes naturally occurring antibiotics as well as semisynthetic formulations [142]. Aquaculture and agriculture contribute to tetracyclines in the environment because they are the most widely used antibiotic in food-producing animals [136]. And as a result, they have been detected in high concentrations in the environment, including marine water [143].

A group of ARGs known as FCA resistance genes confer resistance to antibiotics within 2 different sub-types: amphenicols and quinolones. Amphenicols are broad spectrum antibiotics with extensive inhibitory effects on both gram-negative and gram-positive bacteria [144]. This sub-type contains chloramphenicol (naturally occurring), florfenicol (synthetic) and other derivatives. Historically, chloramphenicol was widely used in animal agriculture, but due to the toxicity to humans, was banned from use in food-producing animals and is no longer used in human medicine except in very rare and life-threatening situations [144,145]. Quinolones and fluroquinolones are broad-spectrum antibiotics often prescribed due to their oral formulations and successful treatment outcomes [146]. However, overuse has significantly contributed to antibiotic resistance [146]. Quinolones are not produced by bacteria but instead are chemically synthesized; even so, bacteria have developed resistance.

Aminoglycosides are among some of the first antibiotics ever used in clinical medicine. Due to toxic side effects and the development of newer antibiotics, their usage had decreased over the years. However, in light of the current increase in antibiotic resistant organisms, they are being used mainly for resistant gram-negative bacterial infections [147]. Aminoglycosides have

natural origins as they are produced in some soil-dwelling bacterial species. Not only are these bacteria resistant to self-produced antibiotics, they have gained resistance mechanisms that protect them from competitive organisms and their antibiotics as well [147].

MLSb genes are grouped together because they are functionally related [148] and cross-resistance due horizontal gene transfer both in the presence and absence of antibiotic pressure, is common[149]. Macrolides, lincosamides and streptogramin b include naturally-occurring and chemically modified antibiotics commonly used for gram-positive bacterial infections [148][150]. The relatively uncontrolled use in animal agriculture and prescribed administration in human and veterinary medicine has led to increased resistance in human and animal isolates and increased ARGs in the environment [149].

Organisms that produce antibiotics also contain ARGs as self-resistance mechanisms to protect themselves from the antibiotic produced [151]. Organisms that contain antibiotic biosynthesis genes (ABSGs) also contain genes that confer resistance to that specific antibiotic and are often clustered with ARGs [152]. Each biosynthesis gene cluster usually encodes for one or more ARGs able to protect the bacteria from the biosynthesized antibiotic [152,153].

Virulence factor genes (VFGs) are genes that encode virulence factors that positively correlate to bacterial survival and can predict pathogenesis [154,155]. These include genes that encode for characteristics that help bacteria evade host defense mechanisms such as: adherence, colonization, immune evasion, secretion systems, cell invasion, iron uptake and toxin production [154,155]. Much like ARGs, VFGs are easily transferred between genera through HGT [156], and have been discovered in many natural environments [157].

Much research has been devoted to the investigation of environmental reservoirs of resistance genes, known as resistomes [20,133,135,158]. However, the human skin resistomes

have not been investigated despite the importance of understanding human skin infection and mitigating the risk of ARG acquisition from the environment. This present study focuses on the diversity and abundance of ARGs and VFGs present on human skin and the changes in the genomic profile associated with ocean water exposure. We make comparative investigations using predicted profiles from 16s rRNA gene results and metagenomic sequencing data to help understand the role of marine environments in the distribution and acquisition of ARGs. The results of the study shed light on the prevention and management of antibiotic resistant skin infections.

Materials and Methods

Sample collection

This study was approved by the University of California, Irvine Institutional Review Board (IRB #2017-3751). Two separate sample collection events occurred in April 2018 and September 2018 in Huntington Beach, CA. The sample collection procedure was described in detailed in our previous paper [92]. In brief, a poster summarizing the study was displayed at the collection site and was used to recruit volunteers. Interested participants were given a detailed study description if they met the participant criteria. Only those who were 18 years of age or older, could speak and read English, and could swim were allowed to enroll. Verbal consents were collected from participants before registration. Skin microbiome samples were obtained from twelve participants including four males and eight females, age ranges from 24-39, with no sunscreen application, infrequent exposure to the ocean and beach (once per month or less), no shower/bath in the past 12 hours, no antibiotic usage in the past 6 months, and no active infections. Microbiome samples were assigned a number (1 through 12) with no identifying

information from the participants. Rayon-tipped swabs moistened in sterile saline were used to swab the skin on the back of the participants' calves before the individuals swam in the ocean. Participants were then instructed to swim or wade in the ocean for 10 minutes and the second set of samples was collected after they completely air-dried, which took approximately 20-30 minutes. Samples were then taken at 6 hours and 24 hours post-swim on sections of the calf skin that was not previously swabbed using the same sampling procedure as previously described [92].

16S rRNA gene sequencing and analysis

All samples were kept on ice until vortexed to loosen the bacteria from swab, and then centrifuged to pellet the bacteria. Cell pellets were frozen at -80°C within 24 hours of collection. DNA was extracted from the cell pellets, and a single-step 30 cycle PCR was performed for the 16S rRNA gene V4 variable region using PCR primers 515F/806R (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′; and 806R: 5′-GGACTACVSGGGTATCTAAT-3′). PCR conditions were: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes. All samples underwent DNA extraction, PCR and analysis by NGS using the 16S rRNA gene V4 variable region on an Ion Torrent PGM at MRDNA/Molecular Research LP (Shallowater, TX.).

Sequence data were analyzed using QIIME [118] at The University of California, Irvine. Raw sequencing reads were demultiplexed; primers, barcodes, short sequences, sequences with ambiguous base calls, and sequences with homopolymers exceeding 6 bp were removed using QIIME default settings. After removing chloroplast sequences from the data, sequences were filtered using a cut-off quality score of 25, clustered (using Uclust at 97% sequence similarity)

into an open reference operational taxonomic unit (OTU) table and taxonomically classified (using Uclust consensus taxonomy assigner).

PICRUSt was used to predict the functional profiles of the bacterial communities in 65 samples with the following scripts: normalize_by_copy_number.py, predict_metagenomes.py, categorize_by_function.py and metagenome_contributions.py. The gene counts from PICRUSt, known as KOs (Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs) [159–161], were compared with the KO database and previously published literature to ensure maximum detection of genes associated with antibiotic resistance [133,135,162] and virulence factors [163]. Weighted nearest sequenced taxon index (NSTI) scores for each sample were calculated to assess prediction accuracy using the predict_metagenomes.py with the -a option. In order to obtain OTU-specific gene counts for ARGs, we used the metagenome_contributions.py script with -l option for each KO of interest detected in the predicted metagenomes [135,164]. The current version of PICRUSt uses a KO database that does not include recently discovered ARGs and associated KOs. The antibiotics and their correspondence genes in KOs investigated in this study are summarized in supporting Table 3.1. The antibiotics of interest include vancomycin, tetracycline, FCA, beta-lactams, multidrug resistance, aminoglycosides, and MLSb, which correspond to 65 ARGs (Table 3.1) in KOs based on the 16s rRNA genes. Data were exported to R Studio (R Studio Inc., Boston, MA) and Excel for boxplot and heatmap generation and further statistical analyses. P values were calculated using the Welch's T-Test in R Studio. Cytoscape version 3.7.2 was used for network analyses [165].

Table 3.1 Antibiotics and their corresponding ARGs and KOs

Antibiotic	Gene (KO)
vancomycin	vanX (K08641); vanY (K07260); vraR (K07694); vraS (K07681)
tetracycline	tetA/tetG/H/J (K08151); tetK (K08168)
FCA	adeA/cmeA (K03585); catB3 (K00638); basR (K07771); qepA (K08167)
beta-lactams	acrA (K03585); ampC (K01467); ampG (K08218); blaI (K02171); blaR1 (K02172); cfxA (K01624); ftsI (K03587); mecA (K02545); mecR1 (K02547); metallo-beta-lactamase family protein (K07576); mrcA (K05366); mrdA (K05515); nagZ (K01207); ompU (K08720); ompC (K09475); ompF (K09476); pbpA (K12552); pbp1b (K03693); pbp2A (K12555); pbp2B (K00687); pbp2X (K12556); pbp3 (K12553); penA (K03587); tolC (K12340)
multidrug	emrE/qac/mmr/smr(K03297); MATE family (K03327); emrB(K03446); emrA(K03543); marC(K05595); mdtB(K07788); mdtC(K07789); mdtA(K07799); lmrP(K08152); blt(K08153); mdfA/cmr(K08160); mdtG(K08161); mdtH(K08162); mdtL(K08163); yebQ(K08169); norB/C(K08170); yitG/ymfD/yfmO(K08221); oprJ(K08721); ebrA(K11814); ebrB(K11815)
aminoglycosides	aacC1(K03395); aacC2(K00662); aacC4(K00663); aadA1(K00984); aadE(K05593); ybcL(K08164)
MLSb	ermC/A(K00561); ereA_B(K06880); mph(K06979); mef(K08217); macA(K13888)

Metagenomic sequencing and data analysis

In addition to 16S rRNA gene analysis, two samples collected before the subject swam in the ocean (1B, 3B), and two samples after the subject swam in the ocean (1A and 3A) were sequenced using shotgun metagenomic analysis. Due to the low concentration of DNA, linear amplification was applied with REPLI-g Mini Kit (QIAGEN) to enhance the amount of DNA while limiting the addition of bias. Sequencing libraries were made with Nextera DNA Sample Preparation Kit (Illumina) according to manufacturer's instructions. Paired-end sequencing was done using MiSeqc (Illumina) and 150 bp length reads were generated for each end at MRDNA/Molecular Research Laboratory (Shallowater, TX.).

FastQC (v0.11.7) was used to analyze the quality of the metagenomic reads. Optical duplicates were first removed with BBMap/clumpify.sh (v38.32, set as dedupe optical). Then the adapters and the potential contaminants indicated in fastQC report were removed, and the 8 bases from the start of the reads were cut with trimmomatic (v0.35)[166]. After quality filtering, clean reads of two sub-Before samples and two sub-After samples were co-assembled into contigs separately using MEGAHIT (v1.1.1, set as --k-step 10)[167]. The N50 of the contigs are 1607bp for before samples and 1913bp for after samples.

ARGs were determined by mapping the reads to comprehensive non-redundant databases or corresponding gene sets. Comprehensive Antibiotic Resistance Database (CARD) protein homolog model version 3.0.0 was used for ARGs [168]. Bowtie2 [170] mapping was done with options -D 20 -R 3 -N 1 -L 20 -i S,1,0.50 and was used to map reads to the CARD database. GenomeCoverageBed tool in Bedtools [171] was used to count number of reads mapping to the gene, length of reads and length of gene. The coverage of a gene was normalized with the coverage of 16s rRNA gene.

Results

Change in diversity and abundance of organisms on the skin after ocean water exposure

The results of 16S rRNA gene analysis showed that several bacterial phyla predominated the skin microbiome at all timepoints regardless of ocean water exposure. They are Bacteroides, Proteobacteria, Firmicutes, and Actinobacteria (Fig. 3.1). However, their relative abundances were different before and after swimming. For example, Bacteroidetes had a higher relative abundance in the after swimming samples as compared to the before samples, while Firmicutes and Actinobacteria had a higher abundance in the before samples. The after samples also

contained more organisms from several phyla seen in the ocean water samples, including Verrucomicrobia, Cyanobacteria and several others associated only with marine environments. Ocean water was predominated by Proteobacteria, followed by Bacteroidetes with a lower abundance of Firmicutes and Actinobacteria and also contained several phyla that were not detected in the other samples. At 6 hours post-swim, the samples contained more Bacteroidetes, Verrucomicrobia, and Cyanobacteria, and less Firmicutes and Actinobacteria than the before samples. At 24 hours post-swim, the microbiomes appeared to trend toward baseline due to the further reduction in Bacteroidetes and increase in Firmicutes and Actinobacteria. These results are similar to previous research that has characterized the change of the human skin microbiome in response to ocean water exposure [92].

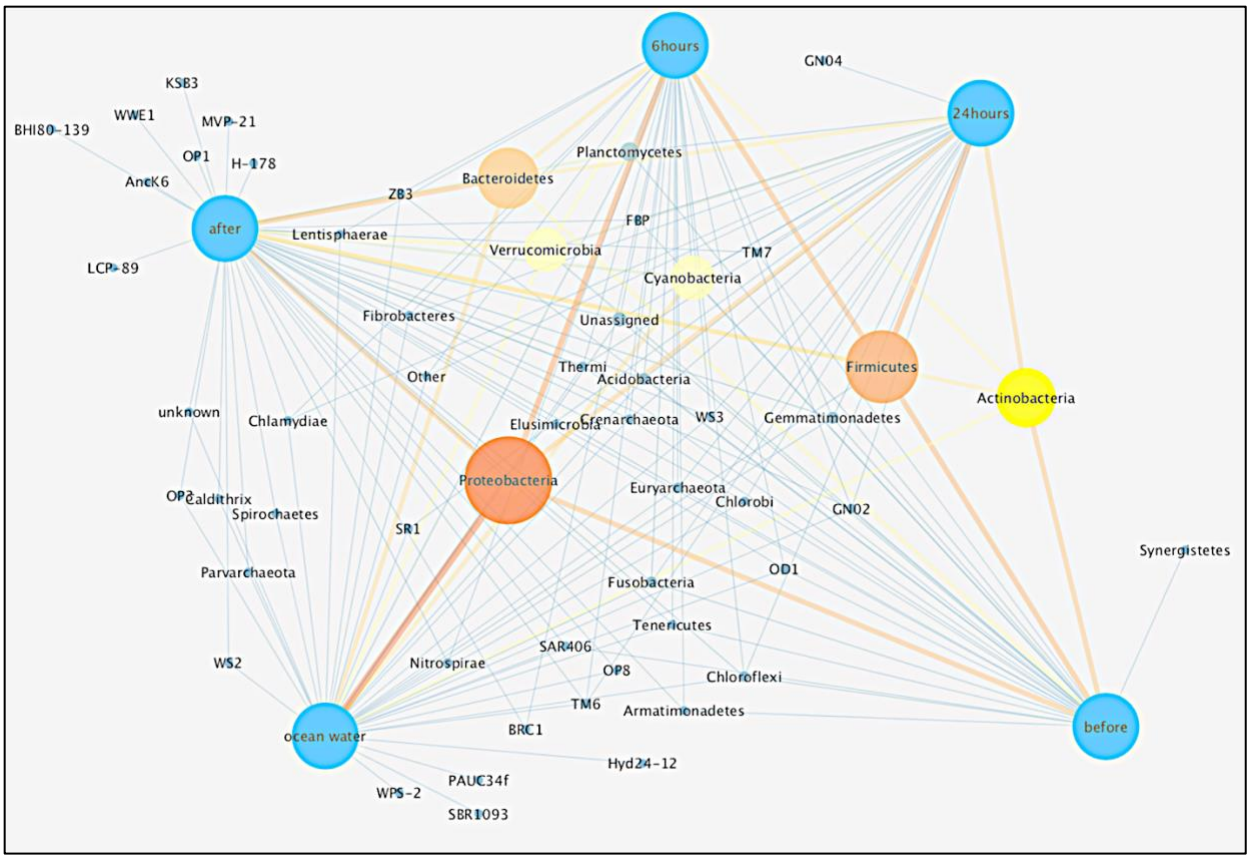


Figure 3.1. Network analysis showing the relationship among bacterial phyla and ocean water exposure in human skin microbiome samples. The relationships between bacterial phyla and the human skin microbiome samples at various time points (before swimming, after swimming, 6 hours and 24 hours post-swim). The strength of the relationship is indicated by the thickness and darkness of the line connecting sample categories to phyla.

ARGs acquired from the ocean are present on the skin after ocean water exposure

PICRUSt analyses showed that ARG counts increased significantly after swimming in the ocean (Fig. 3.2) for both the total number of ARGs and all three sub-groups of ARGs. These changes were statistically significant comparing before and after ocean exposure with the following p-values: 0.005476 for total ARGs, 0.001458 for beta-lactam, 0.02183 for multidrug and 0.03212 for vancomycin resistance genes, respectively. At 6 hours, the median ARG counts for each class remain slightly increased, while the range between individual samples increased as well indicated by the wider range of 25 and 75 quartile values. At 24 hours, the ARG counts

appear to return to pre-swim levels. For comparison, the ocean water at the time of the skin microbiome sampling contained a much higher number of ARG counts (Fig 3.2). The median values for total and each sub-group of ARGs at each sampling point were summarized in Table 3.2.

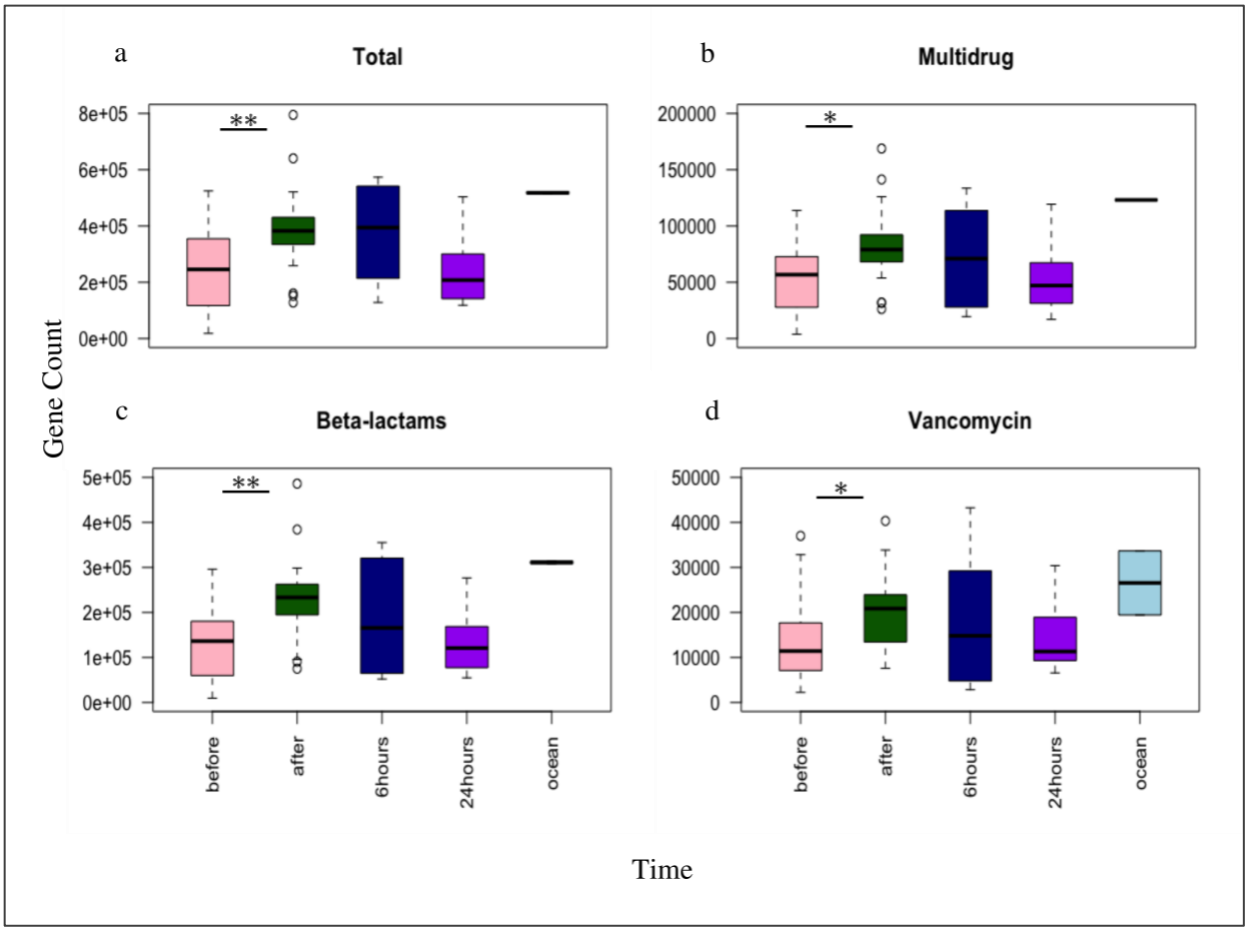


Figure 3.2. Sub-types of predicted ARGs before and after ocean water exposure. Changes in the number of total ARGS (a), multidrug resistance genes (b), beta-lactam resistance genes (c), and vancomycin resistance genes (d) at the following collection times: before, after, 6 hours and 24 hours post-swim.

Table 3.2. Median values for ARG count in each sub-type

ARGs	Before	After	6 hours	24 hours	Ocean
beta-lactam	136,289	233,239	165,709	120,904	311,089
multidrug	56,751	79,000	71,020	47,084	123,153
vancomycin	11,419	20,848	14,809	11,326	26,553
Total	245,705	382,637	393,533	207,799	518,107

Seven sub-types of ARGs that pose important challenges in public health management were investigated further in the samples collected at each timepoint (Fig 3.3). Overall, there was an increase in total ARGs by 70.6% from before to after swimming, and over a 300% increase from before to 6 hours post-swim. Beta-lactam resistance genes were the most abundant ARGs in all of the samples, accounting for nearly 55% of the total ARGs detected (Fig 3.3). In all of the sub-types investigated, with the exception of tetracycline, there was a large increase in gene count in the 6 hour group. ARGs returned to pre-swim levels after 24 hours, although comparatively slightly higher in all sub-types except for MLSb and tetracycline. In fact, total MLSb was the only sub-type that decreased in number immediately after swimming. MLSb genes are mainly associated with *Streptococcus spp*, and according to previous research, these organisms dramatically decrease in abundance within the skin microbiome after swimming in the ocean [92].

sub-type	gene	before	after	6 hr	24 hr	ocean	total
beta-lactams	mrcA	1.250	2.196	2.692	1.312	2.828	10.278
	cfxA	0.642	1.082	1.911	0.706	1.658	5.999
	nagZ	0.568	0.577	1.062	0.512	1.050	3.769
	ftsI	0.531	0.998	1.512	0.508	1.672	5.220
	penA	0.531	0.998	1.512	0.508	1.672	5.220
	ampC	0.482	1.064	1.594	0.693	1.018	4.850
	acrA	0.417	1.005	1.864	0.566	0.678	4.529
	PBPs	0.403	0.195	0.595	0.603	0.004	1.801
	mrdA	0.328	1.030	1.330	0.362	1.604	4.654
	tolC	0.323	0.803	1.184	0.320	1.214	3.845
	ampG	0.205	0.259	0.787	0.229	0.400	1.880
	metallo-beta-lactamase	0.191	0.577	0.404	0.185	0.289	1.647
	mecR1	0.009	0.007	0.010	0.005	0.000	0.031
	mecA	0.007	0.002	0.010	0.011	0.000	0.029
	blaR1	0.002	0.003	0.003	0.002	0.000	0.010
	blaI	0.002	0.000	0.003	0.001	0.000	0.006
	ompU,C,F	0.024	0.077	0.590	0.006	0.521	1.218
	Total beta-lactams	5.913	10.874	17.062	6.531	14.608	54.988
vancomycin	vanY	0.355	0.453	0.514	0.353	0.620	2.294
	vraS	0.098	0.031	0.142	0.090	0.002	0.362
	vraR	0.101	0.032	0.143	0.089	0.002	0.367
	vanX	0.092	0.442	0.468	0.120	0.624	1.746
	Total vancomycin	0.646	0.958	1.267	0.652	1.247	4.769
multidrug	emrE/qac/mmr/smr	0.362	0.380	0.654	0.253	1.283	2.933
	MATE family	0.491	1.131	1.853	0.478	2.394	6.347
	emrB	0.280	0.284	0.929	0.293	0.236	2.022
	emrA	0.382	0.357	1.306	0.428	0.259	2.732
	marC	0.390	1.285	1.262	0.401	1.451	4.790
	mdtB	0.060	0.037	0.553	0.090	0.006	0.746
	mdtC	0.069	0.082	0.584	0.075	0.065	0.875
	mdtA	0.118	0.109	0.605	0.119	0.075	1.025
	lmrP	0.003	0.004	0.009	0.010	0.000	0.027
	blt	0.119	0.066	0.209	0.112	0.003	0.509
	mdfA/cmr	0.010	0.006	0.295	0.020	0.001	0.331
	mdtG	0.044	0.048	0.682	0.034	0.001	0.809
	mdtH	0.001	0.001	0.313	0.001	0.000	0.316

	mdtL	0.001	0.001	0.284	0.001	0.001	0.287
	yebQ	0.068	0.016	0.451	0.060	0.002	0.597
	norB/C	0.192	0.058	0.565	0.163	0.005	0.984
	yitG/ymfD/yfmO	0.008	0.016	0.034	0.014	0.000	0.072
	oprJ	0.005	0.002	0.025	0.009	0.000	0.043
	ebrA	0.000	0.002	0.000	0.000	0.000	0.003
	ebrB	0.000	0.002	0.000	0.000	0.000	0.003
	Total multidrug	2.603	3.890	10.616	2.561	5.783	25.453
tetracycline	tetA/G/H/J	0.201	0.257	0.219	0.185	0.636	1.498
	tetK	0.000	0.001	0.000	0.000	0.000	0.002
	Total tetracycline	0.201	0.258	0.220	0.186	0.636	1.500
FCA	catB3	0.117	0.232	1.287	0.099	0.623	2.357
	adeA/cmeA	0.439	1.005	1.089	0.566	0.678	3.777
	basR	0.003	0.003	0.366	0.006	0.000	0.378
	qepA	0.238	0.133	0.417	0.196	0.023	1.007
	Total FCA	0.797	1.372	3.160	0.866	1.324	7.519
aminoglycosides	aacC2	0.033	0.171	0.209	0.045	0.010	0.468
	aacC4	0.039	0.022	0.117	0.037	0.009	0.224
	aadA1	0.103	0.037	0.145	0.083	0.002	0.370
	aacC1	0.002	0.002	0.002	0.000	0.000	0.007
	aadE	0.046	0.030	0.063	0.091	0.003	0.233
	ybcL	0.027	0.018	0.083	0.026	0.001	0.154
	Total aminoglycosides	0.249	0.280	0.620	0.282	0.025	1.456
MLSb	ermC/A	0.094	0.031	0.138	0.079	0.002	0.344
	ereA_B	0.001	0.002	0.005	0.001	0.000	0.009
	mph	0.430	0.420	0.525	0.362	0.863	2.601
	mef	0.056	0.052	0.158	0.096	0.003	0.364
	macA	0.090	0.051	0.722	0.105	0.029	0.997
	Total MLSb	0.671	0.556	1.548	0.644	0.897	4.315
	Total ARGs	11.080	18.188	34.492	11.722	24.519	100

Figure 3.3. Heatmap of ARGs, categorized by antibiotic sub-type, present on human skin before and after ocean water exposure. The numbers represent the percentages of the total ARGs detected in all samples.

The analysis of four antibiotic biosynthesis gene (ABSG) sub-types showed that ocean water contains many organisms that harbor ABSG and they are deposited onto human skin during swimming (Fig. 3.4). The median ABSG count increased after swimming as compared to before in all four antibiotic groups investigated (Table 3.3). The highest ABSG counts on the skin were found in the samples collected after swimming, and the lowest were found in the samples collected at 24 hours post swim. Ocean water had a median value of two to four times higher for each sub-type of ABSG.

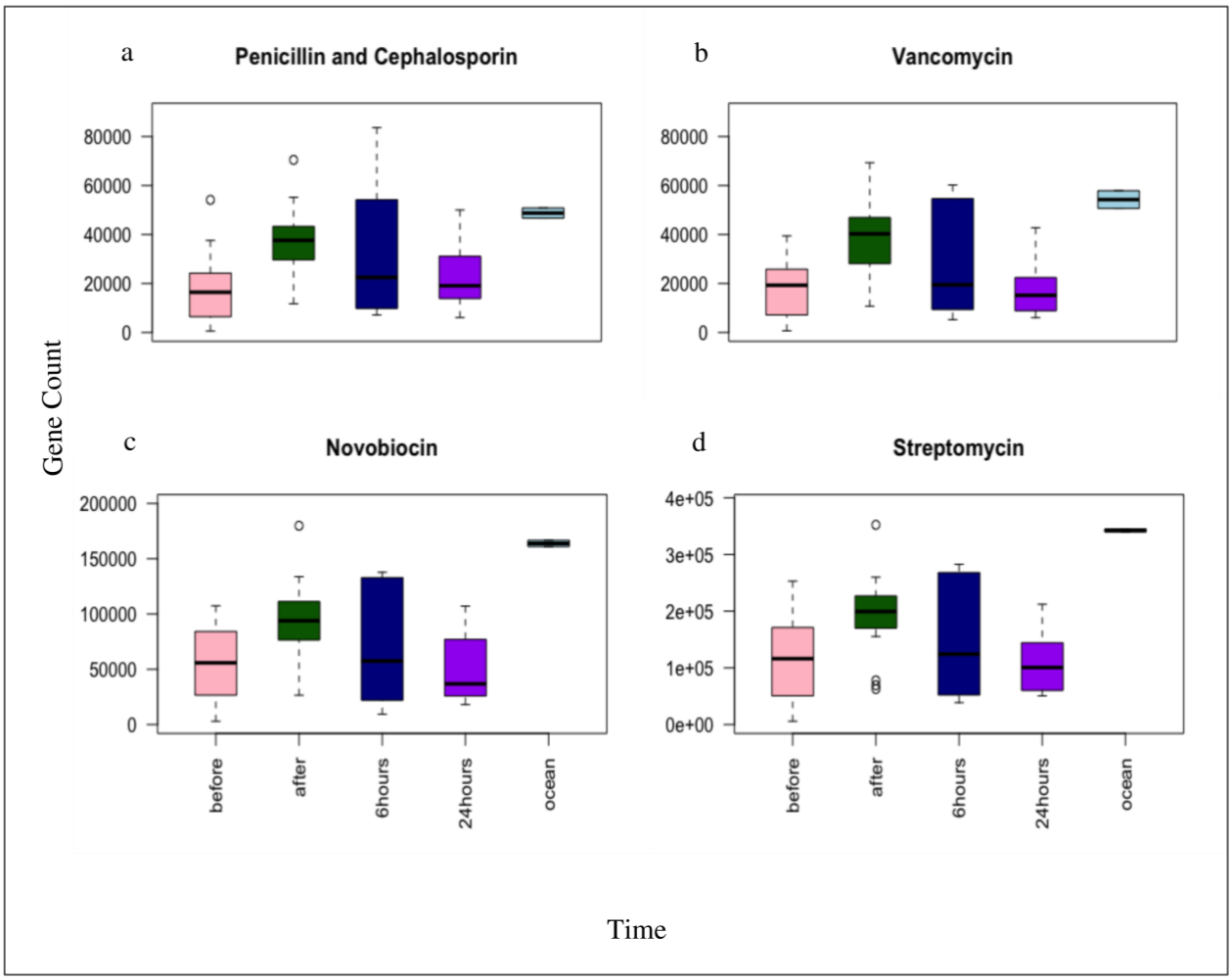


Figure 3.4. Predicted antibiotic biosynthesis gene count. The number of antibiotic biosynthesis genes present on the skin and associated with ocean water are shown for the following antibiotic classes: penicillin and cephalosporins (a), vancomycin (b), novobiocin (c) and streptomycin (d).

Table 3.3. Median values for ABSG count in each sub-type

ABSG sub-type	Before	After	6 hours	24 hours	Ocean
penicillin and cephalosporin	16,440	37,618	22,488	19,056	48,768
vancomycin	19,304	40,278	19,497	15,190	54,274
novobiocin	55,810	93,900	57,496	36,813	163,832
streptomycin	116,194	199,325	124,219	100,659	342,360

There were 897 predicted KO's detected in the samples and each KO corresponds to a specific gene(s) associated with virulence factors. In Figure 3.5, the top 20 most abundant KOs at each time point (28 KOs) are presented along with the total percentage of all VFG KOs present at each time point (Fig 3.5). The selected KOs account for 26.41% of the total VFGs. Of which, the most abundant KO in the samples encodes a sigma-70 factor in the extra-cytoplasmic function (ECF) family. These factors regulate many functions involved in response to stimulus from the environment in which the sigma factor is released, binds to RNA polymerase and promotes gene transcription [172]. Many of the other abundant KO's are associated with transport systems which help transport substrates across cell membranes and modulate bacterial survival [173]. The most abundant KO's increased by 52% after swimming, and by 208% at 6 hours post-swim. Similarly, total VFGs increased after swimming by 52%, and by 242% at 6 hours post-swim. At 24 hours post swim VFGs counts were slightly less than they were before swimming. The ocean water samples contained over 2 times as many VFGs than the human skin microbiome collected before swimming. This demonstrated that ocean water may be a natural reservoir of VFGs as well as ARGs.

KO	before	after	6 hr	24 hr	ocean	total	Description
K03088	0.29	0.78	0.78	0.29	0.58	2.72	RNA polymerase sigma-70 factor, ECF subfamily
K02014	0.17	0.35	0.70	0.22	0.27	1.71	iron complex outer membrane receptor protein
K02004	0.16	0.30	0.30	0.18	0.28	1.21	putative ABC transport system protein
K00059	0.17	0.26	0.35	0.15	0.39	1.32	3-oxoacyl-[acyl-carrier protein] reductase
K06147	0.18	0.25	0.28	0.19	0.48	1.38	ATP-binding cassette, subfamily B, bacterial
K02003	0.16	0.24	0.26	0.17	0.26	1.09	putative ABC transport system protein
K02529	0.12	0.22	0.33	0.12	0.35	1.14	LacI family transcriptional regulator
K03559	0.06	0.20	0.21	0.07	0.18	0.72	biopolymer transport protein ExbD
K03561	0.04	0.20	0.19	0.06	0.12	0.61	biopolymer transport protein ExbB
K01990	0.14	0.20	0.24	0.15	0.13	0.86	ABC-2 type transport system protein
K01992	0.12	0.16	0.19	0.14	0.11	0.72	ABC-2 type transport system permease protein
K03406	0.13	0.16	0.43	0.11	0.22	1.06	methyl-accepting chemotaxis protein
K01784	0.09	0.16	0.17	0.09	0.22	0.71	UDP-glucose 4-epimerase
K02032	0.15	0.15	0.26	0.11	0.39	1.06	peptide/nickel transport system protein
K01915	0.10	0.15	0.19	0.08	0.29	0.80	glutamine synthetase
K07497	0.06	0.14	0.13	0.06	0.29	0.68	putative transposase
K02015	0.17	0.14	0.37	0.15	0.17	1.00	iron complex transport system protein
K03496	0.09	0.13	0.14	0.08	0.20	0.63	chromosome partitioning protein
K01704	0.08	0.13	0.14	0.08	0.27	0.70	3-isopropylmalate/(R)-2-methylmalate dehydratase
K02016	0.14	0.13	0.29	0.13	0.13	0.82	iron complex transport system protein
K02027	0.13	0.11	0.11	0.08	0.38	0.82	sugar transport system protein
K02026	0.13	0.12	0.12	0.09	0.41	0.87	multiple sugar transport system protein
K02025	0.13	0.12	0.13	0.09	0.39	0.85	multiple sugar transport system protein
K02013	0.11	0.11	0.24	0.11	0.16	0.73	iron complex transport system protein
K02483	0.09	0.06	0.12	0.07	0.12	0.46	two-component system response regulator
K00257	0.09	0.12	0.16	0.09	0.20	0.66	acyl-ACP dehydrogenase
K06148	0.08	0.07	0.18	0.07	0.08	0.48	ATP-binding cassette, subfamily C, bacterial
K02078	0.06	0.12	0.17	0.06	0.16	0.58	acyl carrier protein
Total	3.45	5.26	7.19	3.27	7.24	26.41	
Total VFGs	12.36	18.84	29.97	11.63	27.19		

Figure 3.5. Heatmap of VFG KO's present on human skin before and after ocean water exposure. The numbers represent the percentages of the total VFGs detected in all samples.

Occurrence, abundance and diversity of ARGs using metagenomic sequencing

Based on the calculated Shannon Weiner Index, ARGs diversity increased from 1.68 before ocean swim to 2.08 after swim (Fig 3.6a). Similarly, ARG sub-types increased from 10 to 22 in the before and after samples respectively. The total normalized abundance of ARGs in the before and after samples was 0.32% and 0.67% respectively (Fig 3.6b). Sulfonamide resistance

genes were unique to the before samples, whereas the unique sub-types seen in the after samples were: phenicol, peptide, ansamycin, lincosamides, fusidane, streptogramin, fosfomycin, nucleoside, aminocoumarin, triclosan, pleuromutilin, oxazolidinone, mupirocin as shown in the heatmap of Fig. 3.6c. Among all the detected sub-types, 9 of them were shared by both the before and the after samples (Fig. 3.6c). The dominant ARG sub-types in the before samples were aminoglycoside (0.09%), macrolide-lincosamide-streptogramin B resistance (MLSB) (0.08%), and diaminopyrimidine (0.08%). In the after samples, the dominant sub-type was beta-lactam (.2874%), followed by aminoglycoside (.0734%) and multidrug (.0866%)(Fig. 3.6d). After ocean water exposure, the normalized abundances of macrolide, multidrug, tetracycline, beta-lactam, glycopeptide, fluoroquinolone were two to 14 times higher than the before samples.

For comparison, the abundance of predicted ARGs from the PICRUSt analysis are plotted side by side (Fig 3.6d-e). Similar to the metagenomic results, gene counts (before to after) of total ARGs increased by 64%, beta-lactam by 84%, multidrug by 49%, vancomycin by 48%, tetracycline by 28%, FCA by 72%, and aminoglycosides by 12%. For MLSb ARGs, there was a decrease of 17%.

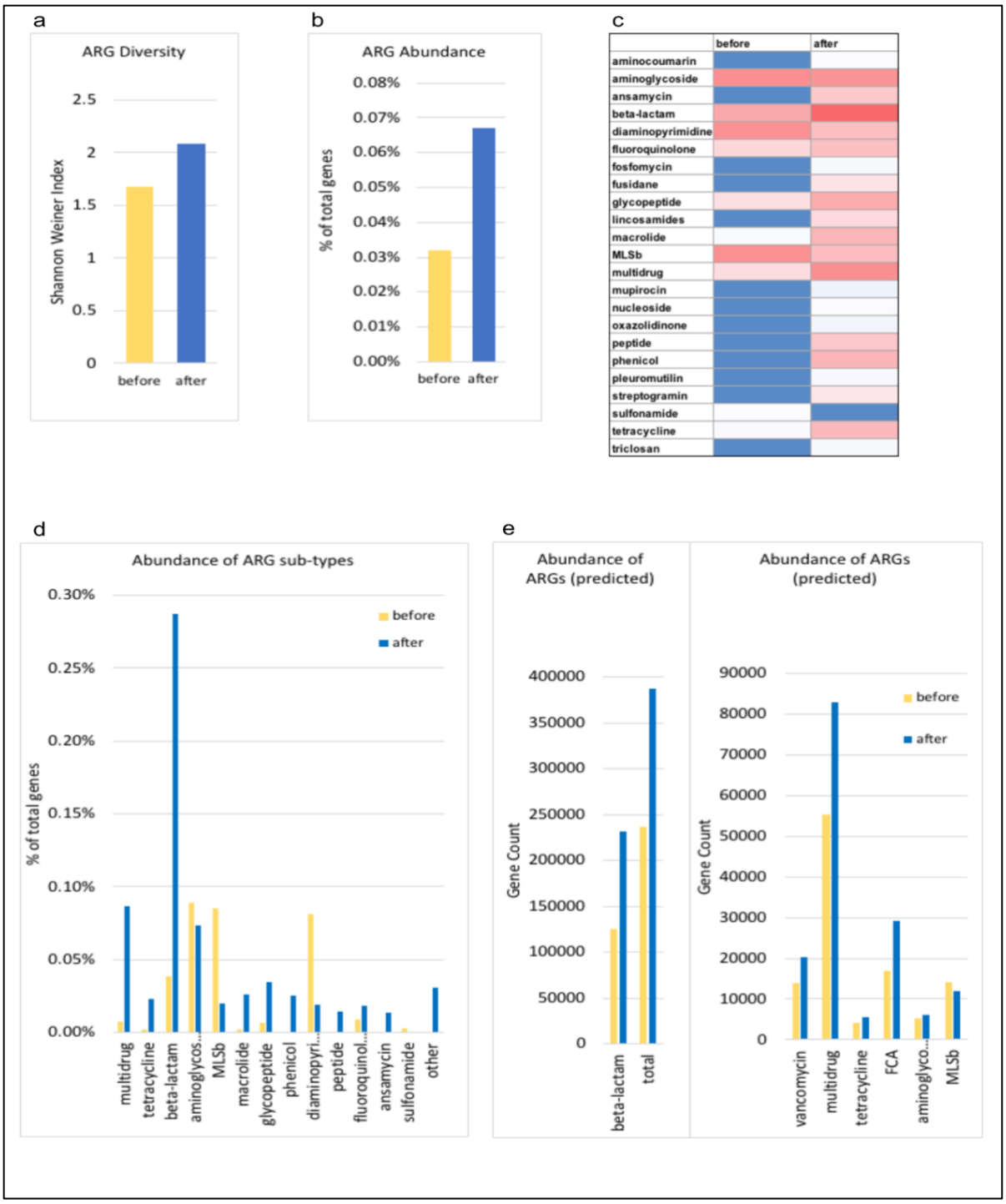


Figure 3.6. Occurrence, abundance and diversity of ARGs. Results from metagenomic sequencing comparing ARGs present in human skin microbiome before and after swimming in the ocean: ARG diversity (a), ARG abundance (b), heatmap comparison of abundance of ARGs where blue is "not detected" and intensity of red color indicates increased abundance (c), and abundance of ARG sub-types (d). For comparison, results from the predicted ARG profiles (PICRUSt) are shown in (e).

Discussion

Study Contribution and Future Research

This is the first known study to investigate the acquisition of ARGs and ABSGs onto human skin after ocean water exposure. Previous studies have shown the presence of antibiotics and ARGs in marine environments and have acknowledged the potential risk of transmission but have not demonstrated deposition or persistence on the skin after exposure [17,129,174,175]. This study reveals that the human skin acquired exogenous ARGs from ocean water and these genes persisted for at least 6 hours post-swim, which may increase the risk of developing antibiotic resistant infections.

It is well-known that horizontal gene transfer (HGT) spreads antibiotic resistance from the environment, yet the dynamics of this process have not been well-characterized [176]. Previous research has shown that ARGs can be acquired through HGT from exposure to antibiotic resistant bacteria in animals and agricultural environments [177,178]. This phenomenon has been well-studied in hospital environments and clinical settings as well [179–181]. While HGT has been shown to occur more often in closely related organisms [182], research suggests that transfer does occur in unrelated organisms including prokaryote to eukaryote transfer [183]. More research is needed to quantify the distribution and acquisition of ARGs through HGT from the natural environment.

Our previous research has shown that exogenous bacteria remain on human skin for at least 24 hours post swim [92]. This study has shown that foreign ARGs, most likely contained in the genomes of exogenous bacteria, increase on human skin after swimming in the ocean. During this time frame, HGT of ARGs to resident organisms may not occur, although this was not addressed by this study. However, the 6 hour time points demonstrated an increase in ARGs for

most of the sub-types and the human skin harbored a significant amount of ocean bacteria, while at the same time normal skin commensals were beginning to predominate once again [92]. This may be one of the reasons for the increase in ARGs seen in most of the sub-types at 6 hours post-swim and may give the bacteria opportunity to exchange genetic information.

Another important aspect of the results indicates that ARGs are not necessarily only associated with polluted water; they are naturally prevalent in waters that are open for public recreation. Since almost all ARGs have a proven environmental origin, even though they are influenced by anthropogenic factors, it is difficult to tell whether these results would change depending on the water quality. Presumably, more contaminated marine environments would contain more antibiotics and ARGs since wastewater treatment plants and storm water run-off are known reservoirs [135,184,185]. Although metagenomic sequencing was not performed on the ocean water in this study, the ARGs detected on the skin after swimming and the PICRUSt predicted ARGs in the ocean water samples paralleled results from previous studies on antibiotics and ARGs present in marine environments [174,186].

Comparing results: PICRUSt vs metagenomic sequencing.

There was an overall increase in ARGs in the human skin microbiome after swimming which was evident in the results from both methods: predictions based on PICRUSt and metagenomic sequencing. Moreover, both methods also revealed similarities in the changes in the sub-types of ARGs present on the skin. One minor difference was found for sulfonamide resistance genes. These decreased in the after samples according to metagenomic analysis; however, this was not specifically addressed in the PICRUSt predictions.

Even though additional metagenomic sequencing may have strengthened the study results, PICRUSt is a proven and valid approach to predictive gene profiling [135,187]. In the absence of resources for metagenomic sequencing capability, PICRUSt is a powerful tool for investigating microbial ecology. In fact, PICRUSt may have underestimated the abundance and diversity of ARGs on the skin demonstrating a conservative prediction of the true changes seen in the metagenomic sequencing results. For example, beta-lactam genes post-swim nearly doubled according to PICRUSt but increased about 7 fold according to the metagenomic data. This study shows strong evidence indicating that there was a significant change in ARGs present on the human skin before and after swimming in the ocean based on both the PICRUSt and metagenomic sequencing methodologies.

Study limitations

Even though a total of 65 samples were analyzed in this study, this only included samples collected from 12 individuals. Due to strict exclusion criteria designed to limit confounding factors and other variables, some individuals were rejected from participation in the study. Therefore, conclusions from the study population may not apply to the general population. However, several highly impactful human skin microbiome studies have utilized small sample numbers [106,121,122]. In our study, each participant served as their own control which allowed for a more accurate analysis of the changes after swimming. This helped to control for individual characteristics which may have influenced the baseline skin microbiome and demonstrated that regardless of the initial ARG profile, ARGs increased in all participants after swimming.

This study only analyzed the microbiome on the calf because this site is large, flat and easily sampled. This site maintained contact with the water without requiring participants to

submerse themselves. Since body sites vary considerably in their microbial community compositions, more ARGs may be detected on body sites that have the ability to trap water (inside the ears and nasal cavity), and/or maintain contact with the ocean sediment (toes). In addition, since HGT is more likely to occur in closely related organisms, the potential for ARG acquisition on body sites with different microbial composition was not addressed in this study but warrants further investigation.

Summary

The environment is a reservoir of ARGs both naturally occurring and anthropogenically selected. It is well-documented that environmental exposures have the capacity to alter the human microbiome [92,188][189][190]. Wastewater, storm-water, hospitals, aquaculture and animal agriculture discharge, contribute to an increase in antibiotics and antibiotic resistant bacteria present in the environment. This presents additional risk for the acquisition of antibiotic resistant infections to those exposed to recreational water [128,130]. Our study demonstrated that exposure to ocean water deposited exogenous ARGs onto our skin and that these changes were detectable for 24 hours post-swim. While it appeared that ARGs gene counts returned to a baseline level, more research is needed to determine if commensals incorporate these exogenous ARGs and the rate of occurrence. Antibiotic resistance in the clinical setting and the occurrence of ARGs in the environment is increasing. It is imperative that we elucidate the role of environmental resistomes in the distribution of ARGs into the human microbiome if we are to continue using antibiotics to treat infectious diseases.

CONCLUSIONS

Climate change, population growth, rapid urbanization, and contaminated water run-off all contribute to the quality of natural waters. Toxic algal blooms pose a significant threat to human health and an increase in pathogenic bacteria, antibiotics and antibiotic resistant bacteria present in the environment increases the risk for acquisition of antibiotic resistant infections to those exposed to recreational water [128,130].

The disparity between the human health effects described in epidemiology case studies and toxicological dermal exposure data may be explained by skin penetration of algal toxins. Based on the physiochemical properties of the toxins and transdermal drug research models, it was predicted that algal toxins may have the potential to penetrate human skin. Since recreational water exposure often involves total body submersion and mucous membrane exposure for extended periods of time, this risk cannot be overlooked.

Alterations of the human skin microbiome have been linked to skin diseases but the impact of recreational ocean water exposure on the human skin microbiome has not been previously studied. This research provides information towards an understanding of the relationship between recreational water exposure, the skin microbiome, and potential skin infection. Ocean water exposure removed normal resident bacteria from human skin, which have been shown to modulate the immune system and provide protection against invading pathogens. Therefore, the removal of these symbiotic organisms could leave the host susceptible to infection. Ocean water exposure simultaneously deposited ocean-borne bacteria onto the skin, including potential pathogens that could cause infection. While the normal skin microflora re-established dominance as time elapsed post-exposure, exogenous bacteria was present on the skin for at least 24 hours after swimming.

Additionally, this research demonstrated that exposure to ocean water deposited exogenous genes onto our skin and that these changes were detectable for at least 6 hours post-swim. While it appeared that gene counts returned to a baseline level, more research is needed to determine if commensals incorporate these exogenous genes into their genomes. Antibiotic resistance in the clinical setting and the occurrence of ARGs in the environment are increasing. While it appears that the microbiome returns to baseline over time, more research is necessary to determine if genomic changes persist. Once ARGs are incorporated into the microbiome, the risks associated with increased antibiotic resistance may be long-lasting. We need to minimize the distribution of ARGs into the human microbiome if we are to continue using antibiotics to treat infectious diseases.

It is imperative that we continue to investigate the effects of recreational water exposure on human health. Cyanotoxin exposure may not be limited to the oral route as we demonstrated the potential for dermal penetration. Increased infection risk may result from incorporation of ARGs and changes in diversity and abundance of bacterial communities in the skin microbiome, and these changes may be long-term. This research demonstrated the importance of the skin's protective functions during recreational water exposure and highlighted that significant changes occurred and persisted post-exposure.

APPENDIX A. Supplemental material from Chapter 2

Table A.1. Summary of alpha diversity metrics.

Sample	Participant	Time	Avg Observed OTUs	Avg Chao 1	Shannon Index	Simpson Index
1B	1	before	1177.6	2310.00	6.297	0.961
1A	1	after	1495.9	3053.40	7.495	0.976
16	1	6 hours	1208.3	2725.21	7.253	0.978
124	1	24 hours	778.3	1083.32	5.660	0.955
2B	2	before	841	1318.36	4.953	0.856
2A	2	after	1352.7	2973.41	7.672	0.988
26	2	6 hours	486.7	736.47	5.188	0.953
224	2	24 hours	849.4	1342.93	5.539	0.940
3B	3	before	1172.1	2276.01	5.648	0.856
3A	3	after	1543.1	3489.04	7.792	0.983
4B	4	before	1120.6	1625.54	7.232	0.982
4A	4	after	1288.6	2686.58	7.355	0.976
5B	5	before	1120.9	2089.82	6.384	0.942
5A	5	after	1943	3488.35	8.038	0.983
56	5	6 hours	943.9	1955.86	6.515	0.975
524	5	24 hours	811.2	1510.60	5.372	0.934
6B	6	before	N/A	N/A	4.486	0.835
6A	6	after	2000.3	3681.70	8.003	0.981
66	6	6 hours	776.6	1429.66	5.349	0.928
624	6	24 hours	803.1	1448.33	5.617	0.936
7B	7	before	285.2	491.58	2.094	0.472
7A	7	after	1319.4	2695.97	7.382	0.982
76	7	6 hours	1394.7	2856.87	7.387	0.977
724	7	24 hours	1380.1	2705.80	6.975	0.966
8B	8	before	697.6	1286.40	5.412	0.956
8A	8	after	1182.7	2417.94	7.429	0.983
824	8	24 hours	850.9	1408.42	5.600	0.943
9B	9	before	765.1	1294.85	5.456	0.934
9A	9	after	997.8	1956.09	5.973	0.948
924	9	24 hours	351.5	510.80	3.897	0.888
Ocean	N/A	N/A	1292.3	2352.78	7.096	0.978

Table A.2. The most abundant phyla expressed as the percentage of the total OTUs for each sample.

	Firmicutes	Proteobacteria	Actinobacteria	Bacteroidetes
1B	53.60%	20.27%	18.49%	7.41%
1A	7.23%	17.31%	4.41%	51.83%
16	13.85%	16.94%	4.69%	47.89%
124	18.41%	38.51%	16.11%	15.15%
2B	48.29%	22.76%	25.05%	1.76%
2A	8.61%	34.49%	2.83%	39.98%
26	15.94%	47.88%	16.36%	13.11%
224	31.12%	31.28%	24.05%	1.14%
5B	43.88%	23.23%	16.23%	13.36%
5A	3.52%	22.55%	4.46%	49.81%
56	8.67%	16.82%	14.33%	44.29%
524	13.38%	27.96%	24.58%	33.71%
Ocean	0.70%	62.94%	7.45%	17.18%

Table A.3. Bacteria detected in ocean water and after swimming samples but not detected on the before samples. Expressed as the percentage of total OTUs.

OTU Identification (genus)	Before (%)	Ocean Water (%)	After (%)	6 hours (%)	24 hours (%)
Pelagibacteraceae (unknown genus)	0.000000	4.309240	0.284843	0.081181	0.004716
Sediminicola	0.000000	2.718191	0.111975	0.033661	0.000214
Pseudoalteromonas	0.000000	2.053771	0.075160	0.000000	0.008360

REFERENCES

1. Lane ME. Skin penetration enhancers. Int J Pharm [Internet]. Elsevier; 2013 [cited 2019 Feb 16];447:12–21. Available from: https://www.sciencedirect.com/science/article/pii/S037851731300184 1?via%3Dihub

2. McGrath JA, Uitto J. Anatomy and Organization of Human Skin. Rook's Textb Dermatology Eighth Ed. Wiley-Blackwell; 2010. p. 34–86.

3. Grice EA, Kong HH, Renaud G, Young AC, NISC Comparative Sequencing Program GG, Bouffard GG, et al. A diversity profile of the human skin microbiota. Genome Res [Internet]. Cold Spring Harbor Laboratory Press; 2008 [cited 2018 Dec 6];18:1043–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18502944

4. National Survey on Recreation and the Environment (NSRE) 2000–2002. The Interagency National Survey Consortium Coordinated by the USDA Forest Service Recreation Wilderness and Demographics Trends Research Group and the University of Tennessee Human Dimens. 2004.

5. Oja P, Titze S, Kokko S, Kujala UM, Heinonen A, Kelly P, et al. Health benefits of different sport disciplines for adults: systematic review of observational and intervention studies with meta-analysis. Br J Sports Med [Internet]. 2015 [cited 2018 Dec 6];49:434–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25568330

6. Paerl HW, Paul VJ. Climate change: Links to global expansion of harmful cyanobacteria. Water Res [Internet]. 2012 [cited 2019 Oct 11];46:1349–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21893330

7. Paerl HW, Huisman J. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microbiol Rep [Internet]. 2009 [cited 2019 Oct 11];1:27–37. Available from: http://doi.wiley.com/10.1111/j.1758-2229.2008.00004.x

8. Cheung MY, Liang S, Lee J. Toxin-producing cyanobacteria in freshwater: a review of the problems, impact on drinking water safety, and efforts for protecting public health. J Microbiol [Internet]. 2013 [cited 2019 Oct 11];51:1–10. Available from: http://link.springer.com/10.1007/s12275-013-2549-3

9. Michalak AM, Anderson EJ, Beletsky D, Boland S, Bosch NS, Bridgeman TB, et al. Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. Proc Natl Acad Sci U S A [Internet]. 2013 [cited 2019 Oct 11];110:6448–52. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1216006110

10. Butler N, Carlisle J, Linville R. Toxicological summary and suggested action levels to reduce potential adverse health effects of six cyanotoxins. Off Environ Heal Hazard Assessment, Calif Environ Prot Agency, Sacramento. 2012;

11. Arnold BF, Schiff KC, Ercumen A, Benjamin-Chung J, Steele JA, Griffith JF, et al. Acute Illness Among Surfers After Exposure to Seawater in Dry- and Wet-Weather Conditions. Am J Epidemiol [Internet]. 2017 [cited 2018 Dec 6];186:866–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28498895

12. Yu S. Uncovering the geographical and host impacts on the classification of *Vibrio*

vulnificus. Evol Appl [Internet]. Wiley/Blackwell (10.1111); 2018 [cited 2018 Dec 6];11:883–90. Available from: http://doi.wiley.com/10.1111/eva.12602

13. Sridhar SCJ, Deo SCR. Marine and Other Aquatic Dermatoses. Indian J Dermatol [Internet]. Wolters Kluwer -- Medknow Publications; 2017 [cited 2018 Dec 6];62:66–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28216728

14. Hannigan GD, Grice EA. Microbial ecology of the skin in the era of metagenomics and molecular microbiology. Cold Spring Harb Perspect Med [Internet]. Cold Spring Harbor Laboratory Press; 2013 [cited 2019 Mar 24];3:a015362. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24296350

15. Naik S, Bouladoux N, Linehan JL, Han S-J, Harrison OJ, Wilhelm C, et al. Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. Nature [Internet]. 2015 [cited 2018 Dec 6];520:104–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25539086

16. Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, et al. Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. Sci Transl Med [Internet]. NIH Public Access; 2017 [cited 2019 Mar 28];9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28228596

17. Martínez JL. Antibiotics and antibiotic resistance genes in natural environments. Science [Internet]. American Association for the Advancement of Science; 2008 [cited 2019 Dec 1];321:365–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18635792

18. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science (80-) [Internet]. 2012 [cited 2019 Dec 2];337:1107–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22936781

19. Riesenfeld CS, Goodman RM, Handelsman J. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol [Internet]. 2004 [cited 2019 Dec 2];6:981–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15305923

20. Segawa T, Takeuchi N, Rivera A, Yamada A, Yoshimura Y, Barcaza G, et al. Distribution of antibiotic resistance genes in glacier environments. Environ Microbiol Rep [Internet]. 2013 [cited 2019 Dec 2];5:127–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23757141

21. Hatosy SM, Martiny AC. The ocean as a global reservoir of antibiotic resistance genes. Appl Environ Microbiol [Internet]. American Society for Microbiology (ASM); 2015 [cited 2019 Dec 1];81:7593–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26296734

22. Buratti FM, Manganelli M, Vichi S, Stefanelli M, Scardala S, Testai E, et al. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch Toxicol [Internet]. 2017 [cited 2019 Oct 11];91:1049–130. Available from: http://link.springer.com/10.1007/s00204-016-1913-6

23. de la Cruz AA, Hiskia A, Kaloudis T, Chernoff N, Hill D, Antoniou MG, et al. A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. Environ Sci Process Impacts [Internet]. 2013 [cited 2019 Oct 14];15:1979. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24056894

24. Paerl HW, Paul VJ. Climate change: Links to global expansion of harmful cyanobacteria. Water Res [Internet]. 2012 [cited 2019 Oct 11];46:1349–63. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0043135411004386

25. Codd GA, Lindsay J, Young FM, Morrison LF, Metcalf JS. Harmful Cyanobacteria. Harmful Cyanobacteria [Internet]. Berlin/Heidelberg: Springer-Verlag; [cited 2019 Oct 11]. p. 1–23. Available from: http://link.springer.com/10.1007/1-4020-3022-3_1

26. Lehman PW, Marr K, Boyer GL, Acuna S, Teh SJ. Long-term trends and causal factors associated with Microcystis abundance and toxicity in San Francisco Estuary and implications for climate change impacts. Hydrobiologia [Internet]. Springer Netherlands; 2013 [cited 2019 Oct 11];718:141–58. Available from: http://link.springer.com/10.1007/s10750-013-1612-8

27. Ho L, Sawade E, Newcombe G. Biological treatment options for cyanobacteria metabolite removal – A review. Water Res [Internet]. 2012 [cited 2019 Oct 11];46:1536–48. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0043135411006907

28. Loftin KA, Graham JL, Hilborn ED, Lehmann SC, Meyer MT, Dietze JE, et al. Cyanotoxins in inland lakes of the United States: Occurrence and potential recreational health risks in the EPA National Lakes Assessment 2007. Harmful Algae [Internet]. 2016 [cited 2019 Oct 11];56:77–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28073498

29. Lopez CB, Jewett EB, Dortch Q, Walton BT, Hudnell HK. Scientific assessment of freshwater harmful algal blooms. Interagency Working Group on Harmful Algal Blooms, Hypoxia, and Human Health of the Joint Subcommittee on Ocean Science and Technology; 2008 [cited 2019 Oct 11]; Available from: http://aquaticcommons.org/14921/

30. Funari E, Testai E. Human Health Risk Assessment Related to Cyanotoxins Exposure. Crit Rev Toxicol [Internet]. 2008 [cited 2019 Oct 11];38:97–125. Available from: http://www.tandfonline.com/doi/full/10.1080/10408440701749454

31. Edwards C, Lawton LA. Chapter 4 Bioremediation of Cyanotoxins. 2009 [cited 2019 Oct 11]. p. 109–29. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0065216408010046

32. Cheung MY, Liang S, Lee J. Toxin-producing cyanobacteria in freshwater: A review of the problems, impact on drinking water safety, and efforts for protecting public health. J Microbiol [Internet]. 2013 [cited 2019 Oct 11];51:1–10. Available from: http://link.springer.com/10.1007/s12275-013-2549-3

33. Ueno Y, Nagata S, Tsutsumi T, Hasegawa A, Watanabe MF, Park HD, et al. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis [Internet]. 1996 [cited 2019 Oct 11];17:1317–21. Available from: https://academic.oup.com/carcin/article-lookup/doi/10.1093/carcin/17.6.1317

34. Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, et al. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol [Internet]. 1992 [cited 2019 Oct 11];118:420–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1618889

35. Campos A, Vasconcelos V. Molecular Mechanisms of Microcystin Toxicity in Animal Cells. Int J Mol Sci [Internet]. 2010 [cited 2019 Oct 11];11:268–87. Available from: http://www.mdpi.com/1422-0067/11/1/268

36. Falconer IR. Algal toxins in seafood and drinking water. Academic Press; 1993.

37. Codd GA (Geoffrey A., International Symposium on Detection Methods for Cyanobacterial (Blue-Green Algal) Toxins (1st : 1993 : University of Bath). Detection methods for cyanobacterial toxins. Royal Society of Chemistry; 1994.

38. Chen Y, Shen D, Fang D. Nodularins in poisoning. Clin Chim Acta [Internet]. 2013 [cited 2019 Oct 11];425:18–29. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0009898113002738

39. Meili N, Christen V, Fent K. Nodularin induces tumor necrosis factor-alpha and mitogen-activated protein kinases (MAPK) and leads to induction of endoplasmic reticulum stress. Toxicol Appl Pharmacol [Internet]. 2016 [cited 2019 Oct 11];300:25–33. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0041008X16300667

40. Christen V, Meili N, Fent K. Microcystin-LR induces endoplasmatic reticulum stress and leads to induction of NFκB, interferon-alpha, and tumor necrosis factor-alpha. Environ Sci Technol [Internet]. 2013 [cited 2019 Oct 11];47:3378–85. Available from: https://pubs.acs.org/doi/10.1021/es304886y

41. Campos F, Durán R, Vidal L, Faro LRF, Alfonso M. In vivo Effects of the Anatoxin-a on Striatal Dopamine Release. Neurochem Res [Internet]. 2006 [cited 2019 Oct 11];31:491–501. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16758357

42. Osswald J, Rellán S, Gago A, Vasconcelos V. Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. Environ Int [Internet]. 2007 [cited 2019 Oct 11];33:1070–89. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17673293

43. Testai E, Scardala S, Vichi S, Buratti FM, Funari E. Risk to human health associated with the environmental occurrence of cyanobacterial neurotoxic alkaloids anatoxins and saxitoxins. Crit Rev Toxicol [Internet]. Taylor & Francis; 2016 [cited 2019 Apr 4];46:385–419. Available from: http://www.tandfonline.com/doi/full/10.3109/10408444.2015.1137865

44. Trainer VL, Hardy FJ. Integrative monitoring of marine and freshwater harmful algae in Washington State for public health protection. Toxins (Basel) [Internet]. 2015 [cited 2019 Oct 14];7:1206–34. Available from: http://www.mdpi.com/2072-6651/7/4/1206

45. O'Neill K, Musgrave IF, Humpage A. Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells. Basic Clin Pharmacol Toxicol [Internet]. 2017 [cited 2019 Oct 11];120:390–7. Available from: http://doi.wiley.com/10.1111/bcpt.12701

46. Jones GJ, Negri AP. Persistence and degradation of cyanobacterial paralytic shellfish poisons (PSPs) in freshwaters. Water Res [Internet]. Pergamon; 1997 [cited 2019 Oct 14];31:525–33. Available from: https://www.sciencedirect.com/science/article/pii/S0043135496001340

47. Froscio SM, Humpage AR, Burcham PC, Falconer IR. Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin. Environ Toxicol [Internet]. 2001 [cited 2019 Oct 14];16:408–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11594027

48. Runnegar MT, Xie C, Snider BB, Wallace GA, Weinreb SM, Kuhlenkamp J. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol Sci [Internet]. 2002 [cited 2019 Oct 14];67:81–7. Available from: https://academic.oup.com/toxsci/article-lookup/doi/10.1093/toxsci/67.1.81

49. Kiss T, Vehovszky A, Hiripi L, Kovács A, Vörös L. Membrane effects of toxins isolated from a cyanobacterium, Cylindrospermopsis raciborskii, on identified molluscan neurones. Comp Biochem Physiol C Toxicol Pharmacol [Internet]. 2002 [cited 2019 Oct 14];131:167–76. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11879784

50. Pichardo S, Cameán AM, Jos A. In Vitro Toxicological Assessment of Cylindrospermopsin: A Review. Toxins (Basel) [Internet]. 2017 [cited 2019 Oct 14];9:402. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29258177

51. Chong MWK, Wong BSF, Lam PKS, Shaw GR, Seawright AA. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon [Internet]. 2002 [cited 2019 Oct 14];40:205–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11689242

52. Torokne A, Palovics A, Bankine M. Allergenic (sensitization, skin and eye irritation) effects of freshwater cyanobacteria--experimental evidence. Environ Toxicol [Internet]. 2001 [cited 2019 Oct 14];16:512–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11769249

53. Stewart I, Seawright AA, Schluter PJ, Shaw GR. Primary irritant and delayed-contact hypersensitivity reactions to the freshwater cyanobacterium Cylindrospermopsis raciborskii and its associated toxin cylindrospermopsin. BMC Dermatol [Internet]. 2006 [cited 2019 Oct 11];6:5. Available from: http://bmcdermatol.biomedcentral.com/articles/10.1186/1471-5945-6-5

54. Bernstein JA, Ghosh D, Levin LS, Zheng S, Carmichael W, Lummus Z, et al. Cyanobacteria: an unrecognized ubiquitous sensitizing allergen? Allergy asthma Proc [Internet]. 2011 [cited 2019 Oct 11];32:106–10. Available from: http://www.ingentaconnect.com/content/10.2500/aap.2011.32.3434

55. Rücker J, Stüken A, Nixdorf B, Fastner J, Chorus I, Wiedner C. Concentrations of particulate and dissolved cylindrospermopsin in 21 Aphanizomenon-dominated temperate lakes. Toxicon [Internet]. 2007 [cited 2019 Apr 9];50:800–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S004101010700222X

56. Runnegar MT, Kong SM, Zhong YZ, Lu SC. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Pharmacol [Internet]. 1995 [cited 2019 Oct 14];49:219–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7840799

57. Froscio SM, Humpage AR, Burcham PC, Falconer IR. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ Toxicol [Internet]. 2003 [cited 2019 Oct 14];18:243–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12900943

58. Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar Drugs [Internet]. 2010 [cited 2019 Oct 14];8:1650–80. Available from: http://www.mdpi.com/1660-3397/8/5/1650

59. Falconer IR. Health effects associated with controlled exposures to cyanobacterial toxins. Cyanobacterial Harmful Algal Bloom State Sci Res Needs [Internet]. New York, NY: Springer New York; 2008 [cited 2019 Oct 14]. p. 607–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18461785

60. Wormer L, Cirés S, Carrasco D, Quesada A. Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study. Harmful Algae [Internet]. 2008 [cited 2019 Oct 14];7:206–13. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1568988307001072

61. Kozdęba M, Borowczyk J, Zimoląg E, Wasylewski M, Dziga D, Madeja Z, et al. Microcystin-LR affects properties of human epidermal skin cells crucial for regenerative processes. Toxicon [Internet]. 2014 [cited 2019 Oct 11];80:38–46. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0041010114000087

62. Pilotto L, Hobson P, Burch MD, Ranmuthugala G, Attewell R, Weightman W. Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers. Aust N Z J Public Health [Internet]. 2004 [cited 2019 Oct 11];28:220–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15707167

63. Stewart I, Robertson IM, Webb PM, Schluter PJ, Shaw GR. Cutaneous hypersensitivity reactions to freshwater cyanobacteria--human volunteer studies. BMC Dermatol [Internet]. 2006 [cited 2019 Oct 11];6:6. Available from: http://bmcdermatol.biomedcentral.com/articles/10.1186/1471-5945-6-6

64. Trevino-Garrison I, DeMent J, Ahmed F, Haines-Lieber P, Langer T, Ménager H, et al. Human Illnesses and Animal Deaths Associated with Freshwater Harmful Algal Blooms—Kansas. Toxins (Basel) [Internet]. 2015 [cited 2019 Oct 11];7:353–66. Available from: http://www.mdpi.com/2072-6651/7/2/353

65. Turner PC, Gammie AJ, Hollinrake K, Codd GA. Pneumonia associated with contact with cyanobacteria. BMJ [Internet]. BMJ Publishing Group; 1990 [cited 2019 Oct 17];300:1440–1. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2116198

66. Testai E, Scardala S, Vichi S, Buratti FM, Funari E. Risk to human health associated with the environmental occurrence of cyanobacterial neurotoxic alkaloids anatoxins and saxitoxins. Crit Rev Toxicol [Internet]. 2016 [cited 2019 Oct 11];46:385–419. Available from: http://www.tandfonline.com/doi/full/10.3109/10408444.2015.1137865

67. Yoshida T, Makita Y, Nagata S, Tsutsumi T, Yoshida F, Sekijima M, et al. Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin, in mice. Nat Toxins [Internet]. John Wiley & Sons, Ltd; 1998 [cited 2019 Oct 17];5:91–5. Available from: http://doi.wiley.com/10.1002/1522-7189%281997%295%3A3%3C91%3A%3AAID-NT1%3E3.0.CO%3B2-H

68. Ito E, Kondo F, Terao K, Harada K-I. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. Toxicon [Internet]. Pergamon; 1997 [cited 2019 Oct 17];35:1453–7. Available from: https://www.sciencedirect.com/science/article/pii/S0041010197000263

69. Fawell JK, Mitchell RE, Everett DJ, Hill RE. The toxicity of cyanobacterial toxins in the mouse: I Microcystin-LR. Hum Exp Toxicol [Internet]. Sage PublicationsSage CA: Thousand Oaks, CA; 1999 [cited 2019 Oct 17];18:162–7. Available from: http://journals.sagepub.com/doi/10.1177/096032719901800305

70. Wood R. Acute animal and human poisonings from cyanotoxin exposure - A review of the literature. Environ Int [Internet]. 2016 [cited 2019 Oct 11];91:276–82. Available from:

https://linkinghub.elsevier.com/retrieve/pii/S0160412016300642

71. Weirich CA, Miller TR. Freshwater Harmful Algal Blooms: Toxins and Children's Health. Curr Probl Pediatr Adolesc Health Care [Internet]. 2014 [cited 2019 Oct 11];44:2–24. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1538544213001211

72. Heise HA. Symptoms of hay fever caused by algae. J Allergy [Internet]. 1949 [cited 2019 Oct 17];20:383–5. Available from: https://linkinghub.elsevier.com/retrieve/pii/0021870749900295

73. Billings WH. Water-Associated Human Illness in Northeast Pennsylvania and its Suspected Association with Blue-Green Algae Blooms. Water Environ [Internet]. Boston, MA: Springer US; 1981 [cited 2019 Oct 17]. p. 243–55. Available from: http://link.springer.com/10.1007/978-1-4613-3267-1_18

74. Giannuzzi L, Sedan D, Echenique R, Andrinolo D. An Acute Case of Intoxication with Cyanobacteria and Cyanotoxins in Recreational Water in Salto Grande Dam, Argentina. Mar Drugs [Internet]. 2011 [cited 2019 Oct 11];9:2164–75. Available from: http://www.mdpi.com/1660-3397/9/11/2164

75. Backer LC, McNeel S V., Barber T, Kirkpatrick B, Williams C, Irvin M, et al. Recreational exposure to microcystins during algal blooms in two California lakes. Toxicon [Internet]. 2010 [cited 2019 Oct 11];55:909–21. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0041010109003481

76. Rapala J, Robertson A, Negri AP, Berg KA, Tuomi P, Lyra C, et al. First report of saxitoxin in Finnish lakes and possible associated effects on human health. Environ Toxicol [Internet]. 2005 [cited 2019 Oct 21];20:331–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15892061

77. Idson B. Percutaneous Absorption. J Pharm Sci [Internet]. John Wiley & Sons, Ltd; 1975 [cited 2019 Oct 21];64:901–24. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022354915402370

78. Nielsen JB, Benfeldt E, Holmgaard R. Penetration through the Skin Barrier. Curr Probl Dermatol [Internet]. Karger Publishers; 2016 [cited 2019 Feb 5]. p. 103–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26844902

79. Bos JD, Meinardi MMHM. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp Dermatol [Internet]. John Wiley & Sons, Ltd (10.1111); 2000 [cited 2019 Apr 29];9:165–9. Available from: http://doi.wiley.com/10.1034/j.1600-0625.2000.009003165.x

80. N'Da D, N'Da, D. D. Prodrug Strategies for Enhancing the Percutaneous Absorption of Drugs. Molecules [Internet]. Multidisciplinary Digital Publishing Institute; 2014 [cited 2019 Apr 22];19:20780–807. Available from: http://www.mdpi.com/1420-3049/19/12/20780

81. Tiejun Cheng, Yuan Zhao, Xun Li, Fu Lin, Yong Xu, Xinglong Zhang, et al. Computation of Octanol−Water Partition Coefficients by Guiding an Additive Model with Knowledge. American Chemical Society ; 2007 [cited 2019 Apr 26]; Available from: https://pubs.acs.org/doi/full/10.1021/ci700257y?src=recsys

82. Chandrashekar NS, Shobha Rani RH. Physicochemical and pharmacokinetic parameters in

drug selection and loading for transdermal drug delivery. Indian J Pharm Sci [Internet]. Wolters Kluwer -- Medknow Publications; 2008 [cited 2019 Apr 26];70:94–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20390089

83. Nielsen JB, Nielsen F, Sørensen JA. Defense against dermal exposures is only skin deep: significantly increased penetration through slightly damaged skin. Arch Dermatol Res [Internet]. Springer-Verlag; 2007 [cited 2019 Feb 7];299:423–31. Available from: http://link.springer.com/10.1007/s00403-007-0788-z

84. Li, Chan. Transport, metabolism and elimination mechanisms of anti-HIV agents. Adv Drug Deliv Rev [Internet]. 1999 [cited 2019 Apr 22];39:81–103. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10837769

85. Feldmann RJ, Maibach HI. Regional Variation in Percutaneous Penetration of 14C Cortisol in Man**From the Division of Dermatology, Department of Medicine, University of California School of Medicine, San Francisco, California 94122. J Invest Dermatol [Internet]. 1967 [cited 2019 Oct 21];48:181–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6020682

86. Potts RO, Buras EM, Chrisman DA. Changes with age in the moisture content of human skin. J Invest Dermatol [Internet]. 1984 [cited 2019 Apr 22];82:97–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6690633

87. Frantz SW, Ballantyne B, Beskitt JL, Tallant MJ, Greco RJ. Pharmacokinetics of 2-ethyl-1,3-hexanediol. III. In vitro skin penetration comparisons using the excised skin of humans, rats, and rabbits. Fundam Appl Toxicol [Internet]. 1995 [cited 2019 Apr 29];28:1–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8566473

88. Munday R, Reeve J. Risk Assessment of Shellfish Toxins. Toxins (Basel) [Internet]. 2013 [cited 2019 Oct 11];5:2109–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24226039

89. Koppel N, Maini Rekdal V, Balskus EP. Chemical transformation of xenobiotics by the human gut microbiota. Science (80-) [Internet]. 2017 [cited 2019 Nov 27];356:eaag2770. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28642381

90. Noh K, Kang YR, Nepal MR, Shakya R, Kang MJ, Kang W, et al. Impact of gut microbiota on drug metabolism: an update for safe and effective use of drugs. Arch Pharm Res [Internet]. 2017 [cited 2019 Nov 27];40:1345–55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29181640

91. Clarke G, Sandhu K V., Griffin BT, Dinan TG, Cryan JF, Hyland NP. Gut Reactions: Breaking Down Xenobiotic–Microbiome Interactions. France CP, editor. Pharmacol Rev [Internet]. 2019 [cited 2019 Nov 27];71:198–224. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30890566

92. Nielsen MC, Jiang SC. Alterations of the human skin microbiome after ocean water exposure. Mar Pollut Bull. 2019;145.

93. Howard M, Nagoda C, Kudela R, Hayashi K, Tatters A, Caron D, et al. Microcystin Prevalence throughout Lentic Waterbodies in Coastal Southern California. Toxins (Basel) [Internet]. 2017 [cited 2019 Oct 11];9:231. Available from: http://www.mdpi.com/2072-6651/9/7/231

94. McPartlin DA, Loftus JH, Crawley AS, Silke J, Murphy CS, O'Kennedy RJ. Biosensors for

the monitoring of harmful algal blooms. Curr Opin Biotechnol [Internet]. 2017 [cited 2019 Oct 11];45:164–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28427011

95. Collier SA, Wade TJ, Sams EA, Hlavsa MC, Dufour AP, Beach MJ. Swimming in the USA: beachgoer characteristics and health outcomes at US marine and freshwater beaches. J Water Health [Internet]. NIH Public Access; 2015 [cited 2018 Dec 6];13:531–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26042984

96. Dewailly E, Poirier C, Meyer FM. Health hazards associated with windsurfing on polluted water. Am J Public Health [Internet]. American Public Health Association; 1986 [cited 2018 Dec 6];76:690–1. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3706597

97. Hlavsa MC, Roberts VA, Kahler AM, Hilborn ED, Wade TJ, Backer LC, et al. Recreational water-associated disease outbreaks--United States, 2009-2010. MMWR Morb Mortal Wkly Rep [Internet]. 2014 [cited 2018 Dec 6];63:6–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24402466

98. Cao Y, Raith M, Smith P, Griffith J, Weisberg S, Schriewer A, et al. Regional Assessment of Human Fecal Contamination in Southern California Coastal Drainages. Int J Environ Res Public Health [Internet]. Multidisciplinary Digital Publishing Institute; 2017 [cited 2018 Dec 6];14:874. Available from: http://www.mdpi.com/1660-4601/14/8/874

99. Bisharat N, Cohen DI, Harding RM, Falush D, Crook DW, Peto T, et al. Hybrid *Vibrio vulnificus*. Emerg Infect Dis [Internet]. 2005 [cited 2018 Dec 6];11:30–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15705319

100. Dickinson G, Lim K, Jiang SC. Quantitative Microbial Risk Assessment of Pathogenic Vibrios in Marine Recreational Waters of Southern California. Appl Environ Microbiol [Internet]. 2013 [cited 2018 Dec 6];79:294–302. Available from: http://aem.asm.org/lookup/doi/10.1128/AEM.02674-12

101. Dechet AM, Yu PA, Koram N, Painter J. Nonfoodborne *Vibrio* Infections: An Important Cause of Morbidity and Mortality in the United States, 1997–2006. Clin Infect Dis [Internet]. Narnia; 2008 [cited 2019 Apr 1];46:970–6. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/529148

102. Han F, Walker RD, Janes ME, Prinyawiwatkul W, Ge B. Antimicrobial susceptibilities of Vibrio parahaemolyticus and Vibrio vulnificus isolates from Louisiana Gulf and retail raw oysters. Appl Environ Microbiol [Internet]. 2007 [cited 2018 Dec 6];73:7096–8. Available from: http://aem.asm.org/cgi/doi/10.1128/AEM.01116-07

103. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. Am J Respir Crit Care Med [Internet]. American Thoracic Society; 2007 [cited 2019 Apr 1];175:367–416. Available from: http://www.atsjournals.org/doi/abs/10.1164/rccm.200604-571ST

104. Gonzalez-Santiago TM, Drage LA. Nontuberculous Mycobacteria. Dermatol Clin [Internet]. 2015 [cited 2018 Dec 6];33:563–77. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0733863515000303

105. Kaffenberger BH, Shetlar D, Norton SA, Rosenbach M. The effect of climate change on skin disease in North America. J Am Acad Dermatol [Internet]. 2017 [cited 2018 Dec 6];76:140–

7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27742170

106. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. Science [Internet]. NIH Public Access; 2009 [cited 2018 Dec 6];324:1190–2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19478181

107. Schommer NN, Gallo RL. Structure and function of the human skin microbiome. Trends Microbiol [Internet]. NIH Public Access; 2013 [cited 2019 Mar 24];21:660–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24238601

108. Pereira SG, Moura J, Carvalho E, Empadinhas N. Microbiota of Chronic Diabetic Wounds: Ecology, Impact, and Potential for Innovative Treatment Strategies. Front Microbiol [Internet]. Frontiers Media SA; 2017 [cited 2019 Mar 28];8:1791. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28983285

109. Meisel JS, Hannigan GD, Tyldsley AS, SanMiguel AJ, Hodkinson BP, Zheng Q, et al. Skin Microbiome Surveys Are Strongly Influenced by Experimental Design. J Invest Dermatol [Internet]. 2016 [cited 2018 Dec 6];136:947–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26829039

110. Rocha MA, Bagatin E. Skin barrier and microbiome in acne. Arch Dermatol Res [Internet]. Springer Berlin Heidelberg; 2018 [cited 2019 Mar 25];310:181–5. Available from: http://link.springer.com/10.1007/s00403-017-1795-3

111. Gardiner M, Vicaretti M, Sparks J, Bansal S, Bush S, Liu M, et al. A longitudinal study of the diabetic skin and wound microbiome. PeerJ [Internet]. 2017 [cited 2019 Mar 25];5:e3543. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28740749

112. Zeeuwen PLJM, Kleerebezem M, Timmerman HM, Schalkwijk J. Microbiome and skin diseases. Curr Opin Allergy Clin Immunol [Internet]. 2013 [cited 2019 Mar 25];13:514–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23974680

113. Price LB, Liu CM, Melendez JH, Frankel YM, Engelthaler D, Aziz M, et al. Community Analysis of Chronic Wound Bacteria Using 16S rRNA Gene-Based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota. Ratner AJ, editor. PLoS One [Internet]. 2009 [cited 2018 Dec 6];4:e6462. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19649281

114. Chang H-W, Yan D, Singh R, Liu J, Lu X, Ucmak D, et al. Alteration of the cutaneous microbiome in psoriasis and potential role in Th17 polarization. Microbiome [Internet]. BioMed Central; 2018 [cited 2018 Dec 6];6:154. Available from: https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0533-1

115. Williams MR, Gallo RL. Evidence that Human Skin Microbiome Dysbiosis Promotes Atopic Dermatitis. J Invest Dermatol [Internet]. Elsevier; 2017 [cited 2019 Mar 28];137:2460–1. Available from: https://www.sciencedirect.com/science/article/pii/S0022202X17329603?via%3Dihub

116. Gilbert JA, Quinn RA, Debelius J, Xu ZZ, Morton J, Garg N, et al. Microbiome-wide association studies link dynamic microbial consortia to disease. Nature [Internet]. 2016 [cited 2018 Dec 6];535:94–103. Available from: http://www.nature.com/articles/nature18850

117. MR DNA Laboratory. www.mrdnalab.com Shallowater, TX. [Internet]. [cited 2018 Dec 6]. Available from: http://www.mrdnalab.com/

118. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods [Internet]. NIH Public Access; 2010 [cited 2018 Dec 6];7:335–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20383131

119. Zitvogel L, Ma Y, Raoult D, Kroemer G, Gajewski TF. The microbiome in cancer immunotherapy: Diagnostic tools and therapeutic strategies. Science (80-) [Internet]. 2018 [cited 2018 Dec 6];359:1366–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29567708

120. Belkaid Y, Tamoutounour S. The influence of skin microorganisms on cutaneous immunity. Nat Rev Immunol [Internet]. Nature Publishing Group; 2016 [cited 2018 Dec 6];16:353–66. Available from: http://www.nature.com/articles/nri.2016.48

121. Oh J, Byrd AL, Park M, Kong HH, Segre JA, Segre JA. Temporal Stability of the Human Skin Microbiome. Cell [Internet]. 2016 [cited 2019 Mar 28];165:854–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27153496

122. Furue M, Iida K, Imaji M, Nakahara T. Microbiome analysis of forehead skin in patients with atopic dermatitis and healthy subjects: Implication of *Staphylococcus* and *Corynebacterium*. J Dermatol [Internet]. 2018 [cited 2018 Dec 6];45:876–7. Available from: http://doi.wiley.com/10.1111/1346-8138.14486

123. Oliver JD. The Biology of Vibrio vulnificus. Microbiol Spectr [Internet]. 2015 [cited 2018 Dec 6];3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26185084

124. Wang Y, Kao M-S, Yu J, Huang S, Marito S, Gallo R, et al. A Precision Microbiome Approach Using Sucrose for Selective Augmentation of Staphylococcus epidermidis Fermentation against Propionibacterium acnes. Int J Mol Sci [Internet]. 2016 [cited 2018 Dec 6];17:1870. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27834859

125. Tacket CO, Brenner F, Blake PA. Clinical features and an epidemiological study of Vibrio vulnificus infections. J Infect Dis [Internet]. 1984 [cited 2018 Dec 6];149:558–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6725989

126. Baddour LM. Extraintestinal Aeromonas Infections—Looking for Mr. Sandbar. Mayo Clin Proc [Internet]. Elsevier; 1992 [cited 2018 Dec 6];67:496–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0025619612604029

127. Dang H, Ren J, Song L, Sun S, An L. Dominant chloramphenicol-resistant bacteria and resistance genes in coastal marine waters of Jiaozhou Bay, China. World J Microbiol Biotechnol [Internet]. Springer Netherlands; 2008 [cited 2019 Jan 29];24:209–17. Available from: http://link.springer.com/10.1007/s11274-007-9458-8

128. Di Cesare A, Vignaroli C, Luna GM, Pasquaroli S, Biavasco F. Antibiotic-Resistant Enterococci in Seawater and Sediments from a Coastal Fish Farm. Microb Drug Resist [Internet]. 2012 [cited 2019 Jan 29];18:502–9. Available from: http://www.liebertpub.com/doi/10.1089/mdr.2011.0204

129. Leonard AFC, Zhang L, Balfour AJ, Garside R, Gaze WH. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ Int [Internet]. Pergamon; 2015

[cited 2019 Jan 29];82:92–100. Available from: https://www.sciencedirect.com/science/article/pii/S0160412015000409

130. Morroni G, Di Cesare A, Di Sante L, Brenciani A, Vignaroli C, Pasquaroli S, et al. *Enterococcus faecium* ST17 from Coastal Marine Sediment Carrying Transferable Multidrug Resistance Plasmids. Microb Drug Resist [Internet]. 2016 [cited 2019 Jan 29];22:523–30. Available from: http://www.liebertpub.com/doi/10.1089/mdr.2015.0222

131. Davies J. Origins and evolution of antibiotic resistance. Microbiologia. American Society for Microbiology; 1996. p. 9–16.

132. Sommer MOA, Church GM, Dantas G. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence. Taylor and Francis Inc.; 2010;1:299–303.

133. Yan ZZ, Chen QL, Zhang YJ, He JZ, Hu HW. Antibiotic resistance in urban green spaces mirrors the pattern of industrial distribution. Environ Int. Elsevier Ltd; 2019;132:105106.

134. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, et al. Antibiotic resistance is ancient. Nature [Internet]. 2011 [cited 2019 Dec 2];477:457–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21881561

135. Fan X-Y, Gao J-F, Pan K-L, Li D-C, Dai H-H, Li X. Functional genera, potential pathogens and predicted antibiotic resistance genes in 16 full-scale wastewater treatment plants treating different types of wastewater. Bioresour Technol [Internet]. Elsevier; 2018 [cited 2019 Dec 2];268:97–106. Available from: https://www.sciencedirect.com/science/article/pii/S0960852418310496

136. Carvalho IT, Santos L. Antibiotics in the aquatic environments: A review of the European scenario [Internet]. Environ. Int. Elsevier Ltd; 2016 [cited 2020 Mar 11]. p. 736–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27425630

137. Baker-Austin C, McArthur J V., Lindell AH, Wright MS, Tuckfield RC, Gooch J, et al. Multi-site Analysis Reveals Widespread Antibiotic Resistance in the Marine Pathogen Vibrio vulnificus. Microb Ecol [Internet]. Springer-Verlag; 2009 [cited 2019 Dec 2];57:151–9. Available from: http://link.springer.com/10.1007/s00248-008-9413-8

138. Poole K. Resistance to B-lactam antibiotics. Cell Mol Life Sci [Internet]. Birkhäuser-Verlag; 2004 [cited 2019 Nov 30];61:2200–23. Available from: http://link.springer.com/10.1007/s00018-004-4060-9

139. Bush K, Bradford PA. β-lactams and β-lactamase inhibitors: An overview. Cold Spring Harb Perspect Med. Cold Spring Harbor Laboratory Press; 2016;6.

140. Bush K. Past and present perspectives on β-lactamases. Antimicrob. Agents Chemother. American Society for Microbiology; 2018.

141. Binda E, Marinelli F, Marcone GL. Old and new glycopeptide antibiotics: Action and resistance. Antibiotics. MDPI AG; 2014. p. 572–94.

142. Zhanel GG, Homenuik K, Nichol K, Noreddin A, Vercaigne L, Embil J, et al. The Glycylcyclines: A Comparative Review with the Tetracyclines. Drugs. Springer; 2004. p. 63–88.

143. Han QF, Zhao S, Zhang XR, Wang XL, Song C, Wang SG. Distribution, combined pollution and risk assessment of antibiotics in typical marine aquaculture farms surrounding the

Yellow Sea, North China. Environ Int [Internet]. 2020 [cited 2020 Mar 11];138:105551. Available from: https://linkinghub.elsevier.com/retrieve/pii/S016041201933034X

144. Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol. Rev. Oxford Academic; 2004. p. 519–42.

145. Du Z, Wang M, Cui G, Zu X, Zhao Z, Xue Y. The prevalence of amphenicol resistance in Escherichia coli isolated from pigs in mainland China from 2000 to 2018: A systematic review and meta-analysis. Cheungpasitporn W, editor. PLoS One [Internet]. Public Library of Science; 2020 [cited 2020 Mar 17];15:e0228388. Available from: https://dx.plos.org/10.1371/journal.pone.0228388

146. Jacoby GA. Mechanisms of Resistance to Quinolones. Clin Infect Dis [Internet]. Oxford Academic; 2005 [cited 2020 Mar 18];41:S120–6. Available from: http://academic.oup.com/cid/article/41/Supplement_2/S120/307501/Mechanisms-of-Resistance-to-Quinolones

147. Becker B, Cooper MA. Aminoglycoside antibiotics in the 21st century. ACS Chem. Biol. American Chemical Society; 2013. p. 105–15.

148. Arthur M, Brisson-Noël A, Courvalin P. Origin and evolution of genes specifying resistance to macrolide, lincosamide and streptogramin antibiotics: data and hypotheses. J Antimicrob Chemother [Internet]. 1987 [cited 2020 Mar 18];20:783–802. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3326871

149. Marosevic D, Cervinkova D, Vlkova H, Videnska P, Babak V, Jaglic Z. In vivo spread of macrolide-lincosamide-streptogramin B (MLSB) resistance-A model study in chickens. Vet Microbiol. Elsevier; 2014;171:388–96.

150. Schwarz S, Shen J, Kadlec K, Wang Y, Michael GB, Feßler AT, et al. Lincosamides, streptogramins, phenicols, and pleuromutilins: Mode of action and mechanisms of resistance. Cold Spring Harb Perspect Med. Cold Spring Harbor Laboratory Press; 2016;6:a027037.

151. Peterson E, Kaur P. Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. Front Microbiol. Frontiers Media S.A.; 2018;9.

152. Mak S, Xu Y, Nodwell JR. The expression of antibiotic resistance genes in antibiotic-producing bacteria [Internet]. Mol. Microbiol. Blackwell Publishing Ltd; 2014 [cited 2020 Feb 26]. p. 391–402. Available from: http://doi.wiley.com/10.1111/mmi.12689

153. Yushchuk O, Ostash B, Truman AW, Marinelli F, Fedorenko V. Teicoplanin biosynthesis: unraveling the interplay of structural, regulatory, and resistance genes. Appl Microbiol Biotechnol [Internet]. 2020 [cited 2020 Feb 25]; Available from: http://link.springer.com/10.1007/s00253-020-10436-y

154. Zhang B, Xia Y, Wen X, Wang X, Yang Y, Zhou J, et al. The composition and spatial patterns of bacterial virulence factors and antibiotic resistance genes in 19 wastewater treatment plants. PLoS One [Internet]. Public Library of Science; 2016 [cited 2020 May 5];11:e0167422. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27907117

155. Mao C, Abraham D, Wattam AR, Wilson MJC, Shukla M, Yoo HS, et al. Curation, integration and visualization of bacterial virulence factors in PATRIC. Bioinformatics [Internet].

2015 [cited 2020 May 5];31:252–8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4287947/

156. Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? Clin. Microbiol. Rev. American Society for Microbiology (ASM); 2013. p. 185–230.

157. Soborg DA, Hendriksen NB, Kilian M, Kroer N. Widespread occurrence of bacterial human virulence determinants in soil and freshwater environments. Appl Environ Microbiol. American Society for Microbiology (ASM); 2013;79:5488–97.

158. Cantón R. Antibiotic resistance genes from the environment: A perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin. Microbiol. Infect. Blackwell Publishing Ltd; 2009. p. 20–5.

159. Kanehisa M, Sato Y, Furumichi M, Morishima K, Tanabe M. New approach for understanding genome variations in KEGG. Nucleic Acids Res. Oxford University Press; 2019;47:D590–5.

160. Kanehisa M. Toward understanding the origin and evolution of cellular organisms. Protein Sci. Blackwell Publishing Ltd; 2019. p. 1947–51.

161. Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. Oxford University Press (OUP); 2000;28:27–30.

162. Van Hoek AHAM, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. Acquired antibiotic resistance genes: An overview. Front. Microbiol. Frontiers Research Foundation; 2011.

163. Pattaroni CKSMB. Virulence Factor Database for 16S metagenomics. Mendeley; 2018;1.

164. Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM, et al. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. Nature Publishing Group; 2015;9:2490–502.

165. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software Environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13:2498–504.

166. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. Oxford University Press; 2014;30:2114–20.

167. Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K, et al. MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods. Academic Press Inc.; 2016. p. 3–11.

168. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res [Internet]. 2017 [cited 2020 Apr 20];45:D566–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27789705

169. Liu B, Zheng D, Jin Q, Chen L, Yang J. VFDB 2019: A comparative pathogenomic platform with an interactive web interface. Nucleic Acids Res [Internet]. 2019 [cited 2020 May 12];47:D687–92. Available from: https://academic.oup.com/nar/article/47/D1/D687/5160975

170. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods.

2012;9:357–9.

171. Quinlan AR. BEDTools: The Swiss-Army tool for genome feature analysis. Curr Protoc Bioinforma [Internet]. John Wiley and Sons Inc.; 2014 [cited 2020 Apr 20];2014:11.12.1-11.12.34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25199790

172. Heimann JD. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. Academic Press; 2002. p. 47–110.

173. Rice AJ, Park A, Pinkett HW. Diversity in ABC transporters: Type I, II and III importers. Crit. Rev. Biochem. Mol. Biol. Informa Healthcare; 2014. p. 426–37.

174. Su Z, Li A, Chen J, Huang B, Mu Q, Chen L, et al. Wastewater discharge drives ARGs spread in the coastal area: A case study in Hangzhou Bay, China. Mar Pollut Bull [Internet]. Elsevier Ltd; 2020 [cited 2020 Mar 25];151:110856. Available from: http://www.ncbi.nlm.nih.gov/pubmed/32056638

175. Bu Q, Wang B, Huang J, Deng S, Yu G. Pharmaceuticals and personal care products in the aquatic environment in China: A review. J. Hazard. Mater. Elsevier; 2013. p. 189–211.

176. Lerminiaux NA, Cameron ADS. Horizontal transfer of antibiotic resistance genes in clinical environments. Can J Microbiol [Internet]. Canadian Science Publishing; 2019 [cited 2020 Mar 19];65:34–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30248271

177. Hammerum AM, Larsen J, Andersen VD, Lester CH, Skovgaard Skytte TS, Hansen F, et al. Characterization of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. J Antimicrob Chemother [Internet]. 2014 [cited 2020 Apr 14];69:2650–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24908045

178. Deng Y, Zeng Z, Chen S, He L, Liu Y, Wu C, et al. Dissemination of IncFII plasmids carrying rmtB and qepA in Escherichia coli from pigs, farm workers and the environment. Clin Microbiol Infect. Blackwell Publishing Ltd; 2011;17:1740–5.

179. Chotiprasitsakul D, Srichatrapimuk S, Kirdlarp S, Pyden AD, Santanirand P. Epidemiology of carbapenem-resistant Enterobacteriaceae: a 5-year experience at a tertiary care hospital . Infect Drug Resist [Internet]. Dove Medical Press Ltd.; 2019 [cited 2020 Apr 14];Volume 12:461–8. Available from: https://www.dovepress.com/epidemiology-of-carbapenem-resistant-enterobacteriaceae-a-5-year-exper-peer-reviewed-article-IDR

180. Ripabelli G, Tamburro M, Guerrizio G, Fanelli I, Flocco R, Scutellà M, et al. Tracking Multidrug-Resistant Klebsiella pneumoniae from an Italian Hospital: Molecular Epidemiology and Surveillance by PFGE, RAPD and PCR-Based Resistance Genes Prevalence. Curr Microbiol. Springer New York LLC; 2018;75:977–87.

181. Flores C, Bianco K, de Filippis I, Clementino MM, Romão CMCPA. Genetic Relatedness of NDM-Producing *Klebsiella pneumoniae* Co-occurring VIM, KPC, and OXA-48 Enzymes from Surveillance Cultures from an Intensive Care Unit. Microb Drug Resist [Internet]. Mary Ann Liebert, Inc., publishers 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA ; 2020 [cited 2020 Apr 14];mdr.2019.0483. Available from: https://www.liebertpub.com/doi/10.1089/mdr.2019.0483

182. Soucy SM, Huang J, Gogarten JP. Horizontal gene transfer: Building the web of life. Nat.

Rev. Genet. Nature Publishing Group; 2015. p. 472–82.

183. Husnik F, McCutcheon JP. Functional horizontal gene transfer from bacteria to eukaryotes [Internet]. Nat. Rev. Microbiol. Nature Publishing Group; 2018 [cited 2020 Mar 19]. p. 67–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29176581

184. Garner E, Benitez R, von Wagoner E, Sawyer R, Schaberg E, Hession WC, et al. Stormwater loadings of antibiotic resistance genes in an urban stream. Water Res [Internet]. Elsevier Ltd; 2017 [cited 2020 Mar 19];123:144–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28662396

185. Baral D, Dvorak BI, Admiraal D, Jia S, Zhang C, Li X. Tracking the Sources of Antibiotic Resistance Genes in an Urban Stream during Wet Weather using Shotgun Metagenomic Analyses. Environ Sci Technol [Internet]. American Chemical Society; 2018 [cited 2020 Mar 19];52:9033–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30020774

186. Ergie AA, Leng Y, Wang J. Antibiotics and Resistance Genes in Awash River Basin, Ethiopia. Ecohealth. Springer; 2019;16:441–53.

187. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31:814–21.

188. Prescott SL, Larcombe D-L, Logan AC, West C, Burks W, Caraballo L, et al. The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. World Allergy Organ J [Internet]. 2017 [cited 2018 Dec 6];10:29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28855974

189. Cardona C, Lax S, Larsen P, Stephens B, Hampton-Marcell J, Edwardson CF, et al. Environmental Sources of Bacteria Differentially Influence Host-Associated Microbial Dynamics. Adams RI, editor. mSystems [Internet]. 2018 [cited 2018 Dec 6];3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29854953

190. Barnard E, Li H. Shaping of cutaneous function by encounters with commensals. J Physiol [Internet]. 2017 [cited 2018 Dec 6];595:437–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26988937